Faculty of Medicine and Health Sciences
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# Advancing RNA-based T-cell receptor redirection of lymphocytes to improve antitumor responses in adoptive T-cell immunotherapy for acute myeloid leukemia

Optimalisatie van RNA-gebaseerde T-celreceptormodificatie van lymfocyten voor de verbetering van antitumorresponsen in adoptieve T-celimmunotherapie voor acute myeloïde leukemie

**Dissertation**

submitted to obtain the degree of
Doctor in Medical Sciences at the University of Antwerp

To be defended by

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Antwerp, 2021

**The journey of a thousand miles must begin with a single step.**

— Lao Tzu

**Cover illustration:** Diana Campillo Davó.

The research described in this dissertation was performed at the Laboratory of Experimental Hematology of the University of Antwerp and at the Department of Cancer Immunology of the Osaka University Graduate School of Medicine.

This work was supported financially by the Bijzonder Onderzoeksfonds (BOF) of the University of Antwerp, the Research Foundation Flanders (FWO; grant G053518N), the Belgian Foundation against Cancer (Stichting tegen Kanker; grant FAF-C/2016/764), grants from the Fonds Baillet-Latour and the European Association for Cancer Research (EACR).

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*To my parents, Juan and Conchi,*
*the origin of this book.*

*A mis padres, Juan y Conchi,*
*el origen de este libro.*

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# Table of contents

# Summary

# English summary

T cells express T-cell receptors (TCRs) that recognize short peptides derived from the intracellular processing of proteins. These peptides are bound to molecules of the major histocompatibility complex (MHC) and presented on the cell surface as peptide-MHC (pMHC) complexes. During T-cell development, T cells randomly rearrange a specific TCR that will be able to bind and respond to a pMHC on nucleated cells. The TCR diversity that is generated during TCR rearrangement allows an almost infinite set of TCR-pMHC combinations, which can be exploited for therapeutic purposes. Thus, in TCR-engineered T (TCR-T)-cell therapy, T cells are engineered with nucleic acids containing the genetic information of T-cell receptors (TCRs) derived from tumor-specific T-cell clones that specifically target tumor-associated antigens (TAAs). TCR-T-cell therapy has achieved extraordinary results in solid cancers like melanoma; however, the development and use of TCR-T cells in the clinic for the treatment of hematological malignancies, such as acute myeloid leukemia (AML), has been challenging and it is still unsatisfactory.

AML is a heterogeneous disease defined by the clonal expansion of myeloid blasts in the bone marrow, peripheral blood and, in some cases, liver and spleen. It is one of the most common types of leukemia in adults, especially in those older than 65. Relapse rate in AML patients after standard of care is 80% and current rate of survival for relapsed patients is no higher than 10%. As described in **Chapter 1**, there are only twelve clinical trials that use TCR-T cells against AML, all in phase I or I/II, and mostly in cases of relapsed or refractory AML [1]. The majority of studies focus on Wilms' tumor 1 (WT1), on preferentially expressed antigen in melanoma (PRAME), and on minor histocompatibility antigen (MiHa) peptides in the context of HLA-A*02:01 restriction [2]. In particular, WT1 is a zinc-finger translation factor regarded as a "universal target" for its overexpression in both solid and hematological malignancies [3]. In AML, overexpression of WT1 protein occurs in 73-93% of patients at diagnosis [4], making this antigen a very interesting candidate for TCR-T-cell targeting. Therefore, the ultimate goal of this thesis was to explore and develop TCR-T-cell therapies for the treatment of AML using patient-derived WT1-specific TCRs and using non-viral RNA-based engineering methods. Indeed, RNA-based redirection of T cells benefits from a better safety profile than viral transduction methods. Stable modification of T cells with viral particles poses safety concerns related to random integration into the genome that could potentially lead to insertional mutagenesis. This concern motivated us to focus on mRNA electroporation as a safer engineering option for the redirection of T cells with mRNAs encoding WT1-specific TCRs.

Thus, in **Chapter 2**, we defined the main factors involved in mRNA electroporation for gene delivery and how to achieve optimal results in the design and production of RNA-

engineered cellular immunotherapies [5]. This included the physical properties of electroporation, the synthesis and stability of in vitro transcribed mRNA, as well as its clinical production and application. In **Chapter 3**, we exhaustively reviewed the preclinical and clinical advances made in the last years on the usage of mRNA electroporation in immune cell-based therapies for cancer, covering from cellular vaccines based on tumor antigen-loading of dendritic cells and B cells to adoptive cell therapies based on immune receptor-engineering of T cells and natural killer cells.

In **Chapter 4**, we set out to implement a fully RNA electroporation-based redirection of bulk primary human resting cytotoxic CD8 T cells using WT1-specific TCRs derived from an AML patient that showed multiepitope responses after *WT1* mRNA-engineered dendritic cell vaccination [6,7]. Since TCR mispairing between native and transgenic TCRs hinders transgenic TCR expression and, thus, TCR-T-cell antitumor activity, we developed a double sequential electroporation protocol in which Dicer-substrate silencing RNAs (DsiRNAs) targeting wild type sequences of T-cell receptor alpha and beta constant regions were electroporated prior to WT1-specific codon-optimized *TCR* mRNA electroporation. We showed that the reduction of native TCR expression via DsiRNA-mediated downregulation of native TCR transcripts minimized TCR mispairing, which translated into a great improvement in transgenic TCR expression and tumor cell recognition [8].

Next, we observed that, when working on TCR-T-cell therapy development, choosing the right antigen-presenting cell (APC) system requires careful attention. This is of paramount importance when the TCRs (i) are derived from circulating T-cell clones that undergo negative selection in the thymus; (ii) target a self-antigen, such as WT1; and (iii) have not undergone artificial affinity maturation to increase TCR affinity, i.e., the likelihood and strength of a TCR biding the pMHC. The gold standard in T-cell functional assays are cell lines that deviate from a physiological state and may misrepresent the actual functional avidity and antitumor capabilities of TCR-T cells. Therefore, **Chapter 5** explored different cell lines as APC models for rapidly assessing WT1-specific TCR-T-cell functional avidity—that is, the response or functional activity of T cells against a certain cognate epitope density—by using full-length *WT1* mRNA electroporation. We observed that the multiple myeloma U266 cell line was an excellent candidate due (i) the expression of the common MHC class I human leukocyte antigen (HLA) serotype HLA-A*02:01, (ii) the low expression of WT1, (iii) the possibility of exogenously pulsing WT1 peptides, and (iv) the possibility and flexibility of electroporating full-length *WT1* mRNA. Thus, we determined that the use of *WT1* mRNA-electroporated and WT1 peptide-pulsed U266 cells cultured with WT1-specific TCR-T cells was a good model for identifying good TCR candidates, those with higher functional avidities, for TCR-T-cell therapies.

Nevertheless, as WT1 is a self-antigen, T-cell clones of high avidity against WT1 are usually eliminated after negative selection in the thymus, reducing the number of high-affinity WT1-reactive TCRs that could be used in TCR-T-cell therapies. Those that remain usually promote inferior T-cell responses to physiological epitope densities, which would partly explain tumor immune escape [9]. Thus, high-affinity TCRs, with better pMHC binding capabilities, commonly generate better T-cell functional avidities and, consequently, better antitumor responses [10]. TCR affinity can be artificially enhanced by affinity maturation; however, this strategy can render TCRs with supraphysiological affinities that may lead to lethal cross-reactivities [11]. At the same time, T-cell functional avidity can be affected by factors such as TCR expression and stability on the T-cell membrane. Therefore, improvement of T-cell functional avidity can be achieved by increasing TCR production as well as enhancing the expression of TCR co-receptors, such as CD8. Moreover, although traditionally TCR-T-cell therapies have mainly focused on redirecting cytotoxic CD8 T cells specificity, there is growing interest for other T cell subsets such as CD4 T cells and γδ T cells due to their critical role in the generation of effective antitumor immune responses. Therefore, in **Chapter 6**, we explored the redirection of different T-cell subsets with WT1-specific *TCR* mRNA in combination with mRNA coding for TCR co-receptor CD8αβ as a strategy to increase TCR-pMHC interaction without potential toxicities while making use of—more frequent— intermediate-affinity TCRs. We observed that redirection of conventional CD8 and CD4 T cells as well as γδ T cells with WT1-specific TCRs benefits from concomitant upregulation or de novo expression of CD8, which increases TCR-T-cell avidity and tumor cell recognition. In fact, CD4 and γδ T-cell subsets were only capable of recognizing WT1-positive tumor cells when *CD8* mRNA was co-electroporated with *TCR* mRNA. In addition, our results indicated that CD8 T cells can further benefit from the upregulation of CD3 expression via mRNA encoding CD3 δ, γ, ε, and ζ subunits.

We finally summarized all the knowledge gathered on TCR affinity and TCR-T-cell functional avidity—concepts frequently and erroneously swapped—for the purpose of developing improved TCR-T-cell therapies against cancer in the final **Chapter 7**. In general, this thesis provides methodological and empirical evidence that RNA electroporation is a versatile, fast, and suitable strategy for engineering different T-cell subsets with antigen-specific TCRs, in particular against WT1 for the treatment of AML. We also provide an efficient and non-genotoxic protocol to reduce TCR mispairing. Moreover, this work also highlights the importance of the target cells used for the screening and testing TCR-T-cell functionality as well as the key role that expression of TCR co-receptors has in order to improve TCR-T-cell tumor cell recognition.

# Nederlandstalige samenvatting

T-cellen brengen T-celreceptoren (TCR's) tot expressie die korte peptiden afkomstig van de intracellulaire verwerking van eiwitten herkennen. Deze peptiden zijn gebonden aan moleculen van het majeur histocompatibiliteits complex (MHC) en worden op het celoppervlak gepresenteerd als peptide-MHC (pMHC) complexen. Tijdens de T-celontwikkeling zal door middel van een willekeurige gen herschikking een specifieke TCR ontstaan die in staat is te binden aan en te reageren op pMHC op gekernde cellen. De TCR diversiteit die ontstaat tijdens de TCR herschikking maakt een bijna oneindige set van TCR-pMHC combinaties mogelijk, welke benut kunnen worden voor therapeutische doeleinden. Bij TCR-gemanipuleerde T (TCR-T)-celtherapie worden T-cellen dus voorzien van nucleïnezuren die de genetische informatie bevatten van TCR's afkomstig van tumorspecifieke T-celklonen gericht op tumorgeassocieerde antigenen (TAA's). TCR-T-celtherapie heeft buitengewone resultaten geboekt bij solide kankers zoals melanoom; de ontwikkeling en het gebruik van TCR-T-cellen in de kliniek voor de behandeling van hematologische maligniteiten, zoals acute myeloïde leukemie (AML), blijkt echter een uitdaging en laat te wensen over.

AML is een heterogene ziekte die wordt gekenmerkt door de klonale expansie van myeloïde blasten in het beenmerg, het perifere bloed en, in sommige gevallen, de lever en de milt. Het is een van de meest voorkomende vormen van leukemie bij volwassenen, vooral bij mensen ouder dan 65 jaar. Het recidiefpercentage bij AML-patiënten na standaardbehandeling is 80% en het huidige overlevingspercentage voor recidiefpatiënten is niet hoger dan 10%. Zoals beschreven in **Hoofdstuk 1**, zijn er slechts twaalf klinische studies, allemaal in fase I of I/II, die gebruik maken van TCR-T cellen tegen AML, meestal in patiënten met recidiverende of refractaire AML [1]. De meeste studies richten zich op Wilms' tumor 1 (WT1), op preferentieel tot expressie gebracht antigeen in melanoom (PRAME), en op mineur histocompatibiliteits antigeen (MiHA) peptiden in de context van HLA-A*02:01 restrictie [2]. In het bijzonder is WT1 een zink-vinger translatiefactor die beschouwd wordt als een "universeel doelwit" door zijn overexpressie in zowel solide als hematologische maligniteiten [3]. In AML komt WT1 tot overexpressie in 73-93% van de patiënten bij diagnose [4], wat dit antigeen een zeer interessante kandidaat maakt voor TCR-T-cel hernkenning. Bijgevolg was het uiteindelijke doel van dit proefschrift het onderzoeken en ontwikkelen van TCR-T-celtherapieën voor de behandeling van AML door gebruik te maken van patiënt-afgeleide WT1-specifieke TCR's en met behulp van niet-virale RNA-gebaseerde manipulatietechnieken. RNA-gebaseerde modificatie van T-cellen heeft immers een beter veiligheidsprofiel dan virale manipulatiemethoden. Stabiele modificatie van T-cellen met virale partikels brengt veiligheidsproblemen met zich mee in verband met willekeurige integratie in het genoom die mogelijk kan leiden tot insertiemutagenese.

Deze bezorgheid motiveerde ons om ons te richten op mRNA elektroporatie als een veiligere methode voor de modificatie van T-cellen met mRNA's coderend voor WT1-specifieke TCR's.

Dus, in **Hoofdstuk 2** definieerden we de belangrijkste factoren voor gen afgifte via mRNA elektroporatie en hoe optimale resultaten in het ontwerp en de productie van RNA-gemanipuleerde cellulaire immuuntherapieën bereikt kunnen worden [5]. Deze factoren omvatten de fysische eigenschappen van elektroporatie, de synthese en stabiliteit van in vitro afgeschreven mRNA, alsmede de klinische productie en toepassing ervan. In **Hoofdstuk 3** hebben we een uitgebreid overzicht gegeven van de preklinische en klinische vooruitgang die in de afgelopen jaren is geboekt in het gebruik van mRNA elektroporatie in immuuncel-gebaseerde therapieën voor kanker, variërend van cellulaire vaccins gebaseerd tumorantigeen-geladen dendritische cellen en B cellen tot adoptieve celtherapieën gebaseerd op immuunreceptor-gemanipuleerde T cellen en natuurlijke killer cellen.

In **Hoofdstuk 4** hebben we ons gericht op het implementeren van een volledig op RNA elektroporatie-gebaseerde modificatie van bulk primaire menselijke, rustende cytotoxische CD8 T cellen met behulp van WT1-specifieke TCR's afkomstig van een AML patiënt die een multiepitoope activiteit vertoonde na vaccinatie met *WT1* mRNA-gemanipuleerde dendritische cellen [6,7]. Aangezien foutief koppelen van een TCR tussen natieve en transgene TCRs de transgene TCR-expressie en dus TCR-T-cel antitumoractiviteit belemmert, ontwikkelden we een dubbel sequentieel elektroporatie protocol waarin Dicer-substraat silencing RNAs (DsiRNAs) gericht op de wild-type sequenties van de T-cel receptor alfa en beta constante regio's werden geëlektroporeerd voorafgaand aan WT1-specifieke codon-geoptimaliseerde *TCR* mRNA elektroporatie. We toonden aan dat de reductie van natieve TCR expressie via DsiRNA-gemedieerde neerregulatie van natieve TCR transcripten foutieve paringen minimaliseerde, wat zich vertaalde in een sterke verbetering van transgene TCR expressie en de herkenning van tumorcellen [8].

Vervolgens stelden we vast dat bij de ontwikkeling van TCR-T-celtherapie, de keuze van het juiste antigeen-presenterende cel (APC) system bijzondere aandacht vereist. Dit is van het grootste belang wanneer de TCR's (i) afkomstig zijn van circulerende T-cel-klonen die negatieve selectie ondergaan in de thymus; (ii) gericht zijn tegen een lichaamseigen antigeen, zoals WT1; en (iii) geen artificiële affiniteitsmaturatie hebben ondergaan om de TCR-affiniteit, de waarschijnlijkheid en sterkte waarmee een TCR pMHC bindt, te verhogen De gouden standaard in T-cel functionele testen zijn cellijnen die afwijken van een fysiologische toestand en dus een verkeerde voorstelling kunnen geven van de werkelijke functionele aviditeit en antitumor capaciteiten van TCR-T cellen. Daarom werden in **Hoofdstuk 5** verschillende cellijnen onderzocht als APC

modellen voor het vlot beoordelen van WT1-specifieke TCR-T-cel functionele aviditeit, de respons of functionele activiteit van T-cellen tegen een bepaalde epitoopdichtheid - door gebruik te maken van elektroporatie van full-length *WT1* mRNA. Wij stelden vast dat de multiple myeloom cellijn U266 een uitstekende kandidaat was door (i) de expressie van het gemeenschappelijke MHC klasse I humaan leukocyten antigeen (HLA) serotype HLA-A*02:01, (ii) de lage expressie van WT1, (iii) de mogelijkheid tot het exogeen pulsen van WT1 peptiden, en (iv) de mogelijkheid en flexibiliteit tot het elektroporeren met full-length *WT1* mRNA. Aldus stelden we vast dat het gebruik van *WT1* mRNA-geëlektroporeerde en WT1 peptide-gepulste U266 cellen gecultiveerd met WT1-specifieke TCR-T cellen een goed model was voor het identificeren van goede TCR-kandidaten met een hogere functionele aviditeit, voor TCR-T-celtherapieën.

Niettemin, omdat WT1 een lichaamseigen antigeen is, worden T-cel-klonen met hoge aviditeit tegen WT1 gewoonlijk geëlimineerd na negatieve selectie in de thymus waardoor het aantal WT1-reactieve TCRs met hoge affiniteit dat gebruikt zou kunnen worden in TCR-T-cel therapieën afneemt. De TCR's die overblijven resulteren meestal in inferieure T-cel-reacties op fysiologische epitoop dichtheden, wat het ontsnappen van de tumor aan het immuunsysteem gedeeltelijk zou verklaren [9]. Daarom hebben TCR's met hoge affiniteit, die een betere pMHC bindingscapaciteiten hebben, meestal betere T-cel functionele aviditeiten en, bijgevolg, betere antitumor activiteit [10]. TCR-affiniteit kan kunstmatig verhoogd worden door affiniteitsmaturatie; deze strategie kan echter TCR's maken met een suprafysiologisch affiniteit die kunnen leiden tot dodelijke kruisreactiviteit [11]. Tegelijkertijd kan de functionele aviditeit van T-cellen beïnvloed worden door factoren zoals TCR-expressie en stabiliteit op de T-celmembraan. Daarom kan een toename van de functionele aviditeit van T-cellen worden bekomen door de TCR-productie te verhogen en de expressie van TCR co-receptoren, zoals CD8, te verbeteren. Hoewel TCR-T-celtherapieën zich traditioneel vooral richten op het veranderen van de cytotoxische CD8 T-celspecificiteit, is er een groeiende interesse voor andere T-celsubgroepen zoals CD4 T-cellen en γδ T-cellen vanwege hun cruciale rol in het genereren van effectieve antitumor immuunreacties. Daarom onderzochten we in **Hoofdstuk 6** de heroriëntering van verschillende T-cel subsets met WT1-specifiek *TCR* mRNA in combinatie met mRNA coderend voor TCR co-receptor CD8αβ als strategie om de TCR-pMHC interactie te versterken zonder potentiële toxiciteit, terwijl gebruik wordt gemaakt van TCR's met een gemiddelde affiniteit. We hebben waargenomen dat het heroriënteren van conventionele CD8 en CD4 T-cellen en γδ T-cellen met WT1-specifieke TCR's baat heeft bij gelijktijdige verhoging of de novo expressie van CD8, wat de TCR-T-cel aviditeit en tumorcelherkenning verhoogt. In feite waren CD4 en γδ T-cel subsets alleen in staat om WT1-positieve tumorcellen te herkennen wanneer *CD8* mRNA gecoëlektroporeerd werd met *TCR* mRNA. Bovendien gaven onze resultaten

aan dat CD8 T-cellen verder kunnen profiteren van de verhoging van CD3 expressie via mRNA dat codeert voor de CD3 δ, γ, ε, en ζ subeenheden.

Tenslotte vatten we alle verzamelde kennis samen over TCR affiniteit en TCR-T-cel functionele aviditeit—concepten die vaak en per vergissing verwisseld worden—met als doel het ontwikkelen van verbeterde TCR-T-celtherapieën tegen kanker in het laatste **Hoofdstuk 7**. In het algemeen levert dit proefschrift methodologisch en empirisch bewijs dat RNA elektroporatie een veelzijdige, snelle en geschikte strategie is voor het modificeren van verschillende T-cel subsets met antigeen-specifieke TCRs, in het bijzonder tegen WT1 voor de behandeling van AML. We bieden ook een efficiënte en niet-genotoxische protocol om foutief koppelen van TCR's te verminderen. Bovendien benadrukt dit werk ook het belang van de doelwitcellen die gebruikt worden voor het screenen en testen van TCR-T-cel functionaliteit, alsook de sleutelrol die expressie van TCR co-receptoren speelt bij het verbeteren van TCR-T-cel tumorcelherkenning.

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# Prologue

## The relevance of translational research for AML in a nutshell: figures on incidence, mortality, and economic burden

With the announcement of "Europe's Beating Cancer Action Plan" on World Cancer Day 2020, cancer research, prevention, and care became a core priority for the European Commission in the healthcare domain [1]. In 2018 and Europe alone, more than 3.9 million new cases of cancer were diagnosed, and more than 1.9 million people died from cancer [2]. Although cancer incidence is rising and mortality rates are high, cancer-related mortality is slightly falling, especially in leukemias, partially due to recent therapeutic developments [3]. Lung and colorectal cancer have the dubious distinction of having the highest incidence rates among the different types of cancer in Europe for men and women together [2]. In the third position, all malignant blood disorders have a combined incidence rate of 7.6% for both sexes in 2018 in the European Union (EU) [2]. The economic burden of blood malignancies in the EU remained very high and has been calculated at €6.8 billion, 12% of the total healthcare expenditure on cancer, only behind breast cancer [4]. The Belgian Cancer Registry indicated in their "Cancer burden in Belgium 2004-2017" report [5] that almost 69.000 individuals were diagnosed with invasive tumors (excluding non-melanoma skin cancer) in 2017 in Belgium, and more than 27.000 people died from cancer in 2016. To put these figures in perspective, according to Statbel, the Belgian statistical office, the population in Belgium was 11.322.088 on 1 January 2017 (statbel.fgov.be). Among malignant blood disorders, the incidence rate of leukemia alone was estimated at 2.5% for both sexes in the EU in 2018 [2]. Lithuania, Belgium, and Luxembourg led the list of countries with the highest incidence of leukemia [2]. More updated and general information on estimates of cancer incidence and mortality can be found on the website of the European Cancer Information System (ecis.jrc.ec.europa.eu/). This institution estimates an incidence of 14.1 cases of leukemia and a mortality of 9.1 per 100,000 individuals in 2020 in the EU, including both sexes. In Belgium, three different blood malignancies (leukemia, non-Hodgkin lymphoma, and multiple myeloma) were among the 15 most frequently diagnosed cancers according to the "Cancer burden in Belgium 2004-2017" report [5] (**Figure 1**).

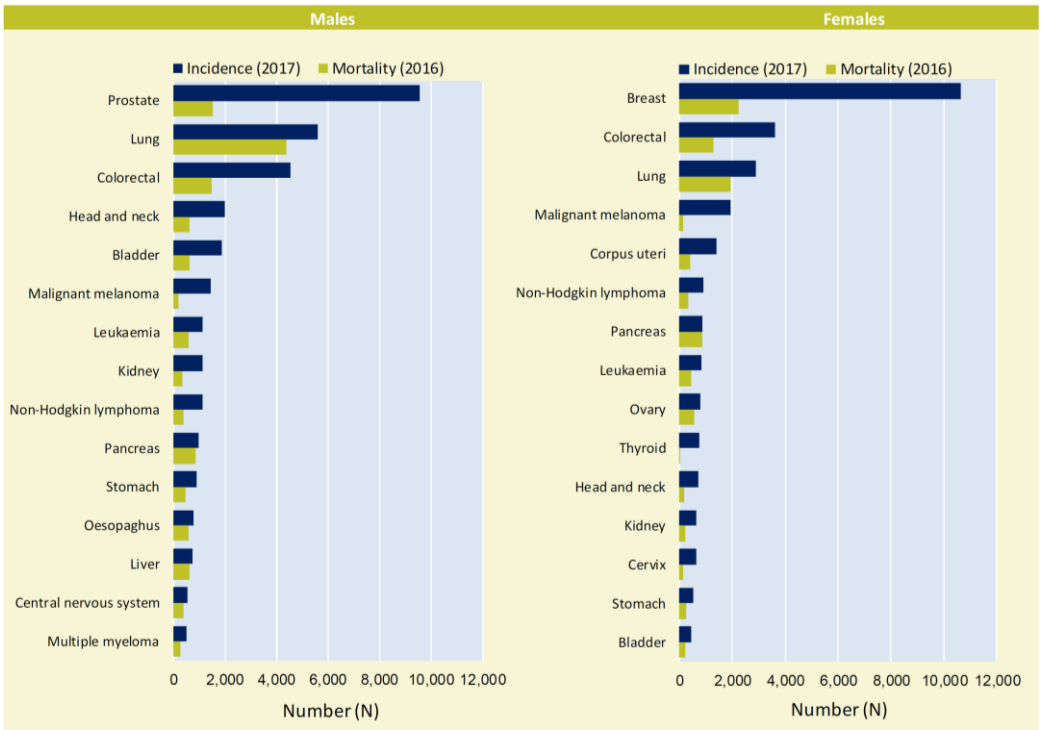


**Figure 1. Incidence and mortality for the 15 most frequently diagnosed malignancies (excluding non-melanoma skin cancer) by sex in Belgium.** Figure adapted from "Cancer burden in Belgium 2004-2017", Belgian Cancer Registry, Brussels, 2020.

In 2015, the Belgian Cancer Registry published a special issue on the incidence of hematological malignancies in Belgium in 2012 [6]. This report shows that all hematological malignancies account for 10% of all malignant tumors in both men and women, making them the fourth most frequent cause of death from cancer for males and the third for females [6]. The Belgian Cancer Registry published an update in 2021 on the epidemiological status of hematological malignancies spanning 15 years of data (from 2014 to 2018) [7]. In this second report, data show that incidence of hematological malignancies has increased over the years. A total of 7.562 people living in Belgium faced the diagnosis of a hematological malignancy in 2018, of which 55% were males [7]. Compared to data from 2012, with an incidence of 6.524 cases [6], this represents an almost 16% increase in the number of cases between 2012 and 2018. Although hematological malignancies are diagnosed in any age group, the average age at diagnosis is set at around 65 years. Among hematological malignancies, leukemia has been traditionally divided into acute or chronic, depending on disease's onset, and into myeloid or lymphocytic, depending on the cell lineage involved. These two classifications combined give the four main types of leukemia: acute lymphocytic leukemia, acute myeloid leukemia (AML), chronic lymphocytic leukemia, and chronic

myeloid leukemia. In 2004-2012, myeloid disorders accounted for almost 30% of the malignancies diagnosed (**Figure 2**), whereas this percentage increased slightly in the period between 2013 and 2018 for some age groups (**Figure 3**).

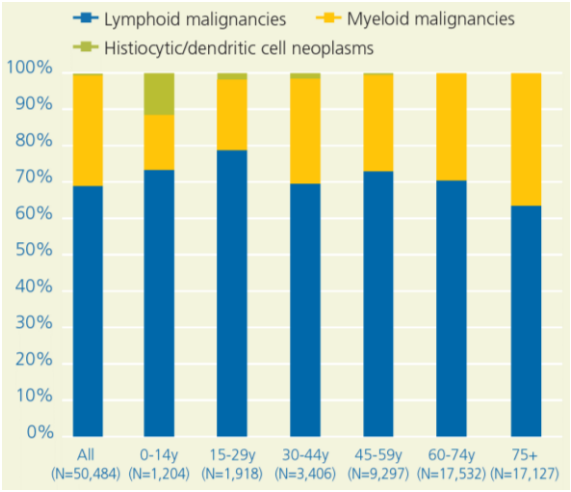


**Figure 2. Hematological malignancies: incidence by cell lineage and age group in Belgium in 2004-2012.** Figure adapted from "Hematological malignancies in Belgium", Belgian Cancer Registry, Brussels, 2015.

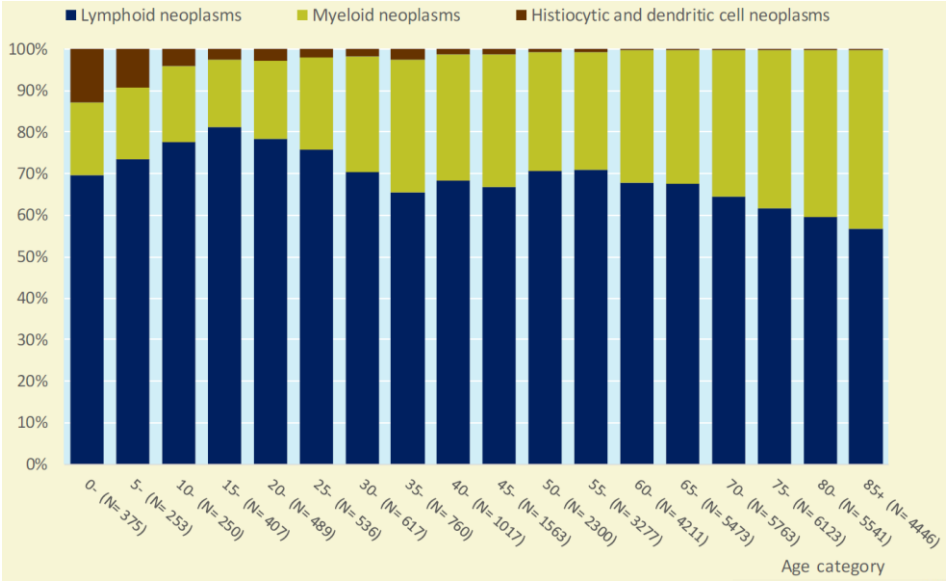


**Figure 3. Hematological malignancies: incidence by cell lineage and age group in Belgium in 2013-2018.** Figure adapted from "Hematological malignancies in Belgium 2004-2018", Belgian Cancer Registry, Brussels, 2021.

In terms of incidence of myeloid malignancies, 2,130 new cases were diagnosed in 2012, of which 53% were males [6]. The average age at diagnosis was 67 years for both sexes [6]. Age-specific incidence rates of myeloid malignancies increased for all age groups, especially in those older than 50 years [6]. Data in the 2020 report by the Belgian Cancer

Registry was, however, mainly classified into four main subtypes: mature lymphoid neoplasms, precursor neoplasms (including precursor lymphoid neoplasms and AML, among other neoplasms), chronic myeloid neoplasms and histiocytic and dendritic cell neoplasms [7]. A total of 4.367 cases of precursor neoplasms were diagnosed between 2013 and 2018, of which 76% were AML and related precursor neoplasms [7]. According to Orphanet, AML is considered a rare disease due to its annual incidence of 1/33.000-1/25.000 people in Europe—based on the European Union Regulation on Orphan Medicinal Products from 1999, a rare disease is described as one that occurs in 1 person every 2000. In Belgium in 2018 alone, annual incidence of AML (and related precursor neoplasms) was higher—approximately 1/19.000—with 595 new cases for a population of approximately 11.4 million inhabitants [7,8]. Moreover, despite being considered a rare disease, AML is the most common type of acute leukemias in adults, especially in adults older than 65. As with myeloid malignancies in general, a rapid increase of AML incidence rates occurs after the age of 50 years. Despite its relatively low numbers compared to other cancers, AML incidence rates are increasing, especially in males older than 75 years of age [6,7]. More importantly, AML has a poor prognosis and has an average 5-year relative survival rate of 20-30% in older patients [6,7] (**Figure 4**). Furthermore, this value drops to less than 10% in patients older than 70 years of age [7].

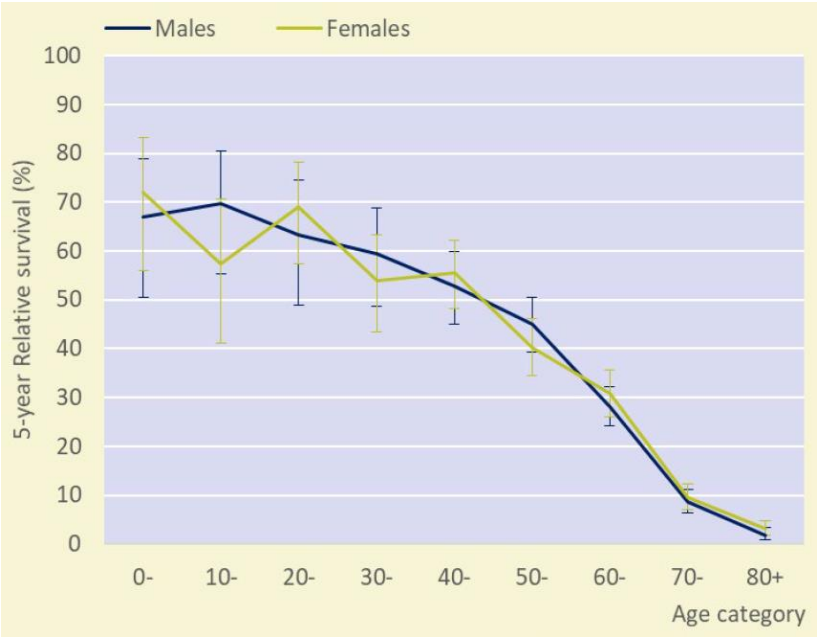


**Figure 4. Acute myeloid leukemia: age-specific 5-year relative survival by sex in Belgium in 2009-2018.** Figure adapted from "Hematological malignancies in Belgium 2004-2018", Belgian Cancer Registry, Brussels, 2021.

Induction therapy of de novo AML normally consists of rounds of chemotherapy to achieve complete remission (CR). After remission, initial induction therapy is usually followed by consolidation therapy consisting of hematopoietic stem-cell transplantation (HSCT) or intermediate doses of chemotherapeutic agents combined with the oral BCL-2 inhibitor venetoclax [9]. However, relapse in AML patients is highly frequent after complete remission due to the persistence of residual AML blasts, an issue defined as measurable residual disease (MRD, previously known as minimal residual disease). Due to its low incidence, heterogeneity, and differences in treatment and cost calculation across countries, the general economic burden associated with AML alone has been poorly characterized, and available data usually dates more than 10 years [10,11]. Even fewer studies focus on those AML patients that are ineligible for high-intensity chemotherapy [12]. However, recent data from the United States (US) and the United Kingdom (UK) showed that AML treatment is associated with high direct costs for the healthcare systems with the current standard of care [13]. A study conducted in the Netherlands and published in 2013 showed that the median costs of first course of induction treatment were higher than those of a second course of induction chemotherapy (€43,355 versus €36,827, respectively), which is administered to those patients that fail to achieve CR after first course [14]. Median costs of consolidation therapy were dependent on the type of therapy administered. Thus, consolidation therapies consisting of high-dose chemotherapy (€33,031) or HSCT, either autologous (€31,951) or allogeneic from a sibling (€37,394) had lower median costs than first course of induction therapy [14]. However, allogeneic HSCT from a matched unrelated donor was the most expensive therapeutic regimen overall (€83,165), due to general costs of donor search and HLA-typing but also to greater hospital visits, diagnostic procedures, medication and longer treatment duration [14]. Our research group recently analyzed the medical costs of treatment and survival of patients with AML in Belgium [15]. This study compared different treatment groups, in which patients were treated with induction and consolidation chemotherapy (ICT) alone, with ICT plus allogeneic HSCT, or with ICT plus immunotherapy using autologous dendritic cells (DC) engineered to express Wilms' tumor 1 protein (WT1). Median total costs were lower for ICT treatment (€32,649; ranging from €4,760 to €140,383), whereas HSCT-related median total costs were around €134,112, ranging from €122,325 to €378,117. Interestingly, median total costs of personalized AML immunotherapy based on WT1-loaded autologous DC were €109,856, lower than HSCT, and ranging from €45,114 to €207,732. More importantly, values of median overall survival (OS) after HSCT (339 days) were lower than those of DC therapy (477 days), which indicates that personalized immunotherapies may be more advantageous in terms of prognosis without incurring in greater costs related to treatment than those of standard of care.

In summary, despite the fact that AML is considered a rare disease, the high percentage of relapse, low survival of relapse and older patients, and high costs associated to HSCT treatment in consolidation therapy within standard of care warrant the development of innovative and more personalized (immuno)therapies, especially those that are highly specialized in detecting residual AML blast for eradicating MRD.

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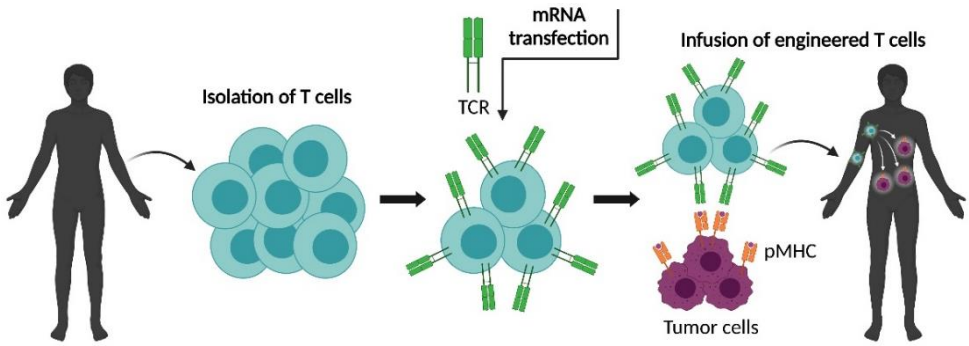
# Outline and objectives

Acute myeloid leukemia (AML) is a heterogeneous disease and the most common type of acute leukemia in adults. Although complete remission is initially achieved in many cases, a high number of AML patients relapse after remission, which demands new therapeutic options. In recent years, adoptive transfer of lymphocytes has marked a turning point in cancer treatment. This type of immunotherapy uses the patient's lymphocytes to induce a targeted attack on cancer cells. To improve the accuracy of the attack, T cells can be armed with T-cell receptors (TCRs) to specifically target peptides from tumor-associated antigens (TAAs). Different technologies can be used to genetically engineer immune cells. These can be categorized as viral/non-viral and transient/stable technologies. The Laboratory of Experimental Hematology has a long track record in transient non-viral RNA-based genetic engineering of immune cells for personalized immunotherapies. The main expertise of the group lies in a type of mechanical non-viral transfection method called electroporation to engineer dendritic cells (DCs) with messenger RNA (mRNA) for transient gene transfer, in particular, Wilms' tumor 1 (*WT1*) gene. WT1 is one of the most prominent AML-associated antigens and a post-diagnosis prognostic factor in AML. In fact, detection of *WT1* expression levels has been proposed as a marker for measurable residual disease (MRD, previously known as minimal residual disease) if no other markers are available. In a clinical trial run in collaboration with the Centre for Cell Therapy and Regenerative Medicine (CCRG in its Dutch acronym), AML patients were vaccinated with *WT1*-encoding mRNA-electroporated DCs. In some patients, WT1-specific multi-epitope T-cell responses were detected.

**We hypothesized that WT1-specific TCRs can be isolated from AML patients and used to engineer different T-cell populations safely and optimally via RNA electroporation. By doing so, WT1-specific TCR-engineered T cells would induce a specialized attack on WT1-positive tumor cells in the context of adoptive T-cell immunotherapy for hematological malignancies and, in particular, for AML.**



**Therefore, this doctoral thesis aims to further improve WT1-specific TCR-based adoptive T-cell therapies for AML using electroporation as a safe engineering method.**

In close collaboration with the Department of Cancer Immunotherapy of the Osaka University Graduate School of Medicine, we isolated and cloned WT1-specific TCRs derived from a leukemia patient who demonstrated clinical benefit after receiving a WT1-targeted DC vaccine (**Figure 1**; **Objective 1 – Chapter 4**). However, mispairing between endogenous and transgenic TCR chains may result in a reduction of transgenic TCR expression levels and can also lead to potentially harmful off-target reactivities. In order to suppress the translation of endogenous *TCR* mRNA in TCRαβ-positive T cells, a safe and RNA-based technology was designed to specifically target the endogenous TCR in TCRαβ $CD8^+$ T cells (**Objective 2 – Chapter 4**). To analyze the impact that different tumor cell lines can have as antigen-presenting cells in evaluating TCR-engineered T-cell avidity, we investigated different tumor cell lines for their antigen-presenting capacity and developed a full-length *WT1* mRNA tumor APC model (**Objective 3 – Chapter 5**). To take advantage of the full potential of T-cell subtypes other than cytotoxic CD8 T cells, we explored the electroporation of TCRαβ CD4 and TCRγδ T cells (**Objective 4a – Chapter 6**). However, WT1 is a self-antigen, i.e., a protein naturally expressed in normal human cells, and WT1-specific TCRs may have a low binding affinity due to the elimination of highly reactive T-cell clones in negative T-cell selection processes. Therefore, we evaluated whether the addition of TCR co-receptors could impact the functional avidity (antigen-specific TCR activation threshold) of engineered T cells with low-affinity TCRs (**Objective 4b – Chapter 6**).

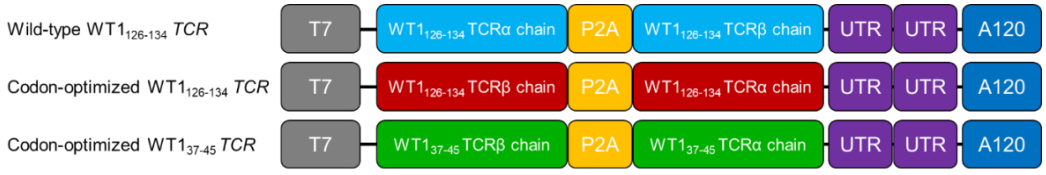
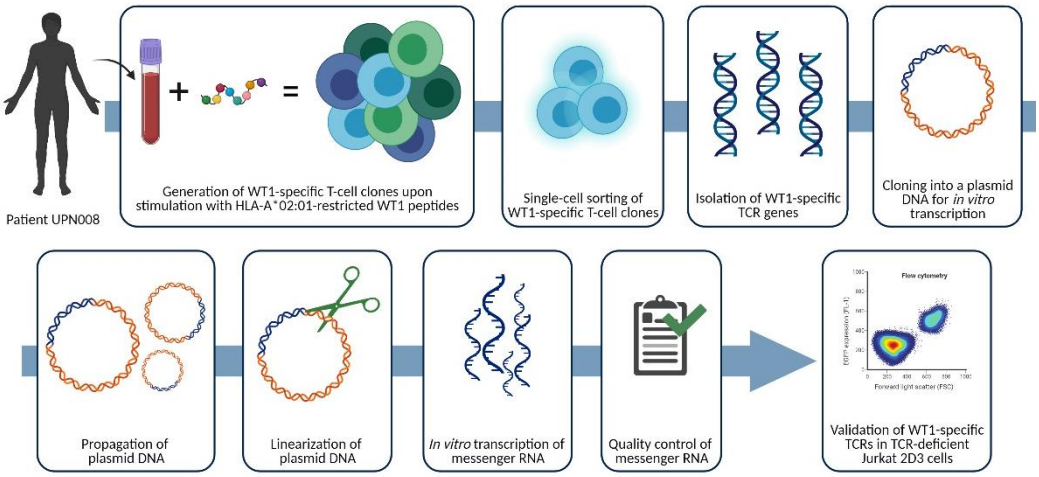



**Figure 1. Constructs containing the TCR α and β chains of WT1-specific TCRs isolated in collaboration with the Department of Cancer Immunotherapy of the Osaka University Graduate School of Medicine.** A120, poly(A) tail comprised of 120 adenosine nucleotides; P2A, "self-cleaving" 2A peptide (ribosomal skipping sequence) derived from porcine teschovirus-1; T7, bacteriophage T7 promoter; TCR, T-cell receptor; UTR, untranslated region; WT1, Wilms' tumor 1.

# Graphical overview of the objectives

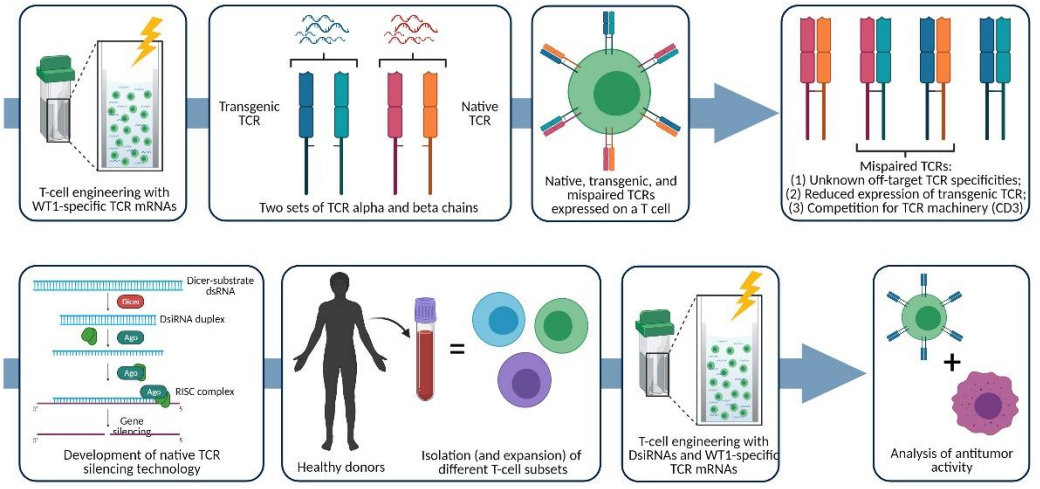
## Objective 1

Isolation, cloning, and in vitro characterization of AML patient-derived WT1-specific TCRs introduced into T cells by mRNA electroporation.



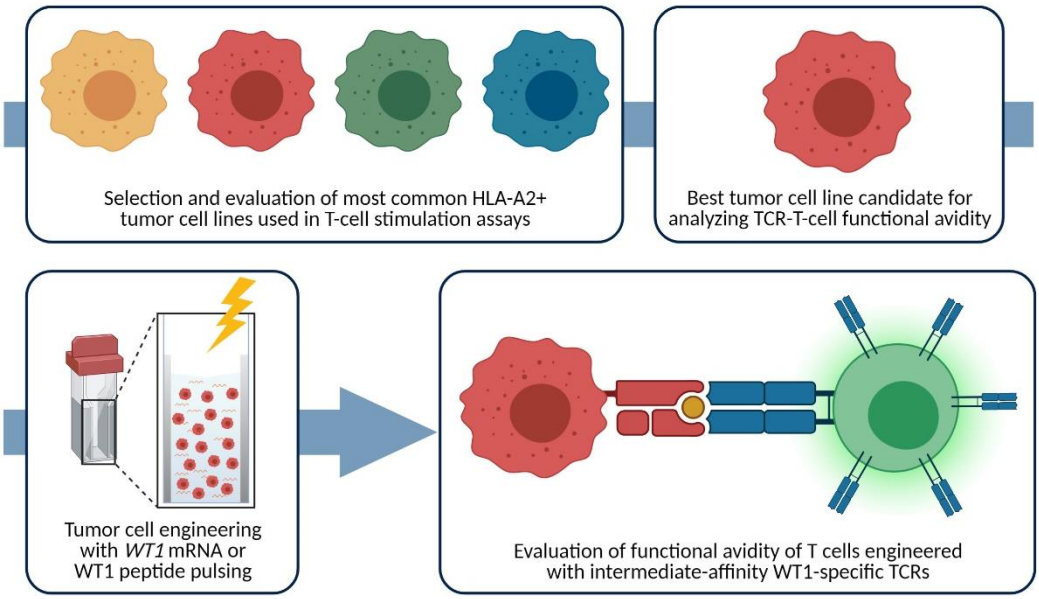

## Objective 2

Prevention of TCR mispairing between endogenous and introduced TCRαβ chains to increase the expression and functionality of the introduced TCR.



## Objective 3

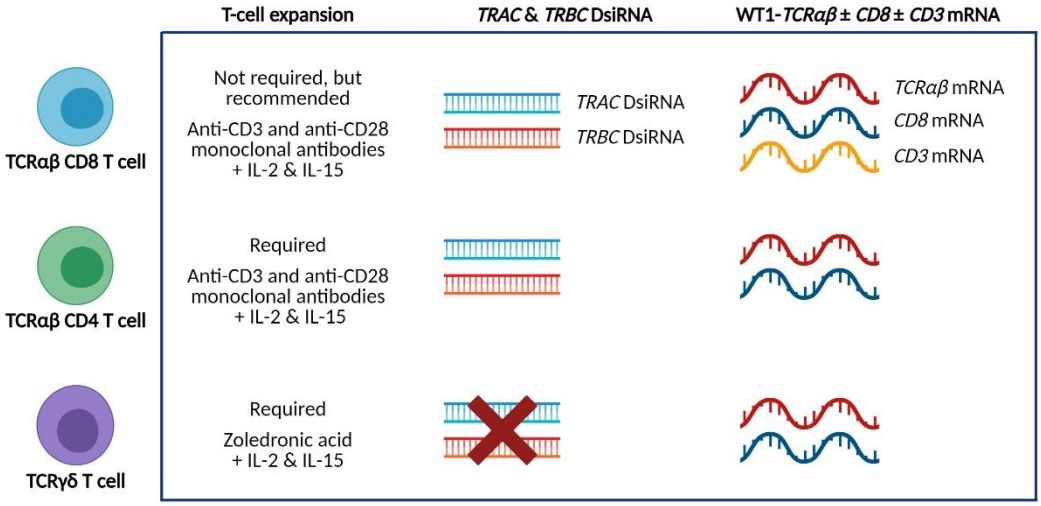
Evaluation of tumor cell lines as antigen-presenting cells (APC) in the context of TCR-T-cell avidity and development of a full-length *WT1* mRNA tumor APC model.




## Objective 4

**a)** TCR-engineering of conventional and unconventional T cells.

**b)** Improvement of functional avidity of T cells engineered with low-affinity WT1-specific TCRs.



**When in doubt, go to the library.**

— Ron Weasley (*Harry Potter and the Chamber of Secrets*, J.K. Rowling)

# 1

# Trial watch: Adoptive TCR-engineered T-cell immunotherapy for acute myeloid leukemia

This chapter has been published in:

Campillo-Davo D, Anguille S, Lion E. *Cancers* (2021);13(18):4519.

# Abstract

Despite the advent of novel therapies, acute myeloid leukemia (AML) remains associated with a grim prognosis. This is exemplified by 5-year overall survival rates not exceeding 30%. Even with frontline high-intensity chemotherapy regimens and allogeneic hematopoietic stem cell transplantation, the majority of patients with AML will relapse. For these patients, treatment options are few, and novel therapies are urgently needed. Adoptive T-cell therapies represent an attractive therapeutic avenue due to the intrinsic ability of T lymphocytes to recognize tumor cells with high specificity and efficiency. In particular, T-cell therapies focused on introducing T-cell receptors (TCRs) against tumor antigens have achieved objective clinical responses in solid tumors such as synovial sarcoma and melanoma. However, contrary to chimeric antigen receptor (CAR)-T cells with groundbreaking results in B-cell malignancies, the use of TCR-T cells for hematological malignancies is still in its infancy. In this review, we provide an overview of the status and clinical advances in adoptive TCR-T-cell therapy for the treatment of AML.

# Introduction

Acute myeloid leukemia (AML) is a type of blood cancer that carries a grim prognosis, despite considerable therapeutic advances in the last decade. Current treatment of newly diagnosed AML generally consists of intensive chemotherapy (IC) followed by allogeneic hematopoietic stem cell transplantation (allo-HSCT) in younger and fit patients, and low-intensity chemotherapy (e.g., hypomethylating agents) combined with—depending on the availability of the drug—the oral BCL-2 inhibitor venetoclax in older and/or less fit patients. Allo-HSCT, which is considered an immunotherapeutic strategy, since part of its mode of action involves the administration of T-lymphocytes that can recognize and eliminate the leukemic cells, is the gold standard post-remission treatment in AML. As discussed above, it is generally reserved for younger patients. Only a small population of elderly AML patients actually receives allo-HSCT [1]. Nevertheless, even with allo-HSCT, the majority of AML patients will relapse, explaining the poor 5-year overall survival rate of only 30% [2]. This explains why there is still a high unmet need to treat relapsed (or refractory) disease or to prevent relapse by strategies aimed at eradication of measurable residual disease (MRD; known before as minimal residual disease), which is the primary cause of relapse [3,4].

It is within this context that immunotherapy comes to the fore. So far, most clinical trials with immunotherapies in AML have focused on monoclonal antibodies or checkpoint inhibitors. Cell-based immunotherapies for AML, except for allo-HSCT, are still experimental and have not yet surpassed the clinical trial stage [5]. Among the cell therapies that are currently being studied in AML are dendritic cells (DC) loaded with leukemia-associated antigens to stimulate anti-leukemia (T-cell) immunity; DC "vaccination" has shown promising results, especially as maintenance therapy in a low-disease burden setting [6]. Still in their infancy but promising are clinical trials that explore adoptive cell therapy (ACT) with leukemia-reactive T lymphocytes. AML cells are known to express a broad range of tumor antigens, including—but not limited to—overexpressed leukemia-associated antigens (LAAs), leukemia-specific antigens (LSAs) or neoantigens and cancer-testis antigens (reviewed by [7,8]). Target antigens are either molecules expressed on the surface ("extracellular") or internally processed ("intracellular") proteins that are present on the AML surface in the form of peptides bound to human leukocyte antigen (HLA)/major histocompatibility complex (MHC) molecules, which can be recognized by T cells via their T-cell receptor (TCR) (**Figure 1**). Surface ("extracellular") antigens are targeted by chimeric antigen receptor (CAR) T-cell therapy. This form of therapy, which involves the adoptive transfer of lymphocytes genetically modified to express a CAR, has become an established treatment option in

different hematological malignancies [9–11]. CAR-T-cell therapy has also been aimed at treating AML (reviewed by [12–14]). The first clinical report of CAR-T-cell therapy in AML dates back to 2013; in that study, a second-generation CAR against Lewis Y antigen showed limited clinical efficacy, but showed T-cell biological activity in terms of trafficking to the BM and in vivo persistence without hematopoietic toxicity [15]. Other target antigens in anti-AML CAR T-cell therapy include CD33, CD123, and C-type lectin-like molecule-1 (CLL-1) [16]. Unfortunately, most of the "extracellular" target antigens in AML are also expressed on normal hematopoietic cells, posing an important barrier to the applicability of CAR-T-cell therapies in AML.

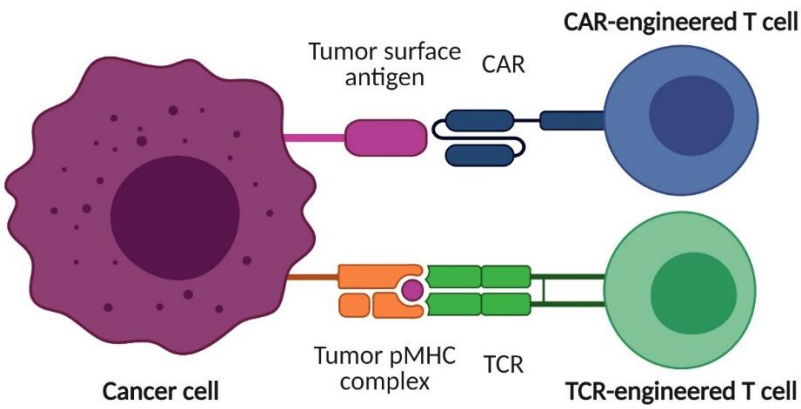



**Figure 1. Chimeric antigen receptor (CAR) and T-cell receptor (TCR) engineered T cells.** CAR-T cells usually target surface antigens, whereas TCR-T cells recognize internally processed proteins presented by molecules of the major histocompatibility complex (MHC) as peptide-MHC (pMHC) complexes. Each TCR is specific for a pMHC, allowing an infinite set of pMHC combinations that can be exploited for TCR-T-cell therapy. Created with BioRender.com.

Although intracellular antigens can also be targeted by CARs using antibodies recognizing peptide-MHC (pMHC) complexes [17], traditionally, this has been the role of TCRs. Thus, in TCR-T-cell therapies, and similar to CAR-T-cell therapies, lymphocytes for adoptive transfer are genetically engineered with tumor antigen-specific TCRs to redirect their specificity towards pMHC complexes (reviewed by [18] in solid tumors and by [19,20] in hematological malignancies). Conventional αβ T cells are the most frequent TCR-engineered cell type. More recently, other lymphocytes, such as γδ T cells, have garnered attention due to their excellent natural antitumor properties that can be exploited as cellular immunotherapy [21]. γδ T cells are a subset of unconventional T cells that express γδTCRs instead of αβTCRs and compose up to 10% of peripheral T cells [22]. Despite the low frequency in peripheral blood, these cells can be easily expanded ex vivo [23]. Given the numerous intracellular antigens identified

in AML and the presence of leukemia-associated surface antigens in healthy cells [7,8], AML might be more suited for TCR-T-cell therapies. Moreover, compared to CARs, TCRs also require lower epitope densities to function [24]. Although there are certain drawbacks to the use of TCR-T cells, including their HLA restriction, which limits their broad applicability, TCR-based T-cell therapies have produced encouraging results, especially in patients with melanoma and certain other solid tumors [24]. In this review, we summarize the status and clinical advances in the use of TCR-engineered T cells for the treatment of AML.

# Target antigens in TCR-T-cell therapies for AML

Cancer immunotherapies using TCR-T cells require TCRs that recognize tumor antigens in a specific manner [25]. Currently, Wilms' tumor 1 (WT1), preferentially expressed antigen in melanoma (PRAME), and minor histocompatibility antigens (MiHA) have been the only AML antigens targeted using TCR-T cells in a clinical setting.

## WT1

WT1 is a zinc-finger translation factor that is overexpressed in AML and other hematological and solid malignancies [26]. It is mainly found in the cytoplasm of tumor cells [27,28], whereas in leukemic cells, it is more frequently found in the nuclei [27,29], although it can shuttle between the nucleus and cytoplasm [30]. In AML, WT1 overexpression occurs in 73–93% of patients at diagnosis (reviewed by [31]). Although other markers and techniques are preferred for MRD measurement (reviewed by [3,4]), *WT1* messenger RNA (mRNA) levels can be used as a marker for MRD and a prognostic factor for relapse in AML if no other markers are available [31]. Somatic *WT1* mutations occur in approximately 6–15% of AML patients at diagnosis (reviewed by [32]) and are more frequent at a younger age and in cytogenetically normal AML (CN-AML) patients [31,32], which account for 40–50% of AML patients [33,34]. *WT1* aberrations usually occur in exons 1, 7, and 9, primarily creating premature stop codons and frameshift mutations affecting the reading frame [32]. These mutations appear to confer a negative prognostic outcome by increasing the risk of relapse and death.

## PRAME

PRAME is a repressor of the retinoic acid receptor [35]. Similar to WT1, PRAME is also overexpressed in different cancer types, including AML [36]. Approximately 30–87% of patients at diagnosis overexpress *PRAME* mRNA [36–38], and, as with WT1, it could be

used as a surrogate marker of MRD in AML [37]. Combined detection of WT1 and PRAME has been suggested to be a sensitive molecular biomarker for monitoring MRD in AML [39]. CD8 T-cell responses against WT1 and PRAME are detected in healthy individuals and AML patients [40–43]. More importantly, multi-epitope WT1-specific and PRAME-specific T-cell reactivities have also been confirmed in clinical studies after vaccination with WT1-loaded DC [44] or PRAME peptide vaccination [45].

### MiHA

MiHA antigens are polymorphic peptides presented by HLA molecules, resulting from the degradation of proteins from polymorphic genes with single-nucleotide polymorphisms, base-pair insertions or deletions, or copy number variations [46]. In cancer therapy, allogeneic T cells from an HLA-matched donor—administered in the setting of allo-HSCT or donor lymphocyte infusions—can recognize mismatched MiHA in the recipient patient [46]. When this recognition occurs against MiHA expressed by the leukemic cells, donor T cells induce graft-versus-leukemia (GVL) processes; however, if the polymorphic peptide is also expressed in normal cells, undesirable graft-versus-host disease (GVHD) can also occur [46]. In hematological malignancies, donor T cells from donor lymphocyte infusions targeting the ubiquitously expressed MiHA peptides can induce complete remissions by GVL, albeit frequently accompanied by concurrent GVHD [47]. In contrast, T cells recognizing hematopoiesis-restricted MiHA peptides, i.e., only expressed in cells of hematopoietic origin, such as HA-1 and HA-2, can lead to complete remissions in leukemia patients that relapsed after receiving allo-HSCT by inducing GVL without severe GVHD [47]. This nonameric peptide has two allelic variants codified on chromosome 9, HA-1H and HA-1R, which vary in one single amino acid [48]. Both variants are able to bind HLA-A*02:01 molecules; however, only HA-1H can effectively be expressed on the cell membrane by HLA-A*02:01 and induce T-cell responses in HA-1 R/R homozygous individuals [48,49]. HA-1H variant, which is present in 30% of the population [49], can also be presented by HLA-A*02:06 [50].

## Characteristics and results of clinical trials using AML-directed TCR-T cells

Currently, there are 12 early phase clinical trials with TCR-T cells against relapsed/refractory (R/R) AML, focusing on investigating the feasibility and safety of the therapy (**Tables 1–4**). So far, 38 AML patients have been treated out of 57 hematological cancer patients recruited, and approximately 250 patients are intended to be recruited in total (status of trials listed in **Table 1**). Most of these clinical trials

genetically engineer conventional autologous T cells with αβ TCRs to target WT1, PRAME, and MiHA in the context of HLA-A*02:01 restriction, which is expressed in approximately 50% of the European population (characteristics of T-cell products are listed in **Table 2**).

Five studies have focused on targeting WT1, of which three have been recently completed. Two of these three completed clinical trials (clinicaltrials.gov identifier: NCT01621724, NCT02550535) used escalating doses of HLA-A*02:01-restricted WT1-specific TCR-T cells (patient characteristics and treatment regimens are listed in **Table 3**) [51–53]. In both cases, single doses of either $2 \times 10^7$ TCR-T cells/kg or $1 \times 10^8$ T cells/kg were administered per cohort, accompanied by an injection of interleukin (IL)-2. In NCT01621724, WT1-specific T cells persisted one year after infusion in four out of a total of seven patients with AML and chronic myeloid leukemia (CML) (outcomes of clinical trials using TCR-T cells for AML are listed in **Table 4**) [51]. Complete responses were also observed in four out of seven patients; however, it is unclear whether those with complete responses presented persistent WT1-specific T cells in peripheral blood. In NCT02550535, a total of six AML patients, three patients with myelodysplastic syndrome (MDS), and a patient with tyrosine kinase inhibitor-resistant CML were treated with a $WT1_{126\text{-}134}$-specific TCR-T-cell product [52,53]. AML patients in complete morphological remission before treatment were administered a single dose of TCR-T cells accompanied with IL-2. TCR-T cells proliferated in vivo and were detectable 28 days following infusion in all patients. Median overall survival (OS) of AML patients following treatment with TCR-T cells was 12 months. It remains unclear whether patients with the highest median OS received the highest dose in the trial or not. In both studies, TCR-T-cell products were generally well tolerated. No adverse effects were observed, except a case of febrile neutropenia and cytokine release syndrome that was successfully treated.

While the HLA-A*02:01 allele is one the most frequent HLA class I types in European and North American populations, HLA-A*24:02 is the most common HLA-A allele in Japan. Therefore, the third completed phase I dose-escalation trial conducted in Japanese individuals focused on administering two rounds of an HLA-A*24:02-restricted $WT1_{235\text{-}243}$ peptide-specific TCR to HLA-A*24:02-positive patients, followed by WT1 peptide vaccination (umin.ac.jp Identifier: UMIN000011519; [54]). In these three completed studies, TCR-T-cell treatment was well tolerated, with no dose-limiting toxicities related to treatment; however, one instance of cytokine release syndrome was reported, possibly related to treatment (**Table 4**). TCR-T-cell treatment was well tolerated, with seven out of eight patients showing no or only grade 1 adverse events.

Observed adverse events were mostly related to peptide vaccination at the site of injection. WT1-specific TCR-T cells were successfully detected in the peripheral blood of eight treated patients. More importantly, TCR-T cells were still detectable 8 weeks post-administration in five of the eight treated patients. The number of circulating TCR-T cells positively correlated with the number of cells administered, especially during the first 14 days after treatment. WT1-specific TCR-T cells from one patient responded to $WT1_{235-243}$ peptide, indicating that TCR-T-cell antitumor activity was still intact despite decreasing circulating T-cell numbers. A transient decrease in peripheral blood or BM blasts was observed in three cases after treatment, whereas the disease progressed in four patients. Median OS was 15.9 months, with four out of five patients with persistent T cells surviving longer than 12 months, compared to only one out of three patients without detectable engineered T cells surviving beyond 12 months. Further studies including more patients would be needed to completely ascertain the hematologic benefit of these WT1-specific TCR-T cells, especially since T cells were engineered with a TCR with a physiological affinity. It is also important to note that patients with decreasing blast numbers in BM after treatment were those with a higher percentage of WT1-specific TCR-T cells in the T-cell product. Therefore, achieving sufficient TCR-engineered T cells in the medicinal product is crucial in this type of therapy. Two other studies focusing on WT1 are still ongoing in the United States of America (USA; **Table 1**; NCT01640301 and NCT02770820) [55,56]. Both are centered on high-risk AML and use the same HLA-A2-restricted T-cell product called WT1-$T_{TCR-C4}$ (**Table 2**), followed by subcutaneous injection of IL-2 (**Table 3**). Preliminary results from 12 treated patients (NCT01640301) indicate that treatment was generally well tolerated (**Table 4**) [55]. GVHD was observed in some patients, including a case of grade 3 acute GVHD; however, since the onset of GVHD events occurred at a median of 123 days after infusion, GVHD was likely not caused by the T-cell product. TCR-T cells persisted in 75% of patients until day 28 post-administration and were detected after 12 months in 33% of patients. More importantly, clinical efficacy following WT1-specific TCR-T-cell treatment was demonstrated by a relapse-free survival (RFS) of 100% at a median of 44 months, which was significantly higher than a comparable group of high-risk AML patients that did not receive TCR-T-cell therapy [55]. Results from trial number NCT02770820 regarding the persistence of T cells and disease response are currently not available. Preliminary data on adverse events indicate that TCR-T cells were well tolerated, with no severe adverse effects (**Table 4**) [56]. Only four out of seven patients completed treatment. One patient died during treatment; however, due to the absence of data on the cause of death, it is difficult to determine whether it was related to treatment or not.

With regard to PRAME-specific TCR-T cells, two different trials are ongoing for relapsed AML in the context of HLA-A2 restriction (**Table 1**). Both NCT02743611 and EudraCT-2017-000440-18 trials are analyzing increasing doses of autologous TCR-T-cell products (BPX-701 and MDG1011, respectively) up to $5 \times 10^6$ T cells/kg (**Tables 2 and 3**) [57]. In addition, in a third clinical study (EudraCT-2018-000717-20), patients enrolled in trial EudraCT-2017-000440-18 will be followed up for up to 14 years. Results from these clinical trials are pending and will provide the field with information about the future of PRAME-specific TCR-T-cell therapies in AML.

Two studies carried out in the Netherlands and one in the USA have targeted MiHA HA-1H peptide following allo-HSCT (**Table 1**). In the completed study (EudraCT-2010-024625-20), donor-derived Epstein-Barr virus (EBV) and/or cytomegalovirus (CMV)-specific T cells were engineered with an HLA-A*02:01-restricted MiHA HA-1H peptide-specific TCR [49,58], which contained an additional disulfide bond to improve TCR pairing and stability, in which the sequence was codon optimized [59]. Donor-derived EBV- or CMV-specific T cells can be safely used for adoptive transfer because (i) the reactivity of these T cells is known, (ii) these T cells help to prevent EBV and CMV infections, and (iii) they do not induce GVHD (**Figure 2**) [55,58,60]. A drawback of this strategy is that patients may not be seropositive for EBV and CMV. Even if they are, sufficient T cells for TCR-T-cell development may not be available when using autologous EBV- or CMV-specific T cells. As illustrated in EudraCT-2010-024625-20, in nine recruited patients, only two were EBV and CMV seropositive [49]. Moreover, although seven donors were EBV seropositive, HA-1H TCR-transduced CMV or EBV-specific T cells could be produced in five individuals [49]. Four out of five treated patients received two administrations of engineered T cells. TCR-transduced donor-derived EBV-specific T cells were well tolerated, with no toxicities related to treatment nor GVHD [49]. A study conducted in the Netherlands is currently investigating this strategy (trialregister.nl identifier: NTR6541). The T-cell product called TEG001 is based on retroviral transduction of a high-affinity Vγ9Vδ2 TCR derived from the natural repertoire of a healthy individual [61,62]. Although there are no clinical results available, a preclinical in vivo evaluation of TEG001 demonstrated that TEG001 eradicated primary AML blasts [63]. More importantly, after administration, TEG001 persisted up to 50 days in mice but did not target human cord blood-derived healthy hematopoietic cells.

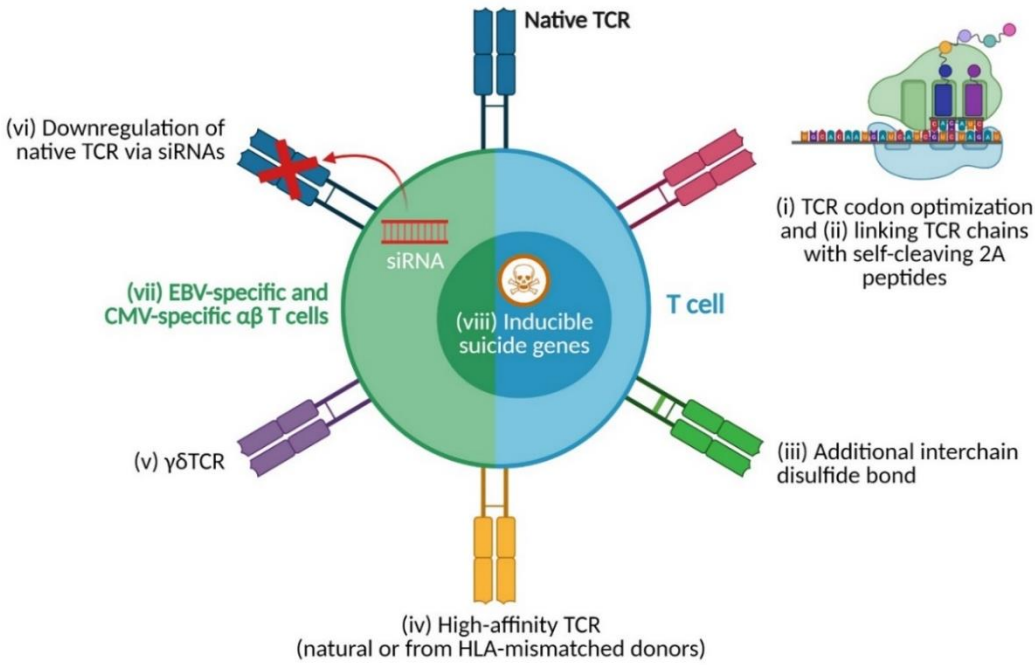



**Figure 2. TCR-directed optimization strategies that have been clinically tested in AML.** Different strategies to enhance TCR-T-cell therapies have been used in clinical trials for the treatment of AML. These include (i) codon optimization of transgenic TCR sequences to improve protein translation; (ii) linking transgenic TCR alpha and beta chains via self-cleaving 2A peptides for equimolar expression of TCR chains; (iii) addition of cysteine residues that bind covalently to provide a second interchain disulfide bond and improve pairing of transgenic TCR chains; (iv) usage of high-affinity TCRs, either derived from normal TCR repertoires after screening of multiple TCR candidates or from HLA-mismatched donors to generate allo-restricted TCRs, to enhance antigen recognition; (v) TCR-engineering with γδTCRs derived from γδ T cell clones, or (vi) downregulation of native TCR chains via introduction of small interfering "silencing" RNAs (siRNAs) targeting native TCR transcripts to prevent TCR mispairing; (vii) usage of autologous donor-derived Epstein–Barr virus (EBV) and cytomegalovirus (CMV)-specific T cells to prevent EBV and CMV infections and graft-versus-host disease in TCR-T-cell treated patients; and (viii) introduction of inducible suicide genes that would allow the elimination of TCR-engineered T cells in case of treatment-related toxicities. Created with BioRender.com.

**Table 1.** Clinical trials using TCR-T cells for AML

| Clinical trial identifier | Location | Status (First posted) | Phase | AML status | Prior treatment | AML patients recruited or treated (intended) | Reference |
|---|---|---|---|---|---|---|---|
| **WT1-specific** | | | | | | | |
| NCT01621724 EudraCT-2006-004950-25 | UK | Completed (2012, completed in 2018) | I/II | AML | n.d. | 7 treated (18) | [51] |
| NCT02550535 EudraCT-2014-003111-10 | Belgium Germany UK | Completed (2015, completed in 2018) | I/II | Relapsed/ stable AML | HAT | 10 pt. treated: 6 AML, 3 MDS and 1 TKI- resistant CML (25-30) | [52,53] |
| UMIN000011519 | Japan | Completed (2013, completed in 2018) | I | R/R AML | n.d. | 12 recruited, 8 treated | [54] |
| NCT01640301 | USA | Active, not recruiting (2012) | I/II | High-risk/ relapsed AML | allo-HSCT | 12 treated (45) | [55] |
| NCT02770820 | USA | Active, not recruiting (2016) | I/II | High-risk non-M3 AML | Consolidation chemotherapy | 7 treated (9) | [56] |
| **PRAME-specific** | | | | | | | |
| NCT02743611 | USA | Active, not recruiting (2016) | I/II | Relapsed AML | n.d. | (28) | [57] |
| NCT03503968 EudraCT-2017-000440-18 | Germany | Recruiting (2018) | I/II | R/R AML | HAT and/or allo-HSCT | (92) | n.d. |
| EudraCT-2018-000717-20 | Germany | Ongoing (2019) | Long-term follow-up of phase I | R/R AML | HAT and/or allo-HSCT | (52) | n.d. |
| **MiHA HA-1H-specific** | | | | | | | |
| EudraCT-2010-024625-20 NTR3454 / NL3307 | Netherlands | Completed - (2012, prematurely ended in 2018) | I | High-risk AML | allo-HSCT | 9 recruited, 5 treated (20) | [49,58] |
| NCT04464889 EudraCT-2019-002346-20 | Netherlands | Active, not recruiting (2020) | I | R/R AML | allo-HSCT | (29) | n.d. |
| NCT03326921 | USA | Recruiting (2017) | I | Recurrent AML | allo-HSCT | (24) | n.d. |
| **Other** | | | | | | | |
| NTR6541 / NL6357 | Netherlands | Recruiting (2017) | I | R/R AML | n.d. | (18) | [61-63] |

**Abbreviations:** allo-HSCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; HAT, hypomethylating agent therapy; MDS, myelodysplastic syndrome; MiHA, minor histocompatibility antigen; n.d., no data; PRAME, preferentially expressed antigen in melanoma; R/R, relapsed/refractory; TKI, tyrosine kinase inhibitors; UK, United Kingdom; USA, United States of America; WT1, Wilms' tumor 1. Last search on 25 July 2021.

**Table 2.** Characteristics of T-cell products

| Clinical trial identifier | Name of T-cell product | TCR | | T-cell population | Reference |
|---|---|---|---|---|---|
| | | Restriction | High affinity/avidity | | |
| **WT1-specific** | | | | | |
| NCT01621724<br>EudraCT-2006-004950-25 | WT1 TCR-001 | HLA-A2 | n.d. | Autologous T cells | [51] |
| NCT02550535<br>EudraCT-2014-003111-10 | n.d. | HLA-A2 | n.d. (allo-restricted TCR) | Autologous T cells | [52,53] |
| UMIN000011519 | n.d. | HLA-A24 | No | Autologous T cells | [54] |
| NCT01640301 | WT1-$T_{TCR-C4}$ | HLA-A2 | Yes (from healthy individual) | Donor-derived EBV-specific CD8 T cells | [55] |
| NCT02770820 | WT1-$T_{TCR-C4}$ | HLA-A2 | Yes (from healthy individual) | Autologous central memory/naïve CD8 T cells EBV-specific T cells | [56] |
| **PRAME-specific** | | | | | |
| NCT02743611 | BPX-701 | HLA-A2 | Yes (allo-restricted donor) | Autologous T cells | [57] |
| NCT03503968<br>EudraCT-2017-000440-18 | MDG1011 | HLA-A2 | n.d. | Autologous T cells | n.d. |
| EudraCT-2018-000717-20 | MDG1011 | HLA-A2 | n.d. | Autologous T cells | n.d. |
| **MiHA HA-1H-specific** | | | | | |
| EudraCT-2010-024625-20<br>NTR3454 / NL3307 | n.d. | HLA-A2 | n.d. | Autologous donor-derived CMV- and/or EBV-specific T cells | [49,58] |
| NCT04464889<br>EudraCT-2019-002346-20 | MDG1021 | HLA-A2 | n.d. | Autologous T cells | n.d. |
| NCT03326921 | n.d. | HLA-A2 | n.d. | CD4 and CD8 memory donor T cells | n.d. |
| **Other** | | | | | |
| NTR6541 / NL6357 | TEG001 | n.a. (Vγ9Vδ2 TCR) | Yes | Autologous T cells | [61-63] |

**Abbreviations:** CMV, cytomegalovirus; EBV, Epstein-Barr virus; HA-1H, HLA-A*0201-restricted minor histocompatibility antigen 1 peptide variant H; HLA, human leukocyte antigen; HLA-A2, HLA-A*02:01; HLA-A24, HLA-A*24:02; MiHA, minor histocompatibility antigen; n.a., not applicable; n.d., no data; PRAME, preferentially expressed antigen in melanoma; TCR, T-cell receptor; WT1, Wilms' tumor 1. Last search on 25 July 2021.

**Table 3.** Patient characteristics and treatment regimens

| Clinical trial identifier | Age of patients | No. patients per arm or cohort | Dosage per cohort | Additional treatments | Reference |
|---|---|---|---|---|---|
| **WT1-specific** | | | | | |
| NCT01621724<br>EudraCT-2006-004950-25 | 1 pt. 18-64 years<br>6 pt. ≥65 years | Cohort 1: 3 pt.<br>Cohort 2: 4 pt. | Cohort 1: ≤2x10$^7$ T cells/kg<br>Cohort 2: ≤1x10$^8$ T cells/kg | Standard conditioning;<br>10$^6$ units/m$^2$ IL-2 | [51] |
| NCT02550535<br>EudraCT-2014-003111-10 | n.d. | Cohort 1: 7 pt.<br>Cohort 2: 3 pt..<br>(6 AML, 3 MDS and 1 TKI- resistant CML in total) | Cohort 1: ≤2x10$^7$ T cells/kg<br>Cohort 2: ≤1x10$^8$ T cells /kg | Subcutaneous low-dose injections of IL-2 (1x10$^6$ units/m$^2$) | [52,53] |
| UMIN000011519 | 1 pt. 18-64 years<br>7 pt. ≥65 years | Cohort 1: 3 pt. (1 AML and 2 MDS)<br>Cohort 2: 3 pt. (MDS)<br>(+2 pt. extracohort; 1 AML and 1 MDS)<br>Cohort 3: 0 pt. | Cohort 1: two doses of 2x10$^8$ cells<br>Cohort 2: two doses of 1x10$^9$ cells<br>Cohort 3: two doses of 5x10$^9$ cells<br>Cells administered at day 0 and 28 | Subcutaneous injection of 300 µg mutated WT1$_{235-243}$ peptide at day 30 and 44 | [54] |
| NCT01640301 | 8 pt 18-64 years<br>4 pt. ≥65 years | Treatment arm: 12 pt.<br>Prophylactic arm: 12 pt. | 12/12 pt.; one dose of 10$^{10}$ T cells/m$^2$<br>7/12 pt.; second dose of 10$^{10}$ T cells/m$^2$<br>(administered if frequency of TCR-T cells was <3% of total peripheral CD8+ T cells) | Subcutaneous low-dose injection of IL-2 | [55] |
| NCT02770820 | 4 pt. 18-64 years<br>3 pt. ≥65 years | Cohort 1: 7 pt. (4/7 pt. completed treatment) | Cohort 1: Two doses (day 0 and day >21) | Subcutaneous injection of IL-2 | [56] |
| **PRAME-specific** | | | | | |
| NCT02743611 | n.d. | n.d. | Escalating doses from 1.25x10$^6$ T cells/kg up to 5x10$^6$ T cells/kg to be explored | Rimiducid (in response to treatment-related toxicity) | [57] |
| NCT03503968<br>EudraCT-2017-000440-18 | n.d. | n.d. | Cohort 1: target dose of 1x10$^5$ T cells/kg<br>Cohort 2: target dose of 1x10$^6$ T cells/kg<br>Cohort 3: target dose of 5x10$^6$ T cells/kg<br>Optional cohort 4: up to 1x10$^7$ T cells/kg | n.d. | n.d. |
| EudraCT-2018-000717-20 | n.d. | n.d. | Patients that were treated with MDG1011 TCR-T-cell product in EudraCT-2017-000440-18 trial | n.d. | n.d. |
| **MiHA HA-1H-specific** | | | | | |
| EudraCT-2010-024625-20<br>NTR3454 / NL3307 | 4 pt. 18-64 years<br>1 pt. ≥65 years | Cohort 1: 5 pt. (4 AML and 1 B-LBL) | Cohort 1: two doses of ≥3x10$^6$ T cells<br>(day 8 and 14 after allo-HSCT) | n.d. | [49,58] |
| NCT04464889<br>EudraCT-2019-002346-20 | n.d. | n.d. | Cohort 1: target dose of 0.3x10$^6$ T cells/kg<br>Cohort 2: target dose of 1x10$^6$ T cells/kg<br>Cohort 3: target dose of 3x10$^6$ T cells/kg | n.d. | n.d. |
| NCT03326921 | n.d. | n.d. | n.d. | n.d. | n.d. |
| **Other** | | | | | |
| NTR6541 / NL6357 | n.d. | n.d. | n.d. | n.d. | [61-63] |

**Abbreviations:** allo-HSCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; B-LBL, B-cell lymphoblastic leukemia; CML, chronic myeloid leukemia; HA-1H, HLA-A*0201-restricted minor histocompatibility antigen 1 peptide variant H; IL-2, interleukin 2; MDS, myelodysplastic syndrome; MiHA, minor histocompatibility antigen; n.d., no data; PRAME, preferentially expressed antigen in melanoma; TKI, tyrosine kinase inhibitors; WT1, Wilms' tumor 1. Last search on 25 July 2021.

**Table 4.** Outcomes of clinical trials using TCR-T cells for AML

| Clinical trial identifier | Treatment-related toxicities (Grade 1-2) | Treatment-related serious adverse events (Grade 3-4) | Persistence of T cells | Disease response | Reference |
|---|---|---|---|---|---|
| **WT1-specific** | | | | | |
| NCT01621724<br>EudraCT-2006-004950-25 | No dose-limiting toxicity | Cohort 1: febrile neutropenia (1/3 pt.) | Cohort 1: 2/3 pt. at day 365<br>Cohort 2: 2/4 pt. at day 365 | Cohort 1: CR (1/3 pt.); no response (2/3 pt.)<br>Cohort 2: CR (3/4 pt. .); no response (1/4 pt.) | [51] |
| NCT02550535<br>EudraCT-2014-003111-10 | No dose-limiting toxicity | Possibly treatment-related cytokine release syndrome (1/10 pt.) | 10/10 pt. at day 28<br>7/10 pt. at day 29-365 | 6 AML pt.: median survival of 12 months | [52,53] |
| UMIN000011519 | No dose-limiting toxicity; Facial edema, dermatitis, fever, phlebitis, arrhythmia, stomatitis (1/8 pt.); Skin reaction at peptide injection site (7/8 pt.) | None | Cohort 1: 2/3 pt. at day 58<br>Cohort 2: 3/5 pt. at day 58 | Decrease of abnormal erythroblasts in PB (1/8 pt.); Decrease of blasts in BM (2/8 pt.); Stable disease (1/8 pt.); Progressive disease (4/8 pt.) | [54] |
| NCT01640301 | None disclosed | Cytokine release syndrome (2/12 pt.)<br>Lymphopenia (12/12 pt.)<br>Trombocythopenia (2/12 pt.)<br>Neutropenia (2/12 pt.)<br>Anemia (7/12 pt.) | 9/12 pt. at day 28<br>4/12 pt. at day >365 | No evidence of disease (AML recurrence) at median follow-up of 44 months (12/12 pt.) | [55] |
| NCT02770820 | Not disclosed if treatment related: Fatigue, alanine aminotransferase increased, hyperglycemia (1/6 pt.); Anemia, thrombocytopenia (2/6 pt.); Neutropenia, leukopenia (3/6 pt.); Hypertension (4/6 pt.); Lymphopenia (5/7 pt.) | Not disclosed if treatment related: Death (1/6 pt.) | n.d. | n.d. | [56] |
| **PRAME-specific** | | | | | |
| NCT02743611 | No results available yet | | | | [57] |
| NCT03503968<br>EudraCT-2017-000440-18 | No results available yet | | | | n.d. |
| EudraCT-2018-000717-20 | No results available yet | | | | n.d. |
| **MiHA HA-1H-specific** | | | | | |
| EudraCT-2010-024625-20<br>NTR3454 / NL3307 | None | None | 3/5 pt. at day 14 after second infusion | Relapsed AML prior to infusion leading to death (1/5 pt.; 1/4 AML pt.); Infections during follow-up leading to death (2/5 pt.; ¼ AML pt.); No AML relapse and alive (2/4 pt.) | [49,58] |
| NCT04464889<br>EudraCT-2019-002346-20 | No results available yet | | | | n.d. |
| NCT03326921 | No results available yet | | | | n.d. |
| **Other** | | | | | |
| NTR6541 / NL6357 | No results available yet | | | | [61-63] |

**Abbreviations:** AML, acute myeloid leukemia; BM, bone marrow; CR, complete response; HA-1H, HLA-A*0201-restricted minor histocompatibility antigen 1 peptide variant H; MiHA, minor histocompatibility antigen; n.d., no data; PB, peripheral blood; PRAME, preferentially expressed antigen in melanoma; TCR, T-cell receptor; WT1, Wilms' tumor 1. Last search on 25 July 2021.

# Strategies for enhancing TCR-T-cell products

Some of the abovementioned clinical trials have used different strategies to optimize antigen recognition, TCR expression, and mechanisms to address potential safety concerns (**Figure 2**). Autologous T cells have been transduced with a codon-optimized $WT1_{126\text{-}134}$-specific TCR construct (NCT02550535) [52,53]. Codon optimization is a technique in which synonymous codons replace codons in coding sequences to improve protein translation rates and enhance protein expression. However, growing evidence indicates that, despite unaltering coding sequences, codon optimization may alter how proteins fold, thus impacting post-transcriptional modifications and protein functionality (reviewed by [64]). In the same study, TCR alpha and beta sequences were linked via a self-cleaving 2A peptide derived from porcine teschovirus-1 [52,53]. Self-cleaving peptides, such as those from the foot-and-mouth disease virus or the abovementioned porcine teschovirus-1, allow the expression of multiple proteins using the same open reading frame [65]. On the one hand, this facilitates cell engineering, as only one vector has to be produced instead of one per sequence. On the other hand, it ensures stoichiometric independent expression of each protein. Nonetheless, careful design of sequences within the vector, including the selection of 2A peptides, the inclusion of additional spacers or furin sequences and the position of the sequences of genes of interest within the open reading frame, is key for correct protein production and function [66,67]. In the case of TCR expression, it has been observed that placing TCR alpha sequences downstream of 2A peptide sequences is preferred [68]. Transgenic TCR expression was further improved in NCT02550535 by an additional disulfide bond between TCR alpha and beta chains [52,53]. This extra bond located within the extracellular domain of the TCR constant regions induces correct pairing of transgenic TCR chains and, therefore, avoids TCR mispairing between native and transgenic TCRs while retaining transgenic TCR functionality [69]. These techniques have also been exploited in other clinical trials (EudraCT-2010-024625-20 [49,59]; UMIN000011519 [54,70]; NCT02743611 [57]).

TCR affinity plays an essential role in TCR-T-cell avidity, which in turn is critical for the efficacy and clinical benefit of TCR-T-cell products [25]. Compared to affinity-matured TCRs, which can lead to supraphysiological affinities and fatal cross-reactivities [71], natural high-affinity TCRs recognize self-antigens within physiological conditions. However, T-cell clones of high affinity against self-TAAs, such as WT1 and PRAME, are usually eliminated after negative selection in the thymus, reducing the number of tumor-reactive TCRs that could be used in TCR-T-cell therapies. Those that remain, normally of low or intermediate affinity, usually promote inferior T-cell responses to

physiological epitope densities, which would partly explain tumor immune escape [25]. Thus, high-affinity TCRs with better pMHC binding capabilities commonly generate better T-cell responses [25]. Techniques to enhance TCR affinity, e.g., artificial affinity maturation, have been developed to improve pMHC binding. TCR-T-cell trials for AML have capitalized on high-affinity HLA-A*02:01-restricted TCRs directed against WT1 (NCT01640301 and NCT02770820) [55,56] and PRAME (NCT02743611) [57] (**Figure 2**). High-affinity WT1-specific TCRs were obtained from an HLA-A*02:01-positive individual (NCT01640301 and NCT02770820) [55,56]. Isolation of high-affinity TCRs from (healthy) donor repertoires is a laborious process, and artificial maturation of TCR affinity can lead to deleterious cross-reactivities. Therefore, an alternative is to isolate high-affinity allo-restricted TCRs from HLA-mismatched donors (**Figure 2**) [72]. In the context of AML therapy, allo-restricted TCRs have been used to recognize HLA-A*02:01-restricted $WT1_{126\text{-}134}$ peptide (NCT02550535) [52,53] and HLA-A*02:01-restricted PRAME peptides (NCT02743611) [57]. In another study, instead of a high-affinity αβTCR, a high-affinity antigen-specific γδTCR was used (NTR6541) [61–63]. As mentioned previously, most studies focus on engineering conventional αβ T cells with αβTCRs. However, unconventional γδTCRs derived from γδ T cells are another source of tumor-specific TCRs (**Figure 2**). These γδTCRs are not HLA restricted and can be safely introduced in αβ T cells instead of classical αβTCRs, since, as opposed to αβTCR gene transfer in αβ T cells, mispairing between transgenic γδTCR chains and native αβTCR chains is unlikely due to preferential pairing [73,74]. With this approach, safety issues associated with mispaired αβ TCR combinations formed from transgenic and native TCRs in TCR-engineered αβ T cells are circumvented. Alternatively, native TCR expression can be eliminated using small interfering RNAs (siRNAs) against native TCR sequences (UMIN000011519; **Figure 2**) [54]. Short hairpin RNAs (shRNAs) were included in the vector containing the HLA-A*24:02-restricted $WT1_{235\text{-}243}$-specific TCR construct to silence the expression of native TCRs and prevent TCR mispairing between native and transgenic TCR chains [54]. Transgenic TCR downregulation was prevented by codon optimization of the TCR sequence. Currently, other techniques such as CRISPR-Cas9 are gaining momentum in TCR-T-cell therapies to completely disrupt native TCR expression or even replace native TCR sequences with transgenic TCR sequences [75–77]. Recently, results from a phase I trial using CRISPR-Cas9-mediated disruption of native TCR and programmed cell death 1 (PD-1) sequences in combination with transduction of a TCR targeting cancer-testis antigen New York esophageal squamous cell carcinoma 1 (NY-ESO-1) have shown that this technique is feasible and safe (NCT03399448) [78]. However, this form of native TCR disruption has yet to be investigated for AML.

Other clinical studies benefit from donor-derived virus-specific T cells for TCR transduction, such as EBV and CMV-specific T cells (**Figure 2**, NCT01640301, NCT02770820, and EudraCT-2010-024625-20) [49,55,56,58]. These T-cell subsets can be commonly isolated, reduce the possibility of graft-versus-host disease (GVHD) by an endogenous TCR, and are naturally enriched for central memory T cells with enhanced in vivo persistence [55]. However, to circumvent the issue of low numbers of CMV seropositive patients, only EBV-specific T cells were used in NCT01640301 and NCT02770820 trials [55,56]. In these two studies, EBV-specific T cells were transduced with a high-affinity WT1-specific TCR derived from HLA-A*02:01-positive healthy donor repertoires [55]. In all current TCR-T clinical trials for AML, T cells are genetically engineered via viral transduction. This technique enables the stable expression of introduced TCRs, which raises concerns about potential insertional mutagenesis and persistent adverse effects caused by the stably engineered TCR-T cells. Results from a recent report in which investigators characterized the genomic integration profile of TEG001 following retroviral transduction showed that this approach does not induce malignant transformation of engineered T cells [79]. However, to address potential safety issues, such as insertional mutagenesis and off-target and on-target off-tumor reactivities, safety mechanisms that can be induced in case of severe toxicities have been introduced in some TCR-T-cell clinical trials (NCT02743611, NCT03326921) [57]. These mechanisms are based on engineering T cells with suicide genes, such as inducible human caspase-9 (iC9; **Figure 2**). This protein is a key initiator of apoptosis and is activated after dimerization. Activation of iC9 can be induced following administration of rimiducid, a chemical compound that induces iC9 dimerization, thus inducing apoptosis in TCR-T cells. This system is not exclusive to TCR-T-cell therapies and can also be used in cases of haploidentical HSCT with alloreplete haploidentical T cells for AML treatment [80]. In this setting, administered haploidentical T cells promote immune reconstitution in patients, while the iC9 system can eliminate the administered T cells in the case of GVHD [80]. Alternatively, the aforementioned CRISPR-Cas9 and other approaches, such as those based on RNA or transposon/transposase systems, which are not per se viral vector-based engineering systems, may be used to replace viral transduction. However, CRISPR-Cas9 and transposon/transposase systems also entail genome editing, similar to viral transduction. In contrast, RNA-based systems, in which transgenic *TCR*-encoding mRNA is transfected into T cells alone or in combination with siRNA-mediated downregulation of native TCR, represent a transient self-limiting approach with a potentially better safety profile [81–83]. Nonetheless, despite extensive preclinical data, these techniques are still emerging in the clinical setting.

# Future directions in TCR-T-cell therapy for AML

Preliminary results indicate that TCR-T therapies for AML are safe and TCR-T cells can persist in most patients. Promising clinical data suggest that this form of therapy may also be efficacious in preventing relapse in AML patients. However, completed and ongoing trials have faced some limitations (**Table 5**). In two instances, investigators were not able to recruit a sufficient number of patients, whereas, in two other cases, planned TCR-T-cell numbers for administration were not achieved. Moreover, in the clinical trial EudraCT-2010-024625-20, the low efficacy of the HA-1H-specific T-cell product observed in treated patients led to the early termination of the study. Therefore, results from ongoing trials from which no results are available will provide more information about the benefit of TCR-T-cell therapy in this context.

Multiple antigens for AML have been described over the years that have yet to be explored in the clinical setting as target antigens in TCR-T-cell therapy [7,8]. For some of these antigens, preclinical and clinical data hinted at their potential role as targets for TCR-T-cell therapy, including proteinase 3 (PR3), hyaluronan-mediated motility receptor (HMMR), and T-cell receptor γ chain alternate reading frame protein (TARP). Together with WT1 and PRAME, PR3 is an AML-associated antigen overexpressed in AML blasts [36,84]. These antigens are differentially expressed in leukemic stem cells (LSCs) compared to hematopoietic stem cells; however, PR3 diverged from the other antigens analyzed in that it was comparatively more expressed on bulk leukemic cells rather than LSC [85]. Cytotoxic T-cell responses against PR3 are spontaneously detected in AML patients [40] and after PR1 vaccination [86,87]. PR1 is a nonameric HLA-A*02:01-restricted peptide derived from PR3 and neutrophil elastase (NE) that is commonly found to be overexpressed in AML [88]. PR3-specific T-cell activity has also been observed after allo-HSCT [89–91], although in this case, LAA-specific T cells are difficult to detect in early phases after transplantation [92]. Results from clinical trials using allo-HSCT demonstrated that donor T cells mediate PR3-directed anti-AML responses, including in treated R/R AML patients [89,90]. Nonetheless, cytotoxic T-cell responses against PR3 were characterized by low-affinity interactions against this self-antigen [89]. Moreover, administration of PR1-specific bulk T cells into an AML xenograft NOD/SCID mouse model led to the reduction of human AML cells in mice [93]. Despite the aforementioned encouraging data, to the best of our knowledge, no TCRs have been isolated from PR1-specific T-cell clones and used in TCR-T-cell therapies. Current strategies targeting PR3 in the context of HLA-A2 restriction are

mostly based on anti-PR1/HLA-A2 antibodies [94,95], including bi-specific antibodies [96] and TCR-like CARs [97]. This warrants the potential of this antigen in fighting AML using TCR engineered T cells.

Greiner and colleagues defined HMMR, also known as RHAMM or CD168, as an immunogenic AML-associated antigen that could be targeted in immunotherapies [98]. Initially considered a promising antigen expressed in blasts in a considerable number of AML patients, HMMR expression was associated with poor overall survival and, therefore, also considered a potential prognostic factor [99]. DCs transfected with *HMMR*-encoding mRNA elicited HMMR-specific TCR responses [100]. However, the upregulation of HMMR via mRNA transfection showed no additional benefit compared to unmodified cells, as DCs presented basal HMMR expression levels to sufficiently activate T cells. T-cell reactivities against HMMR's R3 peptide have been detected in AML patients after standard of care [101–103], including after HMMR-R3 peptide vaccination [104,105]. However, in some cases, HMMR-specific T cells were non-functional [103]. T cells modified to express HMMR-specific TCRs were able to recognize AML target cells in a humanized xenograft mouse model leading to reduced tumor burden [106]. The addition of IL-15 further enhanced the antitumor effect of TCR-T cells. However, HMMR-specific TCR-T cells also recognized hematopoietic stem cells (HSCs), which restricted the use of these cells to MHC-mismatched HSC transplantation. In addition, Snauwaert and colleagues pointed out that HMMR may not be suitable as a candidate antigen in AML due to similar expression levels of HMMR in LSCs and hematopoietic stem cells in healthy individuals and its upregulation in activated T cells [107]. Therefore, the further development of HMMR-specific TCR-T-cell therapies seems unlikely. With regards to TARP, this antigen is upregulated in AML cell lines and de novo pediatric and adult AML cells [108]. Moreover, overexpression of an alternative TARP transcript is specific to AML, being absent in other types of leukemia, such as B-ALL and CML cells [108]. More importantly, TARP-specific TCR-T cells exert cytotoxic activity against TARP-positive AML cell lines and primary cells [108]. Other reports suggest other candidates for TCR-T-cell immunotherapy in AML, such as myeloperoxidase (MPO) [109] or human telomerase reverse transcriptase (hTERT) [110]. In these two cases, high-avidity TCRs were used.

Due to difficulties in isolating high-affinity/high-avidity TCRs for self-antigens, such as those mentioned above, novel target antigens are required. Donor T cells in HSCT can mediate both GVHD and GVL effects. The capacity of these T cells to recognize mismatched HLA molecules makes them a valuable tool in TCR-T therapy for relapsed patients after allo-HSCT. Similar to HA-1 antigen, HLA-DPB1-reactive T cells have been

described as potent GVL inducers [111,112]. To prevent recurrent disease after allogeneic HSCT, T cells engineered with TCRs against HLA-DPB1 antigens, which are mismatched in many allogeneic transplantations, recognized AML cells in vitro; however, only TCR-engineered CD4 T cells were able to effectively mediate leukemic elimination in vivo [113]. Extensive screening and validation of TCRs seem critical in this context to avoid targeting HLA-DP antigens that are also expressed on healthy tissues that could lead to undesirable GVHD [113]. In some cases, due to genetic aberrations that are characteristic of AML, new point mutations may occur in specific genes leading to the formation of neoantigens that the immune system can target more efficiently than self-antigens. An example of a neoantigen in AML is the CBFB-MYH11 fusion protein. A recent report showed that HLA-B*40:01-restricted T-cell clones react with high avidity against a nonameric peptide from the CBFB-MYH11 fusion protein [114]. These T cells showed antitumor reactivity against AML cell lines and primary AML cells both in vitro and in vivo in a humanized xenograft mouse model. Moreover, the introduction of TCRs derived from CBFB-MYH11-specific T-cell clones showed anti-leukemic activity in vitro. T-cell responses have also been observed against nucleophosmin 1 (NPM1) mutations, which occur in approximately 30% of AML patients [115–117]. Isolation and transduction of an HLA-A*02:01-restricted mutated NPM1 peptide-specific TCR into T cells resulted in anti-AML activity in vivo [117]. Finally, the expression of inhibitory immune checkpoints in T cells is a factor to consider in TCR-T-cell therapy. The increased expression of immune checkpoints, such as programmed death 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA4), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and lymphocyte-activation gene 3 (LAG3), contribute to AML immune evasion and are associated with disease progression and relapse (reviewed by [118]). Thus, disruption of inhibitory receptors expressed by TCR-T cells and/or combinatorial immunotherapies based on TCR-T cells and immune checkpoint inhibitors (as seen in the combination of chemotherapy or hypomethylating agents) may pave the way for a higher efficacy and reduced immune evasion in the context of TCR-T-cell therapy for AML [119].

**Table 5.** Limitations of clinical trials using TCR-T cells for AML

| Clinical trial identifier | Description of limitations |
|---|---|
| **WT1-specific** | |
| NCT01621724<br>EudraCT-2006-004950-25 | Enrolment into the study was terminated early due to difficulties in recruitment of patients |
| NCT02550535<br>EudraCT-2014-003111-10 | Enrolment into the study was terminated early in Germany due to difficulties in recruitment of patients |
| UMIN000011519 | T-cell numbers for Arm 2 were not feasible for all patients; T-cell products were not feasible for Arm 3 |
| NCT01640301 | None disclosed |
| NCT02770820 | None disclosed |
| **PRAME-specific** | |
| NCT02743611 | n.d. |
| NCT03503968 / EudraCT-2017-000440-18 | n.d. |
| EudraCT-2018-000717-20 | n.d. |
| **MiHA HA-1H-specific** | |
| EudraCT-2010-024625-20<br>NTR3454 / NL3307 | HA-1H TCR-transduced CMV or EBV-specific T-cell products could not be generated for 4 out of 9 patients; TCR-T cells could not be detected (lack of TCR-T-cell expansion) in peripheral blood in 2 out of 5 treated patients at any time during follow-up; 3 out of 5 treated patients died during follow-up for causes not related to treatment; overall feasibility and efficacy of the procedure was too low to warrant further developments of this therapy |
| NCT04464889 / EudraCT-2019-002346-20 | n.d. |
| NCT03326921 | n.d. |
| **Other** | |
| NTR6541 / NL6357 | n.d. |

**Abbreviations:** AML, acute myeloid leukemia; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HA-1H, HLA-A*0201-restricted minor histocompatibility antigen 1 peptide variant H; MiHA, minor histocompatibility antigen; n.d., no data; PRAME, preferentially expressed antigen in melanoma; TCR, T-cell receptor; WT1, Wilms' tumor 1. Last search on 25 July 2021.

# Concluding remarks

In summary, results from current clinical trials using TCR-T-cell therapy for AML indicate no treatment-related toxicities. This is achieved thanks to the careful selection of high-affinity TCRs derived from natural repertoires that do not rely on artificially enhancing TCR affinity above physiological levels. In terms of efficacy, it is too soon to draw conclusions from these studies, given their early phase design and the results from most of them being pending. However, preliminary results indicate that this type of therapy may be efficacious for AML, especially in cases of relapse, where remaining leukemic blasts may not be eliminated with the standard of care, warranting the development of improved TCR-T-cell strategies to further increase their clinical benefit.

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**The more I read, the more I acquire, the more certain I am that I know nothing.**

— Voltaire

# 2

# The ins and outs of messenger RNA electroporation for physical gene delivery in immune cell-based therapy

This chapter has been published in:

Campillo-Davo D, De Laere M, Roex G, Versteven M, Flumens D, Berneman ZN, Van Tendeloo VFI, Anguille S, Lion E. *Pharmaceutics* (2021);13(3):396.

# Abstract

Messenger RNA (mRNA) electroporation is a powerful tool for transient genetic modification of cells. This non-viral method of genetic engineering has been widely used in immunotherapy. Electroporation allows fine-tuning of transfection protocols for each cell type as well as introduction of multiple protein-coding mRNAs at once. As a pioneering group in mRNA electroporation, in this review, we provide an expert overview of the ins and outs of mRNA electroporation, discussing the different parameters involved in mRNA electroporation as well as the production of research-grade and production and application of clinical-grade mRNA for gene transfer in the context of cell-based immunotherapies.

# Introduction

Since the early experimental application of electric pulses in the field of medicine during the eighteenth century, electroporation has become a universal method for transfecting biological and synthetic compounds into an array of prokaryotic and eukaryotic cells for a wide number of purposes [1]. Electroporation, also called electropermeabilization, is defined as the application of voltage pulses that generate an electric field between two electrodes that disrupts the integrity of a cell membrane, al-lowing the formation of pores. It was first developed as an irreversible process of pore formation that did not allow recovery of the cell membrane, therefore resulting in cell death [1] Reversible electroporation was introduced in 1957 by Stämpfli and Willi [2], but it was not until 1982 that this type of electroporation was described for the transfection of genetic material [3]. In that article, Neumann et al., who also coined the term "electroporation", described how electric pulses enhanced the uptake of extracellular DNA into mouse cells [3]. Since then, the versatility of this technique has been demonstrated in multiple cell types and organisms for the transfection of various molecules in a wide range of applications. The field of cell-based immunotherapy in particular has made enormous progress due to the development and optimization of messenger RNA (mRNA) electroporation for gene transfer. This type of genetic engineering, compared to that of the viral delivery of genes, represents a safer alternative for protein expression with no risk of insertional mutagenesis and lower immunogenicity [4,5]. The superiority of mRNA electroporation over passive pulsing or lipofection of mRNA, and even over plasmid DNA electroporation, in terms of efficiency of gene de-livery was demonstrated by our group two decades ago [6,7]. In contrast to plasmid DNA electroporation, transient gene expression linked to the natural decay of introduced mRNA provides an accurate system to control the synthesis of exogenous proteins. The main factors involved in successful mRNA electroporation for gene transfer can be divided into three main categories, (i) electroporation parameters, (ii) variables of in vitro mRNA synthesis, and (iii) elements used to enhance transfected mRNA stability and transgene expression (**Figure 1**). In this review, we discuss the different parameters marking mRNA electroporation and how to implement them as well as the factors involved in the production of clinical-grade mRNA for electroporation in the context of cell-based immunotherapies.

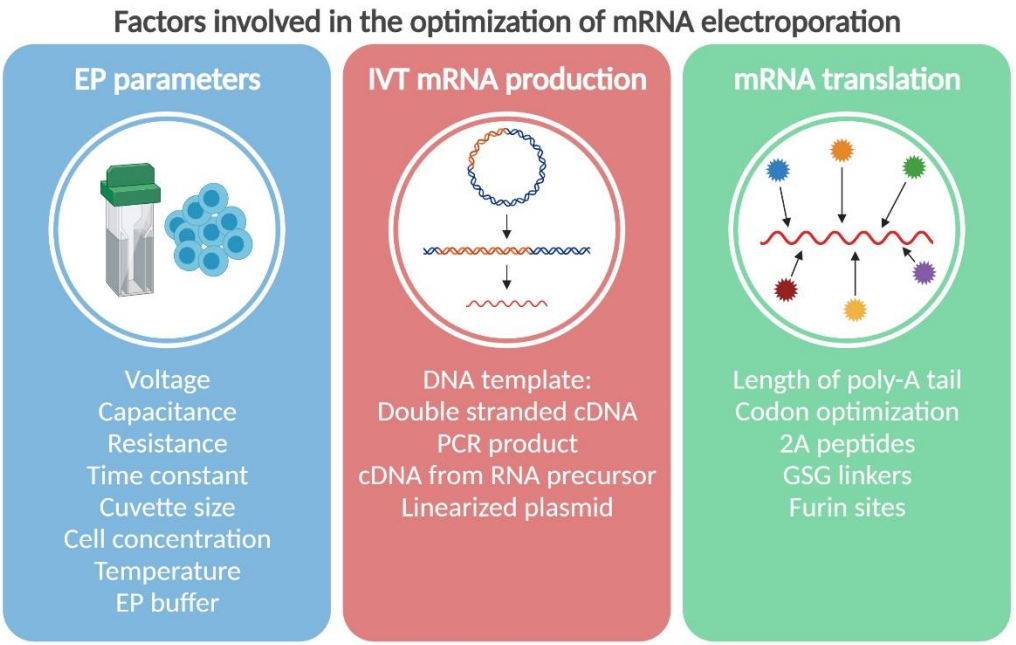



**Figure 1. Overview of the main factors that influence the success of a messenger (mRNA) electroporation-based therapy.** Several factors may influence the transfection efficiency (blue), synthesis (red) and translation (green) of mRNA in electroporation-based therapies. These factors can be individually optimized, combined and tailored for each type of immune cell and target gene to be transferred. EP, electroporation; IVT, in vitro transcription; cDNA, complementary DNA; PCR, polymerase chain reaction. Created with BioRender.com.

# The physics: Parameters of electroporation

Electrical disruption of a cell membrane causes the formation of pores through which nucleic acids, proteins, and other small molecules present in the environment surrounding the cells can permeate, gaining access to the intracellular space (**Figure 2**). In vitro electroporation of immune cells is carried out using a pulse generator (or electroporator). Generally, cells are placed in sterile cuvettes consisting of a cell chamber with two parallel metal electrode plates. Commercially available cuvettes for the transfection of mammalian cells typically have a gap size of 2 or 4 mm. The difference in electric potential between the two electrodes is called voltage ($V$) and it is measured in volts (V). Before electroporation, cell membranes are in a non-permeable state that is characterized by low conductivity, dielectrical constant, and polarizability [8]. As mentioned in the previous section, a voltage pulse is applied during the electroporation process. This generates an electric field that creates a linear strength gradient between the electrodes. The voltage used divided by the gap size of the cuvette determines the electric field strength ($E$), commonly expressed in kilovolts per

centimeter (kV/cm). That electric field will create an induced cell membrane potential. If the field strength is high enough, the induced cell membrane potential will surpass a threshold potential in which the cell membrane will undergo polarization and dielectric breakdown followed by an increase in membrane conductivity and permeability [9]. These changes allow the creation of hydrophilic nanopores through which ions in aqueous solutions may pass [10] (**Figure 2**). That flow of extracellular components will lead to the formation of larger pores allowing the passage of larger molecules such as RNA [10]. Moreover, ions moving from the positive electrode to the negative electrode will create electrophoretic forces that allow RNA (a polyanion) to travel to the positive electrode [8]. The induced cell membrane potential is not uniform nor permeabilization occurs homogenously across the membrane [8,11]. In fact, more pores will be created at the side of the cell membrane that faces the negative electrode, whereas, at the side of the positive electrode, a larger area of the cell membrane will be permeabilized (**Figure 2**). When the electric field is removed at the end of the electroporation process, resealing of the cell membrane occurs. Contrary to pore formation, which takes place within milliseconds, resealing of the cell membrane may take from minutes to hours [8]. When a critical field strength is reached, resealing of the cell membrane may not be possible, leading to cell death.

The electroporation process is also influenced by other parameters, including the capacitance, resistance, time constant, and pulse duration [12]. Capacitance ($C$), measured in microfarads (μF), is defined as the ability of a capacitor—in this case, the membrane of the cells in suspension—to retain a charge ($Q$) in the form of a potential difference or voltage. Accordingly, capacitance follows the equation: $C = Q/V$. Resistance ($R$), expressed in ohms (Ω), is the force against the electrical current, which is influenced by elements such as the cell suspension or electroporation buffer. Taking into account these parameters, two types of pulses (or waveforms) are commonly used for electroporation of immune cells, exponential decay and square waves (**Figure 2**). Exponential decay is a pulse in which the chosen voltage is reached at the beginning of the pulse followed by an exponential and rapid decrease to zero [12]. That decay follows the formula $V_t = V_0\left(e^{-(t/\tau)}\right)$, where $V_0$ is the initial voltage at which the capacitor is charged, $V_t$ is the voltage at a time $t$, and $\tau$ is the time constant at which the voltage of the pulse has decreased from $V_0$ to $V_0/e$ [12]. The time constant results from the combination of the resistance and capacitance ($\tau = R \times C$). The time constant should not be confused with pulse length or duration of the pulse ($\tau$). An alternative form of exponential decay pulse—usually called "time constant"—applies a voltage for a certain amount of time without constraining the capacitance. When the time is kept constant, the capacitance is adjusted to reach a particular (constant) pulse length for all

the test conditions, which is dependent on the resistance of the cell suspension and the volume in the cuvette. In contrast, the square wave pulse, which gets its name from the shape of its electric potential curve, maintains the same voltage for the entire duration of the pulse after which it returns to a voltage of zero [12]. With this type of wave, it is possible to apply multiple and repeated electric pulses during a single electroporation.

The electric field strength, together with the duration of the pulse, is key in maintaining cell viability and transfection efficiency during electroporation (reviewed by [13]). Apart from its implications regarding the field strength, gap size will also determine the electroporation buffer volume and number of cells a cuvette can contain. Thus, widening the gap size will increase the usable volume and number of cells, but it will also reduce the field strength. Other parameters affecting the success of electroporation include the electroporation buffer used, the temperature of the different components during electroporation, and the cell concentration. The conductivity of the electroporation buffer, marked by its salt content, and the cell concentration are two of the main parameters that affect the resistance of the sample during electroporation [14]. Moreover, the composition of the buffer, especially the content in salts and sugars, may have a negative effect on cell viability and transfection efficiency [15–17]. Related to this, the presence of remaining salts in the cell and nucleic acid suspension may increase the final concentration of salts in the electroporation mixture leading to arcing [18]. Arcing is a complete or partial discharge of an electric current in a sample easily recognizable as an audible popping sound. This phenomenon occurs in the presence of high salt concentrations, but also in the presence of bubbles, of oil on the electrodes of the cuvettes due to handling without gloves, or with faulty cuvettes in combination with high voltages, and negatively impacts cell viability. The temperature of the electroporation buffer, cuvette, and cells is another variable to be considered during the optimization of electroporation conditions [12]. For example, keeping the cell suspension on ice or at 4 °C may limit membrane plasticity, reducing electroporation efficiency; however, cell viability and yield are often improved at lower temperatures. With respect to the recovery medium after permeabilization, there is no clear rule; however, a general recommendation to improve cell viability and pore resealing, which occurs within seconds, is the addition of human or non-human serum, depending on the experimental requirements [19]. Taken together, each of these parameters and elements of electroporation can be optimized to improve the efficiency of mRNA delivery while maintaining cell viability and yield [12,20,21].

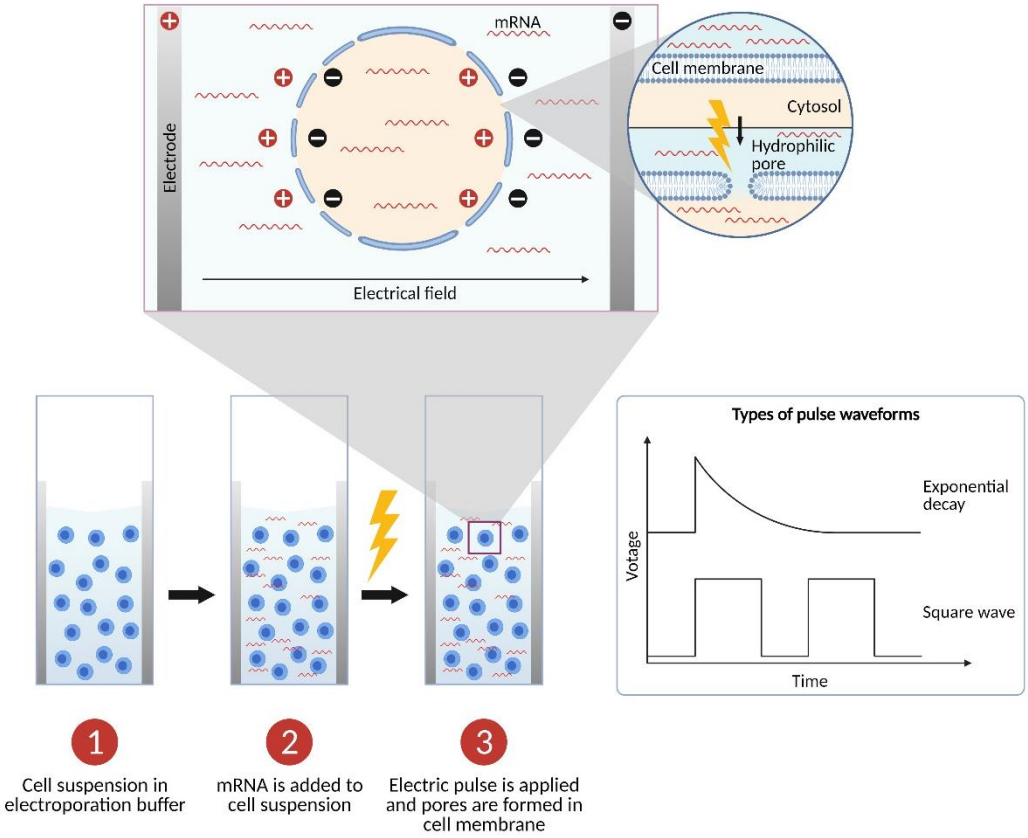



**Figure 2. Elements of the electroporation process.** The electroporation cuvettes contain two parallel electrodes separated by a gap where the cell suspension is placed. The cells that are in suspension in an electroporation buffer (1) are mixed with mRNA (2) and pulsed (3) with one of the two main types of electric waves: the exponential decay or the square wave. During the electric pulse, pores are transiently formed in the cell membrane through which the mRNA can flow into the cytosol. Created with BioRender.com.

# The chemistry: In vitro synthesis of mRNA for electroporation

For mRNA electroporation in gene transfer studies, one of the key factors at the molecular level for efficient and correct protein expression is the synthesis of the mRNA. In eukaryotic cells, the first step of gene expression occurs in the nucleus and consists of the transcription of an mRNA strand from a segment of complementary DNA (cDNA) by RNA polymerase II. Before being transported to the cytoplasm to be translated into protein, the precursor mRNAs (pre-mRNAs) undergo a maturation process in the nucleus that includes modification of the 5′ and 3′ ends and elimination of the non-coding regions (introns). The first modification occurs at the 5′ region where

a methylated guanosine or "cap" is added to the first nucleotide of the pre-mRNA, protecting it from degradation by exonucleases [22]. Next, polyadenylation takes place at the 3′ terminus of the pre-mRNA [23]. Finally, the introns are removed from the pre-mRNA through splicing, leaving a mature mRNA consisting of the protein-coding regions (exons) flanked by untranslated regions (UTRs), the methylated guanosine cap, and a poly(A) tail.

The 3′ UTR region of the mRNA is a primary factor influencing its cellular localization, stability, and translation efficiency [24]. Messenger RNAs encoding the same protein can exhibit different 3′ UTR isoforms depending on the specific intended fate of a particular mRNA. Importantly, the length and composition of the 3′ UTR region help regulate the mRNA, and thereby the protein levels in a cell at any given time. In fact, the 3′ UTR region, together with the 5′ cap, is indispensable for the formation of the stem-loop structure needed to initiate mRNA translation. Shorter 3′ UTRs have an advantage in the formation of the initiation loop compared to that of mRNAs with longer 3′ UTRs. In the nucleus, polyadenylation of mRNAs on their 3′ side is a tightly regulated and standardized process that results in the addition of approximately 200 nucleotides in mammals [25]. The length of the poly(A) tail is usually shortened after the mRNA enters the cytoplasm through a mechanism that is involved in regulating mRNA decay [26]. Actually, the poly(A) tail is a dynamic region of the mRNA sequence that is affected by the processes of adenylation (to lengthen) and deadenylation (to shorten), which are adjusted during different stages of the cell cycle or in response to specific signals. The effect of poly(A) tail length on translational control has been previously reviewed by Weill et al. [27].

Most natural mRNAs are degraded by endonucleases or exonucleases within minutes or hours of being transcribed. However, transcripts that encode proteins which are functionally vital for the cell are usually more stable. An important determinant of mRNA stability lies in the portion of the 3′ UTR preceding the poly(A) tail. In particular, human globin mRNAs have been characterized as being highly stable with half-lives up to 48 h due to their 3′ UTR [28]. Therefore, the addition of these 3′ UTR motifs to synthetic mRNAs benefits their stability, resulting in higher protein expression levels [29]. In situations where increased protein translation is needed without wanting to affect the mRNA half-life, addition of the cytochrome b-245 alpha chain gene 3′ UTR may be a suitable candidate [30]. In the laboratory, mRNA synthesis is commonly performed via in vitro transcription (IVT), a rapid and efficient technique that yields high amounts of mRNA. The open reading frame (ORF) of the therapeutic gene of interest is preceded by a 5′ UTR containing a promoter and the Kozak sequence [31].

The promoter is usually specific for bacteriophage SP6, T3, or T7 RNA polymerase [32–35]. The Kozak consensus elements, called the Shine–Dalgarno sequence in prokaryotes, are the nucleotides preceding and following the AUG start codon. These sequences at the proper position in vertebrates act as enhancers of initiation of translation [36]. The ORF of the gene of interest is followed by a 3′ UTR and a poly(A) tail, depending on the template used. The 3′ UTR and a poly A tail are elements crucial for the stability and translational efficiency of the produced mRNA. To generate IVT mRNA, there is a broad range of commercially available IVT kits; however, the basic requirements to initiate transcription are a purified cDNA template, ribonucleotide triphosphates, distilled water, reaction buffer, and an RNA polymerase. The double-stranded cDNA template is typically a product of polymerase chain reaction (PCR), cDNA from an RNA precursor, or a linearized plasmid DNA (**Figure 3**). In the case of PCR products, the gene of interest is amplified by PCR using a plasmid or genomic DNA as template. Then, through the addition of the appropriate primers and another round of PCR amplification, the cDNA template is linked to a promoter for the ultimate translation of the ORF [37]. This is done by including at the 5′ end of one of the primers the promoter region of an RNA polymerase from one of the bacteriophages. When using cDNA generated from an RNA precursor, the RNA first undergoes a reverse transcription reaction with primers containing the bacteriophage polymerase, resulting in the production of a single DNA strand bound to the RNA precursor. The second cDNA strand is then generated using the complementary RNA as primer to form the double-stranded DNA. For plasmids, the circular DNA is linearized by digestion with a restriction enzyme prior to IVT, to prevent the transcription of the entire plasmid sequence. This results in the creation of either blunt end or sticky 3′-overhanging ends, depending on the enzyme used. Related to this, an important concern after plasmid linearization is the addition of non-adenine nucleotides to the poly(A) tail from the overhanging ends, which otherwise will reduce translation efficiency. To avoid non-adenine nucleotides at the end of the poly(A) tail, type IIS restriction enzymes can be used instead of the classical type II enzymes as type IIS enzymes cleave the DNA sequence outside the recognition site and create blunt ends without 3′ overhangs. A detailed protocol has been previously published [38].

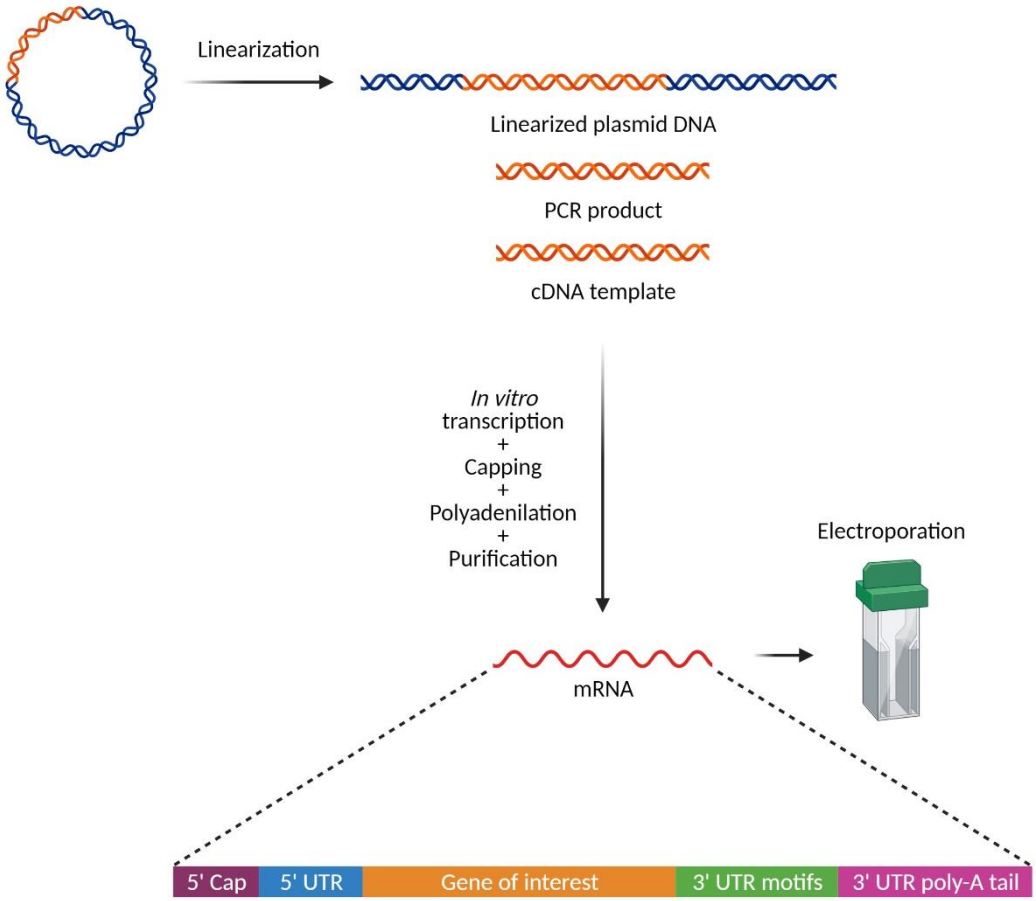



**Figure 3. Basic workflow for mRNA synthesis.** The in vitro synthesis of mRNA starts with the preparation of the DNA template containing the gene of interest (depicted in orange), which can be linearized plasmid DNA, a PCR product, or a cDNA template. These DNA templates will be used for the in vitro transcription of mRNA using an RNA polymerase, followed by mRNA capping at the 5′ untranslated region, addition of a poly(A) tail at the 3′ untranslated region (optional in cases were a poly(A) is included in the DNA template), and purification of the final mRNA. UTR, untranslated regions. Created with BioRender.com.

# The biology: How to improve mRNAs for better stability and translation

Apart from optimizing the electroporation conditions and choosing the best template for mRNA production, other factors also contribute to successful mRNA stability and translation and should be considered to improve protein expression in electroporated cells. As described in the previous section, mRNA capping and polyadenylation are indispensable for successful mRNA translation. The 5′ capping of IVT mRNA can be directly done during RNA generation or done separately. The various options for 5′

capping have been reviewed elsewhere [39]. When polyadenylation is performed separately after IVT, mRNAs are formed with a greater variability in poly(A) tail length. In other cases, the poly(A) tail is cloned into the plasmid and positioned within the construct after the ORF. Since poly(A) tails are shortened in the cytoplasm due to natural mRNA degradation, different plasmids have been developed based on the extension of the poly(A) tails to improve mRNA yield and stability. For example, the pST1-A120 vector includes a poly(A) tail of ~120 base pairs (bp) [29], and the plasmid pEVL can be used to increase the poly(A) tail length up to ~500 bp [40]. Some plasmids for in vitro synthesis of RNA can be purchased from commercial sources, such as pGEM-XZ and pSPXX vector series (Promega), pBluescript II phagemid vectors (Agilent), pCRII and pTRIPLEscript vectors (Invitrogen) [41], pT7-mRNA vector (VectorBuilder Inc.), and pMRNAxp mRNAExpress vector (System Biosciences). Another factor that improves mRNA translation is codon optimization. Some mRNAs may contain "rare" codons that decrease the rate of translation, an issue that has been previously reviewed [42]. Codon optimization involves replacing those codons with more highly expressed synonymous codons, thereby enhancing protein expression compared to that of the native sequences [43].

Gene transfer using mRNAs may encode for multiple proteins at the same time, similar to what can be done using DNA vectors. Over the years, various strategies have been used in gene therapy to yield individual translation products from polycistronic constructs [44]. Two of the most common strategies are the insertion of internal ribosome entry sites (IRES) and self-cleaving 2A peptides sequences between the genes. IRES were first discovered in picornavirus and allow cap-independent translation of proteins (reviewed by [45]). Placed between two independent sequences, IRES are able to recruit ribosomes to initiate the translation of the downstream genes [45]. However, due to the large size and inconsistent translation rates of IRES, this system has become less popular in mRNA gene transfer in favor of 2A peptides [46,47]. Initially found in picornavirus, 2A peptides are 18–22 amino acid-long oligopeptides that are part of the ribosome "skipping" translational mechanism [46]. They allow for the stoichiometric expression of upstream and downstream genes in bicistronic cassettes and exhibit a high cleaving efficiency with minimal addition of amino acids to the translated proteins. Among the various 2A peptides, P2A from porcine teschovirus-1 and T2A from *Thosea asigna* virus usually yield better results in comparative studies than that of other 2A peptides, such as F2A from foot-and-mouth disease virus or E2A from equine rhinitis A virus [46]. Multiple 2A peptides can also be used together in multicistronic constructs, resulting in different gene expression levels depending on the combination of peptides used [48]. An important factor that may limit cleavage efficiency is the C-terminal

sequence preceding the 2A peptide [49,50]. Frequently, 2A peptides are preceded by flexible oligopeptide linkers that are comprised of combinations of glycine and serine, in many cases being the combination Gly-Ser-Gly [50,51]. These spacers improve the cleaving efficiency of the 2A peptides, resulting in the correct expression of the upstream and downstream proteins [50–52]. However, they also add a few more amino acids to the C-terminus of the upstream protein, potentially having functional consequences that must be assessed on a case-by-case situation. A solution to this problem is the addition of furin recognition sites before the 2A peptide [52,53]. Furin is an endoprotease that recognizes RX(K/R)R motifs. The 2A peptides, glycine-serine linkers, and furin cleavage sites can be used simultaneously [51,52]. However, it is important to note that they must be in a single ORF with the genes of interest either before and/or after them. This ensures the correct translation and expression of the transferred proteins.

# Clinical production of mRNA for electroporation

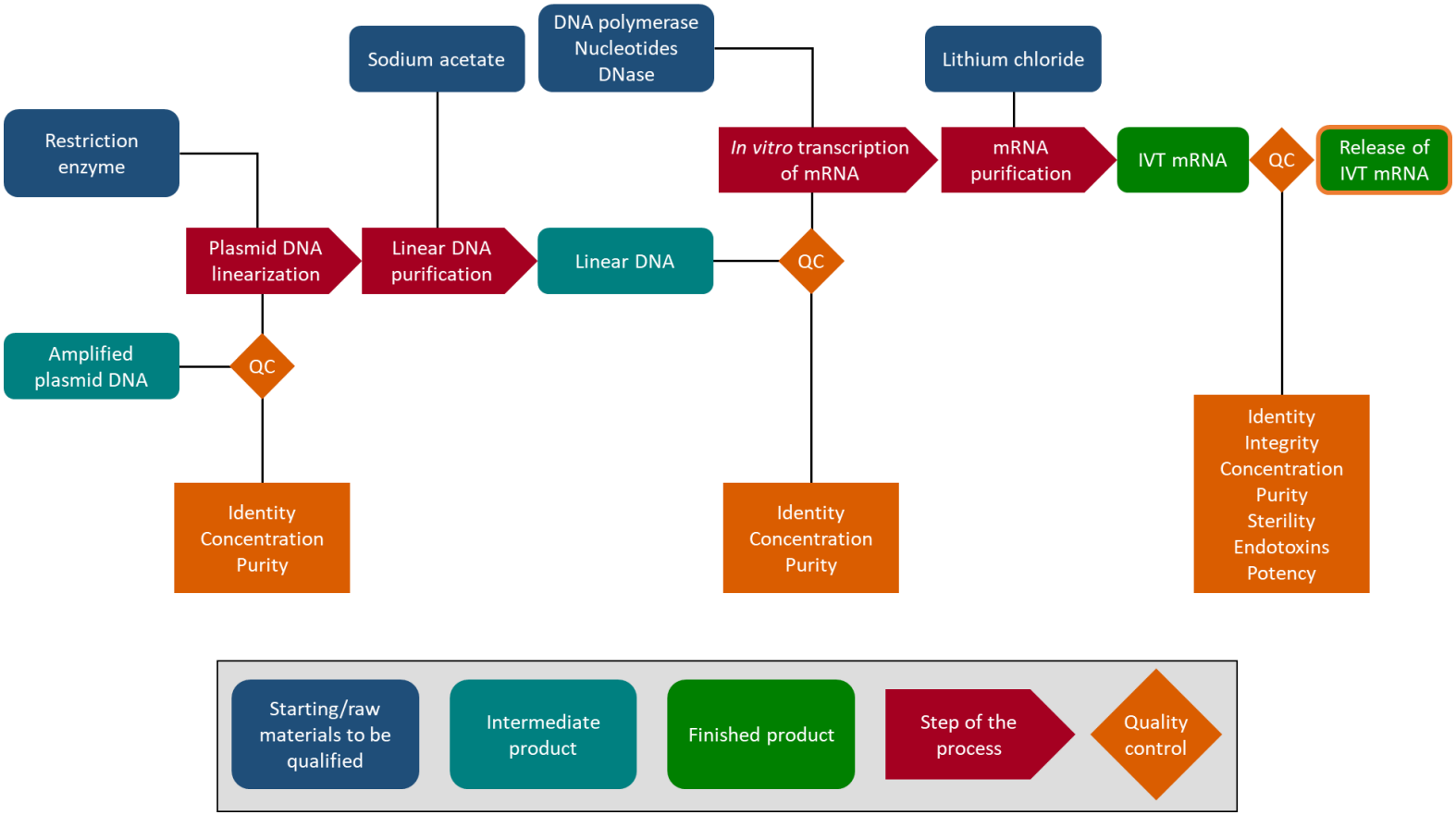
In general, two types of clinical-grade mRNA can be distinguished: documented-grade [54] and good manufacturing practice (GMP)-grade mRNA. These two categories of mRNA vary in the regulatory aspects associated with their production, which are determined by the intended usage of the mRNA (i.e., as a starting material or as a medicinal product), the class of advanced therapy medicinal product (ATMP) the final product belongs to (i.e., cell-based ATMP or gene therapy product), and the stage of development of the medicinal product (i.e., investigational or marketed). In the context of mRNA transfection for immune cell-based immunotherapeutics, mRNA can be considered both starting material and active substance for the generation of a cell-based ATMP. While Directive 2001/83/EC [55], as amended, holds the obligation for the manufacturing authorization holders to use only active substances that have been manufactured in accordance with GMP for starting materials, Directive 2005/28/EC includes no such requirement for manufacturers of investigational medicinal products [56]. For this reason, mRNA not fully complying with the GMP requirements, but of which the quality is controlled and documented in such a way that it justifies its use in the clinical setting (i.e., documented-grade mRNA) is a valid starting material for the production of mRNA-modified cell-based investigational medicinal products. For any other clinical application, GMP-grade mRNA is required, according to the applicable regulatory guidelines. Guidance on the interpretation of the GMP principles and guidelines for active substances used as starting materials are described in “The Rules Governing Medicinal Products in the European Union“ (EudraLex), Volume 4 “Good

Manufacturing Practice", Part II "Basic Requirements for Active Substances used as Starting Materials" as laid down in Directive 2003/94/EC [57].

For the production of GMP-grade mRNA, an extensive documented quality management system needs to be established. This system should cover the complete process of active pharmaceutical ingredient (API) manufacturing, from qualification of raw material suppliers, overproduction, quality control, release of intermediates and the API, to API packaging, labeling, storage, and distribution. The EudraLex GMP guidelines in addition set standards for manufacturing premises, process equipment, and personnel, while also covering administrative aspects such as record keeping, change and deviation management, and corrective action and preventive action (CAPA) system. To ensure the highest quality of the produced mRNA, each batch is subjected to extensive QC testing, which commonly includes assays for integrity, identity, potency, and, as appropriate, sterility and the presence of bacterial endotoxins (Figure 4). QC tests related to detection of relevant impurities, such as residual solvents, proteins, template and/or bacterial DNA, and other mRNA properties (e.g., capping efficiency) depend on the manufacturing process selected and the desired/required degree of control. These procedures should be validated, taking into consideration the relevant guidance and recommendations found in the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 (R1) guidelines (CPMP/ICH/381/95) [58]. The EudraLex GMP guide also includes recommendations (with no obligatory force) for starting materials used in the production of investigational medicinal products. While it is recognized that not all GMP standards are applicable in early clinical development and a certain level of flexibility is required in this phase, manufacturers should still ensure that appropriate GMP concepts are applied in the production of APIs for use in clinical trials and that compliance increases with the stage of development.

From the above, it is evident that producing clinical-grade mRNA requires dedicated infrastructure, equipment, and expertise. Hence, many investigators outsource this activity and purchase customized clinical-grade IVT mRNA from specialized commercial suppliers. Currently, different companies provide these services, which include BioNTech, Biomay CureVac, EtheRNA, and Eurogentec in Europe, and Aldevron, Creative Biolabs, Moderna, and TriLink in the United States of America. Our research group has extensive experience in different clinical trials involving the use of mRNA as API starting material (ClinicalTrials.gov reference number NCT00834002, NCT00965224, NCT01291420, NCT01686334, NCT02649582, NCT02649829). From these clinical studies, we have learned that the service of customized clinical-grade mRNA

production is associated with very high costs and extended turn-around-times for production and delivery. This is at least in part due to the fact that, while the amounts of mRNA as API required in the context of early phase clinical trials are relatively small, substantially higher amounts of mRNA need to be produced, at cost, to comply with GMP quality control and stability testing requirements. In this perspective, in-house production of small to medium batches of documented-grade mRNA, which is less demanding in terms of required infrastructure and overall GMP compliance, may provide clinical research centers with an alternative to support their early clinical development needs. It has to be taken into account, however, that any change to the API at a later stage of development made in view of meeting the increasing regulatory requirements, results in the need for comparability studies to ensure these changes do not alter the final cell therapy product. Still, the significantly reduced cost associated with in-house production of documented-grade mRNA versus custom-produced GMP-grade mRNA may ensure sustainability of research efforts focusing on mRNA-electroporated cell-based immunotherapeutics.


Restriction enzyme
Sodium acetate
DNA polymerase
Nucleotides
DNase
Lithium chloride
Plasmid DNA linearization
Linear DNA purification
Linear DNA
In vitro transcription of mRNA
mRNA purification
IVT mRNA
QC
Release of IVT mRNA
Amplified plasmid DNA
QC
QC
Identity
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Identity
Concentration
Purity
Identity
Integrity
Concentration
Purity
Sterility
Endotoxins
Potency
Starting/raw materials to be qualified
Intermediate product
Finished product
Step of the process
Quality control

**Figure 4. Example of the messenger RNA production processes and quality control testing for the release of IVT mRNA in a clinical setting for human use.** Generally, different reagents, raw materials, and intermediate products are needed to produce any in vitro transcribed (IVT) mRNA. However, compared to research-grade mRNA, manufacture, and final release of IVT mRNA for clinical use in humans usually requires more quality controls (QC). These controls include the quantification of the mRNA concentration, purity, and integrity, but also the confirmation of the identity the mRNA, its sterility, its potency, and the absence of potentially damaging endotoxins.

# Clinical application of mRNA electroporation in cell-based immunotherapies

Electroporation of mRNA as a pharmaceutical tool for transient expression of proteins of interest has been applied as a therapeutic strategy in malignant, infectious, and autoimmune diseases. Loading antigen-presenting dendritic cells (DCs) with tumor-associated antigens (TAAs) alone or in combination with immune-modulating molecules, such as agonists of T-cell activation, is the most common usage of mRNA electroporation in a clinical setting (**Table 1**). This therapeutic modality focuses on promoting multi-epitope antigen-specific T-cell responses to target tumor cells. Taking this idea further, multiple mRNA encoding different TAAs can be co-electroporated in order to improve immune responses and to avoid immune evasion. Another application, as a safer and more versatile alternative than viral transduction, is the redirection of T cells with immune receptors such as T-cell receptors (TCRs) and chimeric antigen receptors (CARs) to specifically and directly target TAAs presented by tumor cells (**Table 2**). Although less popular compared to T cells in a clinical context, peripheral blood mononuclear cells and natural killer cells can also be engineered to express such immune receptors in a transient way, with only a few trials evaluating the former for the treatment of ovarian cancer and malignant peritoneal mesothelioma (NCT03608618; [59]) and the latter for the treatment of colorectal cancer (NCT03415100; **Table 3**).

The use of mRNA electroporation for the treatment of infectious diseases has been less widespread compared to solid and hematological malignancies. DCs have been engineered with human immunodeficiency virus (HIV) antigens alone or in combination with immune-modulating molecules for the treatment of HIV infection (**Table 1**). Furthermore, mRNA electroporation has been used to introduce zinc finger nucleases for the disruption of CCR5, a key chemokine receptor in HIV infection, in CD4 T cells to protect the adoptively-transferred CCR5-edited CD4 T cells from HIV targeting (NCT02388594, **Table 2**). Only one registered clinical study relies on this

technique to redirect T-cell specificity in type 1 diabetes (NCT02117518, **Table 2**). In preparation for clinical translation, tolerogenic DCs electroporated with mRNA-encoding myelin antigens have shown promising results in mouse models for the treatment of multiple sclerosis [60], warranting the exploration of these findings in clinical trials.

**Table 1. mRNA synthesis and electroporation conditions in clinical trials using mRNA electroporation for gene transfer in dendritic cells**

| Disease | Gene(s) | mRNA synthesis | | EP conditions | | Clinical trial identifier and References |
|---|---|---|---|---|---|---|
| | | Template | Production | Device | Settings | |
| **Solid malignancies** | | | | | | |
| Melanoma | TAA (murine TRP2) | Linearized pING vector | mMessage mMachine T7 kit | BTX ECM 830 square wave electroporator | 700 V (two pulses) 2-mm cuvette | NCT01456104 [61] |
| Melanoma | TAA (gp100, tyrosinase) | Linearized pGEM4Z/hgp100/A64 pGEM4Z/tyrosinase/A64 vectors | Produced by CureVac GmbH Purified by PUREmessenger™ (chromatography) | Gene Pulser Xcell (Bio-Rad) | Exponential decay pulse (300 V, 150 µF) 4-mm cuvette | NCT00243529 [62] |
| Melanoma | TAA (h-TERT, survivin) + tumor cell mRNA | ND | T7 mMESSAGE mMACHINE large-scale transcription kit (Ambion) Purified with MEGAclear column (Ambion) | BTX ECM 830 square wave electroporator | Square wave pulse | NCT00961844 [63-65] |
| Melanoma | TAA (gp100 and tyrosinase) + immune modulating molecules (active TLR4, CD70) | Linearized pGEM4Z/hgp100/A64 pGEM4Z/tyrosinase/A64 vectors | Produced by CureVac GmbH Purified by PUREmessenger technology (chromatography) | Gene Pulser Xcell (Bio-Rad) | Exponential decay pulse (300 V, 150 µF) 4-mm cuvette | NCT01530698 NCT00940004 [62,66] |
| Melanoma | TAA (MAGE-A3, MAGE-C2, tyrosinase, gp100) + immune modulating molecules (CD40L, CD70, caTLR4) (TriMixDC-MEL product) | Linearized pGEM-CD40L pGEM-CD70 pGEM-caTLR4 pGEM-sig-MageA3-DCLamp pGEM-sig-MageC2-DCLamp pGEM-sig-gp100-Lamp pGEM-sig-tyrosinase-Lamp vectors | mMESSAGE mMACHINE Ultra T7 Kit Length, concentration and purity evaluated with Agilent 2100 Bioanalyzer (Agilent Technologies) using RNA 6000 Nano LabChip Kit (Agilent Technologies) | EQUIBIO Easyject Plus | 300 V, 450 µF, 99 Ω (pulse time ~5 ms) | NCT01066390 [67,68] NCT01676779 [68] NCT01302496 [68,69] |
| Breast cancer Melanoma | TAA (hTERT, survivin, p53) | Linearized pCI/hTERT/A102 pSP73/p53/A64 pSP73/survivin/A64 vectors | mMESSAGE mMACHINE T7 Ultra kit (Life Technologies) Purified with MEGAclear kit (Ambion) Length, concentration, and purity evaluated with Agilent 2100 Bioanalyzer (Agilent Technologies) using RNA 6000 Nano LabChip Kit (Agilent Technologies) | BTX ECM 830 square wave electroporator | Square wave pulse (500 V, 2 ms) 4-mm cuvette (placed for 5 min on ice) | NCT00978913 [70] |
| Uveal melanoma | TAA (gp100, tyrosinase) | ND | ND | ND | ND | NCT00929019 [71] |
| Renal cell carcinoma | huCD40L + autologous tumor cell mRNA (AGS-003 product) | Linearized pCR2.1/CD40L wt vector from pCR2.1 (Invitrogen) | mMessage mMachine T7 Ultra kits (Ambion) Purified using RNeasy column (QIAGEN) | Bio-Rad | 4-mm cuvette | NCT00272649 [72] NCT00678119 [73,74] NCT01582672 [74,75] |
| Renal cell carcinoma | huCD40L + autologous tumor cell mRNA (AGS-003 product) | ND | ND | ND | ND | NCT02170389 NCT01482949 NCT04203901 |
| Bladder urothelial carcinoma | huCD40L + autologous tumor cell mRNA (AGS-003-BLD product) | ND | ND | ND | ND | NCT02944357 |
| Non-small cell lung cancer | huCD40L + autologous tumor cell mRNA (AGS-003-LNG product) | ND | ND | ND | ND | NCT02662634 |
| Lung cancer | TAA (MIDRIX4-LUNG product) | ND | ND | ND | ND | NCT04082182 |
| Glioblastoma multiforme | CMV pp65-LAMP | pp65-LAMP/A64 | ND | ND | ND | NCT00626483 |
| Glioblastoma multiforme | CMV pp65-LAMP | pp65-LAMP/A64 | ND | ND | ND | NCT00639639 [76,77] |
| Colorectal cancer | TAA (CEA) | ND | ND | Gene Pulser Xcell (Bio-Rad) | Exponential decay pulse (300 V, 150 µF) 4-mm cuvette | NCT00228189 [78] |
| Solid tumors (malignant pleural mesothelioma) | TAA (WT1) | Linearized pGEM/WT1 pST1/sig-WT1-DC-LAMP pST1/sig-WT1-DC-LAMP-OPT (codon-optimized version of pST1/sig-WT1-DC-LAMP) vectors | Produced by CureVac GmbH | Gene Pulser Xcell (Bio-Rad) | Exponential decay pulse (300 V, 7 ms) 4-mm cuvette | NCT01291420 [79] |
| Prostate cancer | TAA (PSA, PAP, survivin, hTERT) | ND | ND | ND | ND | NCT01446731 [80] |

**Table 1. mRNA synthesis and electroporation conditions in clinical trials using mRNA electroporation for gene transfer in dendritic cells (CONT.)**

| Disease | Gene(s) | mRNA synthesis | | EP conditions | | Clinical trial identifier and References |
|---|---|---|---|---|---|---|
| | | Template | Production | Device | Settings | |
| **Hematological malignancies** | | | | | | |
| Hematological malignancies | TAA | ND | ND | ND | ND | NCT02528682 |
| Acute myeloid leukemia | TAA | ND | ND | ND | ND | NCT01686334 |
| Acute myeloid leukemia | TAA (WT1) | ND | Produced by CureVac GmbH | Gene Pulser Xcell (Bio-Rad) | Exponential decay pulse (300 V, 7 ms) 4-mm cuvette | NCT00834002 [81] |
| Acute myeloid leukemia Chronic myeloid leukemia Multiple myeloma | TAA (WT1) | Linearized pGEM/WT1 pST1/sig-WT1-DC-LAMP pST1/sig-WT1-DC-LAMP-OPT (codon-optimized version of pST1/ sig-WT1-DC-LAMP) vectors | Produced by CureVac GmbH | Gene Pulser Xcell (Bio-Rad) | Exponential decay pulse (300 V, 7 ms) 4-mm cuvette | NCT00965224 [82] |
| Acute myeloid leukemia | TAA (hTERT-LAMP-1) | Linearized pGEM4Z/hTERT/LAMP/A64 vector | mMESSAGE mMACHINE high yield capped RNA transcription kit (Ambion) Purified with RNeasy kit (Qiagen) | Gene Pulser II (Bio-Rad) | Cells + mRNA for 5 min on ice Exponential decay pulse (300 V, 150 µF) 4-mm cuvette | NCT00510133 [83,84] |
| Acute myeloid leukemia | TAA (WT1 isoform A, PRAME, CMV pp65) | Codon-optimized mRNA | Produced at Oslo University Hospital | ND | ND | NCT01734304 [85-87] |
| Myelodysplastic syndromes Acute myeloid leukemia | TAA | ND | ND | ND | ND | NCT03083054 |
| Multiple myeloma | TAA | ND | ND | ND | ND | NCT01995708 |
| **Infectious diseases** | | | | | | |
| HIV | HIV antigen (Gag, Nef, Vpr, Rev (GNVR)) + immune modulating molecules (hCD40L) (AGS-004 product) | HIV antigens: PCR fragments hCD40L: Linearized pCR2.1 vector | mMessage mMachine T7 Ultra kit (Life Technologies) Purified with RNeasy columns (QIAGEN) | ND | ND | NCT02042248 [88,89] NCT02707900 [90] NCT00381212 [91] NCT01069809 NCT00672191 [92] |
| HIV | HIV antigen (HIV-1 Gag, Nef) | Codon-optimized coding sequence including endoplasmic reticulum translocation signal peptide, antigen polypeptide, and human lysosome-associated membrane protein-1 targeting sequence | Produced by Asuragen | Gene Pulser II (Bio-Rad) | Square wave pulse (900 V, 0.75 ms) | NCT00833781 [93] |
| HIV | HIV antigen (Tat, Rev, Nef, Gag, NP1) | Linearized pGEM-sig-Tat-DC-LAMP pGEM-sig-Rev-DC-LAMP pGEM-sig-Nef-DC-LAMP pST1-sig-Gag-DC-LAMP pGEM-Sig-Flu-NP1-DC-LAMP vectors | mMESSAGE mMACHINE™ kit (Life Technologies) | EQUIBIO EasyjecT Plus® (EQUIBIO) | $12\times10^6$ DC: 300 V, 150 µF, 99 Ω (pulse time 5–6 ms) $50\times10^6$ DC: 300 V, 450 µF, 99 Ω 4-mm cuvette | VUB-05-001 / MEC-2005-227 [94] |
| CMV | CMV pp65 | ND | Produced by Curevac GmbH | ND | ND | EudraCT 2008-006074-15 EudraCT 2008-000430-45[95] |

**Abbreviations:** CEA, carcinoembryonic antigen; CD, cluster of differentiation; CMV, cytomegalovirus; DC, dendritic cell; EP, electroporation; gp100, glycoprotein 100; HIV, human immunodeficiency virus; hTERT, human telomerase reverse transcriptase; LAMP, lysosome-associated membrane protein; MAGE, melanoma-associated antigen; mRNA, messenger RNA; ND, no data; PAP, prostatic acid phosphatase; PCR, polymerase chain reaction; PRAME, preferentially expressed antigen in melanoma; PSA, prostate specific antigen; TAA, tumor-associated antigen; TLR4, toll-like receptor 4; TRP2, tyrosinase-related protein 2; WT1, Wilms' tumor 1. Last search on clinicaltrials.gov and PubMed: 5 March 2021.

**Table 2. mRNA synthesis and electroporation conditions in clinical trials using mRNA electroporation for gene transfer in T cells**

| Condition | Gene | mRNA synthesis | | EP conditions | | Clinical trial Identifier and References |
|---|---|---|---|---|---|---|
| | | Template | Production | Device | Settings | |
| **Solid malignancies** | | | | | | |
| Malignant peritoneal mesothelioma | CAR | Linearized pDrive vector (Qiagen) (GOI + two repeats of 3'-UTR from beta globulin (2bgUTR) with or without 150 poly(A) tail) | mMESSAGE mMACHINE T7 kit (including regular cap analog; Life Technologies) mMESSAGE mMACHINE T7 Ultra kit (including anti-reverse cap analog; Life Technologies) mScript™ RNA System (including capping enzyme and 2'-O-Methyltransferase capping enzyme to generate Cap 1 IVT RNA; Epicentre) | BTX ECM 830 square wave electroporator / Maxcyte | 2-mm cuvette (BTX) / OC-400 (Maxcyte) | NCT01355965 [96-98] |
| Pancreatic ductal adenocarcinoma Breast cancer | CAR | Linearized pDrive vector (Qiagen) (GOI + two repeats of 3'-UTR from beta globulin (2bgUTR) with or without 150 poly(A) tail) | mMESSAGE mMACHINE T7 kit (including regular cap analog; Life Technologies) mMESSAGE mMACHINE T7 Ultra kit (including anti-reverse cap analog; Life Technologies) mScript™ RNA System (including capping enzyme and 2'-O-Methyltransferase capping enzyme to generate Cap 1 IVT RNA; Epicentre) | Maxcyte | ND | NCT01897415 [98-100] NCT01837602 [98-101] |
| Breast cancer | CAR | ND | ND | ND | ND | NCT03060356 [102] |
| Hepatocellular carcinoma | TCR | Linearized pVAX1 vector | mMESSAGE mMACHINE T7 Ultra kit (including anti-reverse cap analog; Life Technologies) Concentrated by lithium chloride precipitation Dissolved in T4 buffer (BTX) | AgilePulse Max system (BTX) | Manufacturer's recommended protocol | NCT02719782 [103-105] NCT03634683 [103,104] NCT03899415 [103,104,106] |
| Hepatocellular carcinoma | TCR | ND | ND | ND | ND | NCT04745403 |
| Colorectal cancer | TCR | mRNA expression vector Sequence containing 2A construct | Capping: Anti-Reverse Cap Analog (TriLink Biotechnologies Inc.) | BTX ECM 830 square wave electroporator | Square Wave pulse (500 V, 2 ms) 4-mm cuvette | NCT03431311 [107,108] |
| **Hematological malignancies** | | | | | | |
| Hodgkin lymphoma | CAR | Linearized pGEM4-Z/A64 vector | mMESSAGE mMACHINE T7 Ultra kit (including anti-reverse cap analog and *in vitro* poly(A) tailing ("E-PAP"); Life Technologies) | Gene Pulser Xcell (BioRad) | Square wave pulse (500 V, 5 ms) | NCT02277522 NCT02624258 [109-111] |
| B-cell non-Hodgkin's lymphoma B-cell chronic lymphocytic leukemia | CAR | ND | ND | ND | ND | NCT02315118 [112] |
| Acute myeloid leukemia | CAR | Linearized pDA vector | mMESSAGE mMACHINE T7 Ultra kit (including anti-reverse cap analog; Life Technologies) mRNA purified by RNeasy Mini Kit (Qiagen) | BTX ECM 830 square wave electroporator | 2-mm cuvette | NCT02623582 [113-115] |
| Multiple myeloma | CAR | Linearized DNA plasmid Codon-optimized nucleotide sequence containing 3'-UTR, mouse alpha globin 5'-UTR, and poly(A) tail | ND | ND | ND | NCT03448978 [116,117] |
| **Autoimmune diseases** | | | | | | |
| Type 1 diabetes | Peptide-MHC-CD3-zeta construct | ND | ND | ND | ND | NCT02117518 |
| **Infectious diseases** | | | | | | |
| HIV | ZFN | Linearized pDA-A.2bg.150A vector | mMESSAGE mMACHINE T7 Ultra kit (including anti-reverse cap analog; Life Technologies) mRNA purified by RNeasy Maxi kit (Qiagen) | MaxCyte GTTM Flow Transfection System | ND | NCT02388594 [118] |

**Abbreviations:** CAR, chimeric antigen receptor; CD3, cluster of differentiation 3; EP, electroporation; GOI, gene of interest; IVT, *in vitro* transcribed; MHC, major histocompatibility complex; mRNA, messenger RNA; ND, no data; TCR, T-cell receptor; UTR, untranslated region; ZFN, zinc finger nuclease. Last search on clinicaltrials.gov and PubMed: 5 March 2021.

Table 3. mRNA synthesis and electroporation conditions in clinical trials using mRNA electroporation for gene transfer in natural killer cells

| Condition | Gene | mRNA synthesis | | EP conditions | | Clinical trial identifier and Reference |
|---|---|---|---|---|---|---|
| | | Template | Production | Device | Settings | |
| Colorectal cancer | CAR | PCR product from pFBCMV-T7 vector GOI + 5′-UTR with Kozak sequence, and ClaI, the GM-CSF signal peptide encoding sequence (SP) and the alpha-globin 3′-UTR sequence | mMESSAGE mMACHINE T7 Ultra kit (including anti-reverse cap analog (ARCA); Life Technologies) mScript™ RNA system (Epicentre) | NEPA21 electroporator (Nepagene) BTX electroporator (AgilePulse) | 2 or 4-mm cuvette | NCT03415100 [119] |

**Abbreviations:** CAR, chimeric antigen receptor; EP, electroporation; GOI, gene of interest; mRNA, messenger RNA; ND, no data; UTR, untranslated region. Last search on clinicaltrials.gov and PubMed: 5 March 2021.

# Concluding remarks

Electroporation of mRNA is a versatile methodology for the transient expression of proteins of interest. As a highly flexible system, it allows the fine-tuning of transfection conditions for each cell type and to multiplex mRNAs as required. The selection of the best transfection conditions for mRNA ensures maximal transfection efficiency, and thus protein expression, without compromising cell viability. As we have noted, there is a wide variety of options when it comes to improving both the electroporation conditions and stability/translation of the mRNAs for monocistronic and polycistronic gene transfer. These enhancements and different tools can be used either alone or in combination, depending on the needs of the study. Although we have focused on conventional mRNA, similar statements are true for other types of RNA, such as small interfering RNA, guide RNA in a CRISPR setting, or unconventional self-replicating mRNA but also for purposes other than the transient gene transfer, as in gene silencing and gene disruption. The safety of the system due to its transient non-integrative approach together with its simplicity in terms of the basic equipment needed for its application ensure that mRNA electroporation will continue to be an essential method for non-viral genetic engineering in cell-based immunotherapies, especially in a clinical setting.

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**Escribir es siempre muy difícil, sobre todo hacerlo de forma aparentemente sencilla.**

— Ana María Matute

# 3

# Advances in cellular cancer immunotherapy using messenger RNA electroporation for versatile gene transfer

This chapter is under review in:

Campillo-Davo D, Roex G, Versteven M, Flumens D, Berneman ZN, Van Tendeloo VFI, Anguille S, Lion E.
*Molecular Therapy – Nucleic Acids* (2021).

# Abstract

Electroporation of messenger RNA (mRNA) is a widely used ex vivo non-integrating gene transfer technique in immune cell-based trials for cancer to transiently supply immune cells with multiple proteins. This technique has been used to engineer dendritic cells and B cells with tumor-associated antigens to boost the immune system of cancer patients and to redirect the antitumor activity of T cells and natural killer cells with immune receptors. Although this form of gene delivery results in only transient expression of the protein of interest, many investigators and clinicians are turning towards mRNA electroporation as a feasible, flexible, and safe technique over stable expression methods using viral vectors. In this review, we delve into the progress made regarding mRNA electroporation for gene transfer and assess the strengths and limitations of this technique for redirecting and boosting immune responses against different tumor antigens in cell-based cancer immunotherapy.

# Introduction

Over the past decades, gene therapy has revolutionized medicine in an unprecedented way. Among the multiple delivery systems for gene transfer, electroporation of messenger RNA (mRNA) has been widely used in cancer immunotherapy to supply immune cells with a myriad of proteins, since it offers a rapid, safe, and efficient method for genetically engineering immune cells. Electroporation is a non-viral efficient technology based on the formation of pores on the cell membrane by means of an electric field. The transient disruption of the integrity of the cell membrane allows the passage of genetic material and other molecules surrounding the cells into the cytoplasm. In the case of mRNA electroporation, protein-coding synthetic mRNA is added to the electroporation medium to induce the transient expression of proteins of interest. As our group demonstrated twenty years ago, mRNA electroporation is superior to the passive pulsing of peptides and proteins and lipofection of mRNA, and even to electroporation of plasmid DNA [1,2]. The flexibility of mRNA electroporation, its safety profile compared to viral vectors [3,4] as well as its simplicity and minimum equipment required for applying it both in a preclinical and clinical setting have also demonstrated over the years that this tool can be used to engineer multiple immune cell types with different proteins and for diverse purposes. First developed for the activation of the host immune system against tumor cells by loading antigen presenting cells (APCs) with mRNAs coding tumor antigens, this technique is gaining momentum for use in other immune cell types to redirect and bolster their tumor-killing capacities. In this review, we discuss the advances, benefits, and limitations of this type of gene transfer into different human primary cell types in the context of cell-based cancer immunotherapies.

# Messenger RNA electroporation of dendritic cells

As key orchestrators of the immune system, dendritic cells (DCs) have gained substantial attention over the last years for use as a cellular vaccine against cancer. Due to their central role in interactions with a plethora of immune effector cells, optimization of DC vaccines is of strategic interest. Historically this has been done using a range of techniques [5-7]; however, the focus of this section will be on the use of mRNA electroporation. Depending on the stage of DC development and the mRNA constructs used, this approach has been used with DCs for several purposes [8] and has included both human DCs and murine DCs [9-11] (**Table 1**). First, mRNA electroporation can be used in the maturation of immature DCs. Second, mature DCs can be electroporated

with mRNA as a means to load the DCs with tumor-associated antigens (TAA) or third to increase their immunogenicity. Finally, DCs can be electroporated with mRNA encoding secondary molecules. In this section, we will elaborate on these four purposes.

To induce maturation, immature DCs are classically treated with a mixture of cytokines [12]; however, electroporation with specific mRNAs can also be used for this purpose. A combination of three different mRNAs coding for cluster of differentiation (CD)70, CD40 ligand (CD40L), and constitutively active toll-like receptor 4 (caTLR4) have been co-electroporated into immature DCs to induce maturation [13,14]. This combination has been named TriMix and the resulting TriMix-electroporated DCs have been used in a number of studies [15-18]. A major advantage of these DCs is that they do not require a 48-hour maturation period, which can potentially render DCs exhausted. Because of the rapid expression of the maturation molecules, TriMix-electroporated DCs can be delivered to a patient within a few hours after electroporation [15]. Furthermore, additional studies have shown that this protocol is compatible with the simultaneous electroporation of tumor antigen mRNA [15,16,19-21]. In addition, others have combined a short maturation process using pro-inflammatory cytokines interferon (IFN)-$\gamma$ and tumor necrosis factor-$\alpha$, with the co-electroporation with TAA-encoding mRNA and CD40L mRNA [22,23]. DCs treated in this manner show improved interleukin (IL)-12 secretion compared to DCs that are not electroporated with CD40L mRNA. Moreover, DCs treated with both a pro-inflammatory cytokine cocktail and CD40L mRNA demonstrate superior induction of CD8 T-cell cytolytic activity [22]. Taken together, these studies demonstrate the importance of mRNA electroporation-mediated DC maturation. Consequently, several clinical trials have been or currently are being conducted using this maturation approach as a DC vaccine of melanoma (clinicaltrials.gov Identifier: NCT01302496, NCT01066390, NCT01530698).

Antigen delivery is probably the most common application of mRNA electroporation in DCs. It is routinely done by either pulsing DCs with one or more peptides or by electroporating tumor-antigen-specific mRNA. The latter technique offers important advantages over the use of peptide pulsing. For instance, electroporation of the full-length sequence of the TAA results in a multi-epitope DC vaccine, which can also present TAA epitopes that may be currently unknown [24]. Furthermore, this approach offers the advantage that DC vaccines can be generated without prior knowledge regarding a patient's human leucocyte antigen (HLA) haplotype [25]. As DCs electroporated with TAA-encoding mRNA are capable of presenting TAA-epitopes by both HLA class I and class II molecules, they can stimulate both CD8 and CD4 T cells [26,27]. While both these advantages also apply to the transduction of DCs with a TAA

encoding plasmid, mRNA electroporation results in transient expression without the risk of integration into the host genome, making it a clinically safer vaccine. Furthermore, mRNA electroporation has a higher transfection efficiency compared to that of plasmid DNA transduction [1,28]. One study tested sixteen different constructs of several TAAs and found electroporation to be a highly efficient method for introducing TAA-mRNA without affecting the phenotype of the DCs [28]. These observations demonstrate the broad applicability of this technique and explain the wide variability in TAAs that have been chosen by investigators for mRNA electroporation of DCs. While not a TAA on its own, *EGFP* mRNA is frequently used to optimize electroporation settings [29,30]. Frequently used TAAs (previously reviewed by [31]) include Wilms' tumor protein 1 (WT1) [16,32-37], melanoma-associated antigen 3 [26], glycoprotein 100 (gp100) [38], melanoma antigen recognized by T cells 1 (MART-1) [22,23,39] and mucin (MUC) 1 [40]. Several minor histocompatibility antigens have also been electroporated into DCs, including mRNAs encoding for purinergic receptor P2X5 and minor histocompatibility protein HA-1 [41-43]. In addition, mRNA electroporation of DCs has also been applied using mRNA encoding viral antigens including human immunodeficiency virus proteins [44-46] and human cytomegalovirus pp65 antigen [47], and mRNA encoding antigens related to autoimmune disorders [48]. To prevent immune escape by tumor cells via downregulation of TAAs, DCs can be electroporated with multiple mRNAs. For instance, in a pancreatic cancer model, DCs co-electroporated with MUC4 mRNA and survivin mRNA induced stronger cytotoxic T-cell responses compared to that of DCs electroporated with a single mRNA [49]. To further maximize the number of T cells reacting to an antigen-specific DC vaccine and to ensure all tumor-derived antigens are presented by the DCs, several groups have electroporated DCs using whole tumor mRNA [50,51].

TAA-mRNA loaded DCs are currently being used in a large number of clinical trials [52]. However, their antigen-specific immune stimulating properties are not limited to clinical use and may serve as useful research tools shedding light on key aspects of DC electroporation and help enhance their future preclinical development [53]. In this regard, mRNA-electroporated DCs have been used to investigate their long-term immunological impact by means of immunomonitoring [54]. Furthermore, TAA-mRNA electroporated DCs have been used to stimulate and expand CD8 T cells ex vivo in order to generate antigen-specific T-cell clones [55] from which immunodominant T-cell receptors (TCRs) can be isolated for the purpose of adoptive T-cell transfer [56,57].

There remains a need for a consensus regarding the optimal culture stage for TAA-mRNA electroporation. While immature DCs have antigen uptake as their main

function, mature DCs are ideally suited for antigen presentation. Research into the optimal time point for mRNA electroporation of DCs has shown that electroporation using mature DCs results in a higher viability compared to that of using immature DCs [58]. This study also showed that electroporation of DCs after maturation results in greater capability to induce antigen specific T-cell responses [58]. Interestingly, the capacity for transgene expression is dependent on the maturation cocktail used. Schuurhuis *et al.* demonstrated that DC maturation in the presence of poly(I:C) results in decreased protein expression after mRNA electroporation [59]. Another study showed that DCs electroporated after their maturation have a greater capacity to migrate in in vitro migration assays [60]. Despite the observations in favor of electroporating DCs post-maturation, TAA-mRNA electroporation is still performed using DCs during their immature and mature stages. The combination of double electroporation of DCs at both the immature and mature stage has also been tested, but this resulted in a substantial decrease in DC viability and yield [41].

DCs have also been electroporated in effort to increase their immunogenicity with several constructs being used. Co-electroporation of DCs with mRNA coding for IL 15 and the $\alpha$-chain of the IL-15 receptor results in the transpresentation of IL-15 on the membrane of IL-4 DCs. This proved to be a successful strategy for increasing tumor-antigen-specific T-cell activation [61,62]. Furthermore, these so-called IL-15 transpresenting DCs are able to activate natural killer (NK) cells, which is in contrast to conventional IL-4 DCs [63]. Electroporation of DCs with CD40L or OX40 ligand (OX40L) mRNA also fits the increasing immunogenicity category as they deliver important costimulatory signals to T cells during the DC/T-cell interaction [17,23,64]. Another strategy to increase the immunogenicity of DCs is to increase their migratory potential to regional lymph nodes. This can be accomplished by electroporating DCs to express a human chimeric CD62/CD62 ligand (CD62L) protein [65].

A final application of mRNA electroporation of DCs is its use for the delivery of other types of molecules. As DCs possess the capacity to move towards lymph nodes and interact with a multitude of effector cells [66], they are exceptionally well suitable for the production of immune modulatory molecules at these locations. As such, IFN-$\alpha$ mRNA electroporated DCs have been suggested to be potent inducers of adaptive and innate antitumor immunity [67]. Similarly, IL-21 mRNA-electroporated DCs have been shown to increase the cytotoxic capacity of TAA-specific T cells [68]. With the rise in use of immune checkpoint blockade in combination with DC vaccination, in situ delivery of checkpoint inhibitors is an interesting approach. Messenger RNA encoding for the soluble portion of programmed death protein-1 (PD-1) and PD-1 ligand results

in the production of these molecules at the site of T-cell interaction, leading to increases in the number of multifunctional T cells and downstream cytokine secretion [69]. Similarly, DCs have been electroporated with mRNA encoding the heavy and light chain of an antibody specific for DcR3, a decoy receptor for Fas ligand that is overexpressed in a number of tumors and known to promote tumor growth [70]. These DCs, co-electroporated with TAA-encoding mRNA, are able to secrete anti-DcR3 antibody leading to increased tumor-specific T-cell activation [70]. Electroporation of DCs with antibody-encoding mRNA has also been described for anti-glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related (GITR) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [71,72]. Moreover, this strategy has also been evaluated in a clinical trial for patients with metastatic melanoma (clinicaltrials.gov Identifier: NCT01216436).

**Table 1. Electroporation settings for dendritic cells**

| Cell type | Gene(s)/Antigen(s) | EP system (Company) | Type of pulse | Voltage (V) | Capacitance (µF) | Time (ms) | Gap (mm) | Buffer (µL) | Amount of mRn.a. (µg) | Cell number ($x10^6$ cells) | Cell concentration ($x10^6$ cells/mL) | % Protein expression (time point) | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IL-4 DCs | GFP, CHO-GITR, CHO-CTLA-4 | ECM830 (BTX) | SW | 300 | n.d. | 0.5 | 2 | 100-200 | n.a. | n.a. | 25-30 | n.d. | [8] |
| BM-derived mouse IL-4 DCs | EGFP, OVA | EasyJect Plus (EquiBio) | n.d. | 300 | 150 | 6 | 4 | 200 | 20 | n.a. | 20 | 77% EGFP (24h) | [9] |
| BM-derived mouse IL-4 DCs | EGFP, CEA | Gene Pulser (BTX) | SW | 300 | n.a. | 0.5 | 2 | 200 | 20 | n.a. | 5 | 40% EGFP (24h) | [10] |
| IL-4 DCs | TriMix, MelanA-A2, NGFR | EasyJect Plus (EquiBio) | n.d. | 300 | 150 | n.a. | n.d. | n.d. | 10-20 | 4 | n.a. | ~60% CD40L (4h), 78% CD70 (24h) | [13] |
| BM-derived mouse IL-4 DCs | TagBFP | Gene Pulser Xcell (Bio-Rad) | ED | 300 | 150 | n.a. | 4 | 200 | 10 | 4 | 20 | 90.8% (24h) | [14] |
| IL-4 DCs | TriMix, MAGE-A3, MAGE-C2, tyrosinase, gp100 | Gene Pulser Xcell (Bio-Rad) | n.d. | 300 | 450 | n.a. | 4 | 600 | 20 µg TriMix + 60 µg TAA encoding mRn.a. | 50 | n.a. | n.d. | [15] |
| Immature IL-4 DCs | CD40L, caTLR4, CD70 | Gene Pulser Xcell (Bio-Rad) | SW | n.d. | n.d. | n.d. | n.d. | 200 | n.d. | n.d. | n.d. | n.d. | [17] |
| IL-4 DCs | TriMix, Melan-A2, MAGE-A3, MAGE-C2, tyrosinase, gp100 | EasyJect Plus (EquiBio) | n.d. | 300 | 450 | n.a. | n.d. | 600 | 20 µg TriMix + 60 µg TAA encoding mRn.a. | 50 | n.a. | ~80% CD70 (24h) | [18] |
| BM-derived mouse IL-4 DCs | OVA, tyrosinase, WT1, tNGFR, TriMix | EasyJect Plus (EquiBio) | n.d. | 300 | 150 | 6 | 4 | 200 | 20 | n.a. | 20 | n.d. | [19] |
| IL-4 DCs | PSA-1, MART-1, MART-1-APL | n.d. (Bio-Rad) | n.d. | n.d. | n.d. | n.d. | 4 | 500 | 2 µg/10^6 cells TAA + 4 µg/10^6 cells CD40L | n.a. | 40 | Dependent on the protocol | [22] |
| Immature IL-4 DCs | MAGE-A3 with different signal sequences (Ii, LAMP1, DC-LAMP) | EasyJect Plus (EquiBio) | n.d. | 300 | 150 | ~5 | 4 | 200 | 20 or 40 | 4 or 8 | n.a. | 7-17 x 10e3 (relative expression in qPCR) | [26] |
| IL-4 DCs | 16 different constructs | Gene Pulser Xcell (Bio-Rad) | SW | 500 | n.a. | 1 | 4 | 100-600 | 50 µg/mL | n.a. | 30-60 | 65.62% MelanA (24h) | [28] |
| IL-4 DCs | different WT1 constructs | Gene Pulser Xcell (Bio-Rad) | n.d. | 300 | 150 | n.d. | 4 | 200 | 20 | 8 | | n.d. | [32] |
| IL-4 DCs | WT1 | Gene Pulser Xcell (Bio-Rad) | TC | 300 | n.a. | 7 | 4 | 200-500 | 20 | max 50 | n.a. | n.d. | [33] |
| IL-4 DCs | WT1, TriMix (CD40L, caTLR4, CD70) | EasyJect Plus (EquiBio) | n.d. | 300 | 150 | n.a. | n.d. | n.d. | 20 µg WT1 and 5µg of each of the TriMix mRn.a.s | 4-5 | n.a. | n.d. | [34] |
| IL-4 DCs | gp100, Tyrosinase | Gene Pulser Xcell (Bio-Rad) | ED | 300 | 150 | n.a. | 4 | 200 | 20 | 12 | n.a. | n.d. | [38] |
| IL-4 DCs | EGFP, MelanA1, NGFR, IMP-1 | EasyJect Plus (EquiBio) | n.d. | 300 | 150/ 450 | 5-6 | 4 | 200/600 | 30 | 12 or 50 | 60 or 83 | 58% (X-15), 76% (Optimix) | [39] |
| IL-4 DCs | MUC1 | Gene Pulser II (Bio-Rad) | n.d. | 300 V/cm | 150 | n.a. | 4 | 200 | 8 | n.a. | 10-40 | 20 (relative quantification with RT-qPCR) | [40] |
| IL-4 DCs | MiHA mRNA (P2x5, HMHA1) | Gene Pulser II (Bio-Rad) | ED | 200 | 150 | n.d. | n.d. | n.d. | 20 | n.d. | n.d. | n.d. | [41] |
| IL-4 DCs | P2x5 | Gene Pulser II (Bio-Rad) | n.d. | 300 | 150 | n.d. | | 200 | 20 | n.d. | n.d. | 10^2 relative expression (RT-qPCR) (24h) | [42] |
| IL-4 DCs | MiHA mRNA (P2x5, HMHA1) | Gene Pulser II (Bio-Rad) | n.d. | 200-300 | 150 | n.a. | 4 | 200 | 20 | n.d. | n.d. | n.d. | [43] |
| IL-4 DCs | Gag | EasyJect Plus (EquiBio) | n.d. | 300 | 150 | n.d. | n.d. | n.d. | 20 | 4 | n.a. | "all cells displayed strong intracellular Gag expression" | [44] |
| IL-4 DCs | MUC4, surviving | Gene Pulser II (Bio-Rad) | n.d. | n.d. | n.d. | n.d. | 4 | 500 | 20 µg per construct | n.d. | 10-40 | n.d. | [49] |
| IL-4 DCs | whole tumor RNA | Nucleofector (Amaxa) | Program U16 | n.d. | n.d. | n.d. | n.d. | 100 | 2 µL (conc. 500 ng/mL - 20 µg/mL) | 0.5-10 | n.a. | n.d. | [50] |
| Immature IL-4 DCs | whole tumor RNA | ECM830 (BTX) | SW | 500 | n.d. | 0.3 | n.d. | 100 | 1.5 µg/10e6 cells | n.a. | 10 | 87.8% EGFP (24h) | [51] |
| IL-4 DCs | MelanA | n.d. | SW | 500 | n.a. | 0.5 - 1 | 4 | 100-600 | 50 µg/mL | max 60 | n.a. | 89.3% (4h) | [53] |
| DCs | p53, survivin, hTERT, EGFP, CEF | ECM830 (BTX) | SW | 500 | n.d. | 2 | 4 | 400 | 5 | 9 | n.a. | n.d. | [54] |
| Immature IL-4 DCs | FoxP3 | ECM830 (BTX) | SW | 300 | n.a. | 0.5 | 2 | 200 | 15-20 | 5-6 | n.a. | n.d. | [55] |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IL-4 DCs | GPC3, HLA-A2 | Gene Pulser Xcell (Bio-Rad) | ED | 300 | 150 | n.a. | n.d. | n.d. | 35 µg GPC3<br>60 µg HLA-A2 | n.d. | n.d. | HLA-A2: 4% (3h) – 56% (24h)<br>GPC3: 24% (3h) | [56] |
| IL-4 DCs | HLA-A2 | ECM830 (BTX) | SW | 1250 V/cm | n.a. | 3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [57] |
| IL-4 DCs | EGFP, M1 | ECM830 (BTX) | SW | 500 | n.d. | 1 | 4 | 250 | 25 | | 5 | ~90% (24h) | [58] |
| IL-4 DCs | gp100, tyrosinase, CEA | Gene Pulser Xcell (Bio-Rad) | ED | 300 | 150 | n.d. | 4 | 200 | 20 | 10 | n.a. | 81% gp100, 12% tyrosinase, 76% CEA (24h) | [59] |
| | | | SW | 400 | n.d. | 0.6 | | | | | | | |
| IL-4 DCs | EGFP, MAGE-A3, MelanA | EasyJect Plus (EquiBio) | n.d. | 300 | 150 | n.d. | 4 | n.d. | n.d. | n.d. | n.d. | 89.1% (24h maturation post EP) or 93.4% (EP post maturation) | [60] |
| IL-4 DCs | OSP-IL-15, IL-15Ra | Gene Pulser Xcell (Bio-Rad) | TC | 300 | n.d. | 7 | 4 | 200 | 5 µg per construct | n.d. | n.d. | High | [61] |
| IL-4 DCs | IL-15, IL-15Ra | Gene Pulser Xcell (Bio-Rad) | TC | 300 | n.a. | 7 | 4 | 200 | 10 µg per construct | n.d. | n.d. | n.d. | [63] |
| IL-4 DCs | CD62E/CD62L | Gene Pulser Xcell (Bio-Rad) | SW | 500 | n.a. | 1 | 4 | 100-600 | 50 µg/mL | n.a. | 30-60 | 96% (28h) | [65] |
| IL-4 DCs | IFN-α, WT1 | Gene Pulser Xcell (Bio-Rad) | TC | 300 | n.a. | 7 | 4 | 200 | 10 µg per construct | n.d. | n.d. | $>4x10^3$ IU IFN-α /$5x10^5$ DCs | [67] |
| IL-4 DCs | IL-21, MART-1, EGFP, IL-12 | EasyJect Plus (EquiBio) | n.d. | 300 | 150 | n.a. | 4 | 200 | 10 | n.d. | n.d. | 90% EGFP (24h), 600 pg/million cells IL-21 | [68] |
| Immature IL-4 DCs | tNGFR, sPD-1, sPD-L1 | Gene Pulser Xcell (Bio-Rad) | n.d. | 300 | 150 | 5-6 | | 200 | 20 | 4 | n.a. | secretion measured, but mentioned as OD-value | [69] |
| IL-4 DCs | anti-DcR3 mAb | Gene Pulser II (Bio-Rad) | SW | 500 | n.a. | 0.3 | 4 | 500 | 3-10 µg | n.a. | 10-40 | < 15% but high secretion (24h) | [70] |
| IL-15 DCs | EGFP, M1 | Gene Pulser Xcell (Bio-Rad) | ED | 300 | 150 | 7 | 4 | 200 | 20 (EGFP)<br>10 (M1) | 1 | 5 | 50-75% (4h, 24h, 48h) | [29] |

Abbreviations: APL, altered peptide ligand; BM, bone marrow; C, capacitance; caTLR4, constitutively active toll-like receptor 4; CD62E, CD62 antigen-like family member E (E-selectin); CD40L, CD40 ligand; CD62L, CD62 antigen-like family member L (L-selectin); CEA, carcinoembryonic antigen; CEF, cytomegalovirus, Epstein-Barr virus and influenza virus; CHO-GITR, Chinese hamster ovarian derived glucocorticoid-induced tumor necrosis factor receptor; CHO-CTL4, Chinese hamster ovarian derived cytotoxic T-lymphocyte associated protein 4; DC, dendritic cell; DC-LAMP, dendritic cell-lysosome associated membrane protein; ED, exponential decay; EGFP, enhanced green fluorescent protein; EP, electroporation; FoxP3, forkhead box P3; Gag, group specific antigen ; GFP, green fluorescent protein; gp100, glycoprotein 100; h, hours; hTERT, human telomerase reverse transcriptase; IFN-α, interferon alpha; IL-, interleukin; IMP-1, influenza matrix protein 1; LAMP, lysosome-associated membrane protein ; M1, matrix protein 1; mAb, monoclonal antibody; MAGE, melanoma associated antigen; MART-1, melanoma antigen recognized by T cells 1; MelanA, melanoma antigen recognized by T cells ; MiHA, minor histocompatibility antigen; mRNA, messenger RNA; MUC1, mucin1; n.a., not applicable; n.d., no data; NGFR, nerve growth factor receptor; OVA, ovalbumin; P2x5, purinoceptor 5; p53, tumor protein 53; PSA-1, prostate-specific antigen 1; Ref., reference; sPD-1, soluble programmed death protein 1; sPD-L1, soluble programmed death receptor ligand 1; SW, square wave; TagBFP, blue fluorescent protein; TC, time constant; tNGFR, truncated rat nerve growth factor receptor; TriMix, mRNA encoding CD70, CD40L and a constitutively active toll-like receptor 4; WT1, Wilms' tumor 1.

# Messenger RNA electroporation of T cells

## T-cell receptor gene transfer

T cells have been widely exploited in cancer immunotherapy due to their antitumor effector activity [73]. They possess TCRs that are highly specialized in recognizing internally-processed peptides presented by the major histocompatibility complex (MHC), including TAAs. Unfortunately, T cells recognizing TAAs, especially those that are autoantigens, are usually scarce due to the negative selection of auto-reactive T cells. To circumvent this problem, T cells have been engineered with TAA-specific *TCR* mRNAs isolated from TAA-reactive T cells [74,75]. Given the transient nature of mRNA electroporation, strategies to achieve optimal TCR expression are of special importance. TCR expression can be enhanced by increasing the correct TCR pairing, reducing CD3 competition with endogenous TCRs, or by improving TCR affinity. For example, when a secondary TCR is expressed in a T cell, chains from the endogenous and transgenic TCRs can pair incorrectly. This can be prevented by humanized or murinized TCR chains (for murine- or human-derived TCRs, respectively) [76-78], additional interchain disulfide bonds [79], single chain TCRs [80], swapping human TCR chain domains [81], or combination of these techniques [80,82]. Our group has shown that electroporation in a two-step process with silencing RNAs (siRNAs) against T-cell receptor alpha constant and T cell receptor beta constant transcripts downregulates de novo expression of endogenous TCR chains, which improves both CD3 availability and the pairing of transgenic TCR [83]. High-affinity antitumor TCRs, which exhibit improved interaction between TCR and the peptide-MHC (pMHC) complex, rarely occur naturally. However, they can be generated by using humanized mice [84], mismatched HLA donors [85] or phage display techniques [86] and can be further optimized through genetic mutation [87,88].

Different tumor antigens have been targeted with TCR-mRNA electroporated T cells (**Table 2**), including MART-1 [74,77-79,88-90], tumor protein p53 [74,77-80], WT1 [83], carcinoembryonic antigen (CEA) [91], gp100 [77,80,82,92,93], chaperonin containing TCP1 subunit 6A [93], and New York esophageal squamous cell carcinoma 1 (NY-ESO-1) [74,78,79,86,88]. However, other types of epitopes can also be targeted using TCR-mRNA-redirected T cells, such as tumor neoantigens [93,94] and viral antigens, which can be expressed in cancer cells [95-97] or in virus-related complications after allogeneic hematopoietic stem cell transplantation (HSCT) [98,99]. Inderberg and colleagues isolated a TCR directed against a frameshift mutation in transforming growth factor β receptor II (TGFβRII), which is present in the majority of microsatellite instable colon

cancers [94]. In a xenograft transplantation model, anti-TGFβRII TCR-mRNA modified T cells reduced tumor burden and prolonged survival after repeated intraperitoneal injections, which was contrary to what was observed following repeated intravenous (IV) injections [100]. The lack of activity after IV injection suggests that transiently modified T cells may benefit from injection near the tumor site, as trafficking long distances before encountering the tumor cells may result in reduced TCR availability. In 2018, the use of these anti-TGFβRII TCR-mRNA modified T cells received approval for a first-in-human trial involving TCR-mRNA transfection (clinicaltrials.gov Identifier: NCT03431311; **Table 3**). However, the trial was terminated by the sponsor in June 2019 after recruiting only one out of the five intended patients.

Although conventional TCRαβ CD8 T cells have been widely used in TCR engineering experiments, interest has risen over the past years in transfecting unconventional immune cells or other types of immune receptors in addition to TCRs. Kyte and colleagues showed that HLA class II-restricted TCRs against telomerase-positive target cells from helper T cells isolated from long-term cancer survivors can also be successfully used to transiently modify both CD4 and CD8 T cells [101]. Importantly, due to the potential off-target specificities of allo-reactive TCRs, suicide genes such as RQR8 can be included in the mRNA sequence so that toxicities arising from these T cells can easily be abrogated [101]. More recently, Klobuch and colleagues showed the potential of using TCRs for helper T cells against allogeneic HLA class II DPB1 antigens, a rising candidate in graft-versus-leukemia reactions [102]. The mRNAs of these TCRs can also be transfected into both CD4 and CD8 T cells, which then effectively recognize human primary acute myeloid leukemia blasts. However, the reactivity of allogeneic TCRs must be carefully assessed as they can potentially be promiscuous leading to off-target reactivities [85]. Similar to CD4 T cells, other T-cell types have advantageous properties that have not yet been fully exploited. For instance, an interesting approach is the use of γδ T cells. Although γδ T cells only represent a small fraction of peripheral blood T cells, they can be expanded ex vivo to clinically relevant numbers, both from healthy donors and patients [92,103]. These T cells are characterized by TCRγδ, which do not mispair with TCRαβ. In a comparative analysis using a current good manufacturing practice (GMP)-adaptable protocol, Harrer and colleagues showed that γδ T cells are equally capable of expressing TCRαβ as conventional CD8 T cells and are just as efficient in their antitumor capacity [92]. In fact, γδ T cells exhibited an even safer profile for adoptive therapy than CD8 T cells with less background secretion of cytokines, supporting the choice of γδ T cells as an excellent alternative for antitumor TCR engineering [92]. Another attractive approach in TCR engineering is to generate T cells expressing two additional receptors (TETARs) [93]. TETARs provide dual TAA

specificity, which may further address the problem of tumor immunoediting and escape. Höfflin and colleagues successfully transfected two TCRs, one against a common tumor antigen and another against a patient-specific neoantigen [93]. These investigators showed that titration of the mRNAs and murinization of the TCR chains were vital for the success of this technique in order to avoid TCR mispairing between the introduced TCR chains and competition for cellular factors. The transfer of innate receptors, such as semi-invariant natural killer T-cell (NKT) receptors, to conventional and unconventional T cells represents an interesting alternative to conventional TCR engineering by conferring HLA-unrestricted antitumor NKT features [104]. Transfection with NKT receptors may pave the way for implementation of more potent allogeneic and universal T-cell therapy platforms in cancer T-cell immunotherapy, especially in the case of expanded γδ T cells [104]. The flexibility and simplicity of TCR-mRNA electroporation should facilitate the development of off-the-shelf TCR-engineered reference samples (TERS) to control antigen-specific T-cell performance in functional assays compared to other techniques that would entail significantly more work [105,106]. These TERS can be produced in a standardized fashion using multiple electroporation systems across different laboratories, ensuring aliquots of TERS effectively control the performance, sensitivity and deviations of common T-cell assays [107].

**Table 2. Electroporation settings for T cells with T-cell receptors**

| Cell type | TCR specificity | Restriction | Cancer type | EP system (Company) | Type of pulse | Voltage (V) | Time (ms) | EP gap (mm) | EP buffer (µL) | Amount of mRNA (µg) | Cell number (x$10^6$ cells) | Cell concentration (x$10^6$ cells/mL) | % TCR expression (time point) | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stimulated CD8 T cells from melanoma patients | NY-ESO-1, MART-1 and p53 TCR | HLA-A2 | Melanoma | ECM830 (BTX) | SW | 400 | 0.5 | 2 | 50-200 | 2 µg/$10^6$ cells | n.a. | 25 | 45-65% (ON) | [74] |
| CD8 T cells | gp100 | HLA-A2 | Melanoma | Gene Pulser Xcell (Bio-Rad) | SW | 500 | 5 | 4 | 100-600 | 150 µg/mL | n.a. | 80 | 93% EGFP (4h) low % TCR (24h) | [75] |
| Expanded PBLs | MART-1, gp100, p53 | HLA-A2 | Melanoma | ECM830 (BTX) | SW | 400 | 0.5 | 2 | n.d. | 1 µg/$10^6$ cells | n.a. | 20 | 69-72% (24h) | [77] |
| Expanded PBLs from melanoma patients | MART-1, p53, NY-ESO-1 | HLA-A2 (MART-1, p53), HLA-DP4 (NY-ESO-1) | Melanoma | ECM830 (BTX) | SW | 500 | 0.5 | 2 | n.d. | 2 µg/$10^6$ cells | n.a. | 25 | 21-93% (24h) | [78] |
| Expanded PBLs from melanoma patients | MART-1, NY-ESO-1, p53 | HLA-A2 | Melanoma | ECM830 (BTX) | SW | 500 | 0.5 | 2 | n.d. | 1.5 µg/$10^6$ cells | n.a. | 25 | 29-85% (24h) | [79] |
| CD8 T cells | gp100, p53 | HLA-A2 | Melanoma | Gene Pulser Xcell (Bio-Rad) | SW | 495 | 10 | n.d. | n.d. | ≤20 | n.d. | n.d. | 35-77% (20h) | [80] |
| J76 Jurkat cells | gp100 | HLA-A2 | Melanoma | Gene Pulser Xcell (Bio-Rad) | SW | 500 | 4 | n.d. | 200 | 10 | 4 | n.a. | 18.5-99% (next day) | [82] |
| Resting CD8 T cells | WT1 | HLA-A2 | Leukemia | Gene Pulser Xcell (Bio-Rad) | SW | 500 | 5 | 4 | 200 | 1 µg/$10^6$ cells | 10-20 | n.a. | 19-42% (24h) | [83] |
| Stimulated CD4/CD8 T cells from metastatic melanoma patients | NY-ESO-1 | HLA-A2 | Melanoma | ECM830 (BTX) | SW | 400 | 0.5 | 2 | 50-200 | 2 µg/$10^6$ cells | n.a. | 25 | 16-88% (n.d.) | [86] |
| Stimulated CD4/CD8 T cells | NY-ESO-1, MART-1 | HLA-A2 | Melanoma | ECM830 (BTX) | SW | 400 | 0.5 | 2 | 50-200 | 2 µg/$10^6$ cells | n.a. | 25 | n.d. | [88] |
| CD8 T cells | MART-1 | HLA-A2 | Melanoma | Gene Pulser Xcell (Bio-Rad) | SW | 500 | 5 | 4 | 100-600 | 150 µg/mL | n.a. | 80 | n.d. | [89] |
| Expanded T cells | MART-1 | HLA-A2 | Melanoma | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.5-2 | 1 | 25 | 1-25% (2-16h) | [90] |
| Stimulated CD4/CD8 T cells | CEA | HLA-A2 | Colorectal cancer | ECM830 (BTX) | SW | 500 | 0.5 | 2 | 50-200 | 2 µg/$10^6$ cells | n.a. | 20 | 72-78% (24h) | [91] |
| Expanded γδ T cells and CD8 T cells | gp100 | HLA-A2 | Melanoma | Gene Pulser Xcell (Bio-Rad) | SW | 500 | 5 | 4 | n.d. | 15 | n.a. | 1 | 10-28.9% (24h) | [92] |
| CD8 T cells | gp100, patient-specific neoantigen | HLA-A2, HLA-B27 | Melanoma | Gene Pulser Xcell (Bio-Rad) | SW | 500 | 5 | n.d. | n.d. | Variable | n.d. | n.d. | n.d. | [93] |
| Expanded CD8 T cells | HBV envelope | HLA-A2 | HBV-related HCC | Nucleofector 2b (Lonza) | X-001 | n.d. | n.d. | n.d. | 100 | 10-20 | 5-20 | n.d. | 80% (24h) | [95] |
| Expanded CD4/CD8 T cells | TGFβRII frameshift mutation | HLA-A2 | Colorectal cancer | ECM830 (BTX) | SW | 500 | 2 | 4 | n.d. | 100 µg/mL | n.a. | 70 | 60-70% (ON) | [94] |
| Expanded CD4/CD8 T cells | TGFβRII frameshift mutation | HLA-A2 | Colorectal cancer | ECM830 (BTX) | SW | 500 | 2 | 4 | n.d. | 100 µg/mL | n.a. | 70 | 75–85% (18h) | [100] |
| Fresh and thawed expanded T cells | hTERT | HLA-DP4 | Generally expressed in all cancer forms | ECM830 (BTX) | SW | 500 | 2 | 4 | 800 | 100 µg/mL | n.a. | 5 | 90% (17h) | [101] |
| Expanded T cells | allo-HLA-DPB1*04:01, allo-HLA-DPB1*03:01 | | | Gene Pulser Xcell (Bio-Rad) | SW | 500 | 5 | 4 | n.d. | 10 µg per TCR chain | 5-10 | n.d. | 96% (16-20h) | [102] |
| PBMCs. Expanded T cells and γδ T cells | α-GalCer (semi-invariant NKT receptor) | CD1d | n.d. | ECM830 (BTX) | SW | 500 | 3 | n.d. | n.d. | 10 | n.d. | n.d. | 60-70% (12h); 54% (24h) | [104] |
| PBMCs | NY-ESO-1, Tyrosinase | HLA-A2, HLA-B7, HLA-DRB0401 | TCR-engineered reference samples | ECM830 (BTX) | SW | 500 | 3 | 4 | 250 | 30 | 20 | n.a. | 40-60% (18-20h) | [105] |
| PBMCs | NY-ESO-1, Influenza, Tyrosinase | Class I | TCR-engineered reference samples | ECM830 (BTX), Multiporator (Eppendorf), Gene Pulser II (Bio-Rad), Gene Pulser Xcell (Bio-Rad), Nucleofector II (Lonza), Amaxa 4D (Lonza) | SW/ED | 200-1000 | 0.05-15 ms (SW) 150-1500 µF (ED) | 4 (Bio-Rad); Nucleocuvette Vessel/Stripe (Lonza) | 20-250 | 2-10 (Flu-TCR), 10-75 (TAA-TCRs and Flu-TCR in ECM830) | 1-5 (Flu-TCR), 10-60 (TAA-TCR and Flu-TCR in ECM830) | n.a. | 61-90% (ON) | [107] |

Abbreviations: allo-, allogeneic; CEA, carcinoembryonic antigen; ED, exponential decay; EP, electroporation; gp100, glycoprotein 100; h, hours; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HLA-, human leucocyte antigen-type; hTERT, human telomerase reverse transcriptase; MART-1, melanoma antigen recognized by T cells 1; mRNA, messenger RNA; n.a., not applicable; n.d., no data; NKT, natural killer T cell; NY-ESO-1, New York esophageal squamous cell carcinoma 1; ON, overnight; PBMC, peripheral blood mononuclear cell; PBL, peripheral blood lymphocyte; Ref., reference; SW, square wave; TCR, T-cell receptor; TGFβRII, transforming growth factor beta receptor II; WT1, Wilms' tumor 1; α-GalCer, α-galactosylceramide;.

**Table 3. Clinical application of mRNA-electroporated T cells and natural killer cells for cancer therapy.**

| Clinical trial identifier | Type of malignancy | Study phase | Recruitment Status | Cell type | Gene(s) | No. subjects treated/enrolled | Trial treatment | Feasibility | Safety | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| NCT01355965 | MPleM | I | Completed | Autologous T cells | Anti-Meso scFv-BBz CAR | 4/18 | Repeated infusions | CAR-T cell manufacturing was possible for 4 patients. 21 infusions were administered in total. | Well tolerated, except for a case of treatment-related anaphylaxis and cardiac arrest from which the patient recovered. Possibly treatment-related Gr. III-IV AEs. | [141,143] |
| NCT01837602 | Metastatic (triple-negative) BC | 0/I | Completed | Autologous T cells | Anti-cMet scFv-BBz CAR | 6/6 | Single infusion | CAR-T cells were successfully manufactured for all patients. | Well tolerated. No treatment-related Gr. >I AEs. | [145] |
| NCT01897415 | PDAC | I | Completed | Autologous T cells | Anti-Meso scFv-BBz CAR | 6/10 | Repeated infusions | CAR-T cell manufacturing was possible for 10 patients. Manufacture failure in one case. 53 of 54 planned CAR-T cell infusions were administered. | Treatment-related Gr. III-IV AEs. No treatment-related deaths, CRS, neurologic symptoms, pleuro-pericarditis or peritonitis. No DLT. | [144,198] |
| NCT02277522/NCT02624258 | Adult and pediatric HL | I | Terminated | Autologous T cells | Anti-CD19 scFv-BBz CAR | 4/5 | Repeated infusions | CAR-T cells were successfully manufactured for all patients. | Well tolerated. No Gr. III-IV AEs. No CRS. | [146,199] |
| NCT02315118 | B-Cell CLL B-Cell NHL | I//II | Unknown | Autologous T cells | Anti-CD16-BBz CAR | 6/6 | Repeated infusions | CAR-T cells were successfully manufactured for all patients. 14 infusions were administered in total. Estimated minimum target levels of CAR-T cells were met in 13 of 14 products. | No off-target effects, no CRS, no treatment-related AEs. | [200] |
| NCT02623582 | R/R AML | I | Terminated | Autologous T cells | Anti-CD123 scFv-BBz CAR | 5/7 | Repeated infusions | CAR-T cell manufacturing was attempted in 6 of the 7 enrolled patients. CAR-T cell manufacturing was possible in 5 patients. 14 of 24 planned CAR-T cell infusions were manufactured; all planned doses were successfully manufactured in 2/6 patients. | No treatment-related deaths, clinically apparent vascular, neurological or hematological toxicities. All infusions were followed by a fever with CRS (Gr. I-IV). All CRS episodes resolved within 1 day. | [201] |
| NCT03060356 | R/R melanoma Metastatic triple-negative BC | I | Terminated | Autologous T cells | Anti-cMet scFv-BBz CAR | 7/77 | Repeated infusions | 77 subjects were pre-screened for tumor cMet expression. 37 subjects met the eligibility threshold. CAR-T cell infusions were administered to 7 patients. | Well tolerated. Possibly or definitely treatment-related Gr. I-II AEs in 5 patients. No Gr. ≥III toxicities or CRS. | [202] |
| NCT03448978 | Multiple myeloma | I/II | Recruiting | Autologous CD8 T cells | Anti-BCMA scFv-28z CAR | No results available | Repeated infusions | No results available | No results available | |
| NCT03608618 | Ovarian cancer MPerM | I | Recruiting | Autologous PBMC | Anti-Meso scFv-BBz CAR | 11/Recruiting | Repeated infusions | CAR-T cells were successfully manufactured for all patients. | Well tolerated. Gr. I-II AEs. A case of Gr. II pericarditis, fever and transient neutropenia clinically assessed as related severe AEs (resolved without further complications) due to on-target off-tumor effects and possibly Gr. I CRS. No neurotoxicity. No DLT. | [203] |
| NCT03431311 | CRC | I//II | Terminated | T cells | HLA-A2-restricted TGFβII-specific TCR | 1/1 | Repeated infusions | No results available | No results available | |
| NCT03415100 | Metastatic CRC | I | Unknown | Autologous/ allogeneic NK cells | NKG2D-ED-DAP12 or -z CAR | 3/3 | Repeated infusions | CAR-NK cells were successfully manufactured for all patients. | No dose-limiting toxicities. No Gr. ≥III AEs. | [178] |

Abbreviations: AEs, adverse events; AML, acute myeloid leukemia; BB, 4-1BB; BC, breast cancer; BCMA, B-cell maturation antigen; CAR, chimeric antigen receptor; CD, cluster of differentiation; CLL, chronic lymphocytic leukemia; cMet, tyrosine-protein kinase Met; CRC, colorectal cancer; DLT, dose-limiting toxicities; ED, extracellular domain; Gr., grade; HL, Hodgkin lymphoma; HLA, human leukocyte antigen; IL, interleukin; Meso, mesothelin; MPleM, malignant pleural mesothelioma; MPerM, malignant peritoneal mesothelioma; NK, natural killer cell; NKG2D, natural killer group 2 member D; NHL, non-Hodgkin's lymphoma; PBMC, peripheral blood mononuclear cells; PDAC, metastatic pancreatic ductal adenocarcinoma; R/R, relapsed or refractory; scFv, single-chain fragment variable; TCR, T-cell receptor; TGFβRII, transforming growth factor beta receptor II; -28z, CD28-CD3ζ signaling domain; -BBz, 4-1BB-CD3ζ signaling domain; -DAP12, DNAX activation protein of 12 kDa signaling domain; -z, CD3ζ signaling domain.

## Chimeric antigen receptor gene transfer

Chimeric antigen receptors (CARs) are another type of artificial immune receptors that have received significant attention and wide application in the past years. Contrary to TCRs, CARs bind to antigens expressed on the surface of cancer cells. This pattern of recognition is generally independent of MHC expression, circumventing the constraints posed by MHC on TCRs. Therefore, modifying T cells with CARs allows the targeting of virtually any surface antigen, including protein, glycosylated and lipid epitopes. CARs usually consist of several protein regions fused together into one open reading frame. The regions include, (i) an antigen recognition domain, most frequently generated by coupling the variable light and heavy chains of an antibody through a flexible linker forming a single chain variable fragment (scFv), (ii) a hinge/transmembrane domain providing flexibility that allows proper binding of the scFv to target antigen and anchors the protein in the cell membrane, and (iii) a signaling region consisting of one or more costimulatory domains (e.g., CD28, 4-1BB) and the CD3ζ signaling domain of the TCR complex. Over the past two decades, four generations of CARs have been developed. The first three generations incorporated none, one, or at least two costimulatory domains, respectively, with the goal of improving therapeutic efficacy or persistence. Fourth generation CARs are characterized by the co-expression of cytokines following CAR activation. Although viral integration of CAR sequences into the host T-cell genome has been the preferred method for producing CAR-T cells, concerns over potentially serious adverse effects of permanently modified CAR-T cells, such as cytokine release syndrome (CRS) and neurotoxicity, have fostered the exploration of safer alternatives for CAR delivery. Due to their self-limiting nature, CAR-mRNA electroporated T cells may potentially alleviate these concerns and serve as a platform for initial safety and efficacy screening of novel CARs. Thus far, CAR-mRNA T cells have been used to target different antigens and malignancies, including surface antigens CD3 [108], CD19 [109-118], CD20 [109], CD33 [119], CD37 [120], B-cell maturation antigen [121], mesothelin (Meso) [116,122,123], melanoma-associated chondroitin sulfate proteoglycan (MCSP) [92,124-128], human epidermal growth factor receptor 2 (HER2) [129-131], folate receptor (FR)α [113], FRβ [132], GD2 [115], epidermal growth factor receptor [133], CEA [127,131], natural killer group 2 member D (NKG2D) [134-136], epithelial cell adhesion molecule [137], and MART-1 and TGFβRII peptides presented by MHC molecules [138] (**Table 4**). CAR surface expression in CAR mRNA transfected T cells usually peaks between 4 and 24 hours post electroporation and lasts for at least one week. Variations in the expression pattern is attributed to the half-life of the mRNA, dilution of the CAR as a result of T-cell proliferation, protein stability on the cell membrane, and characteristics

of the domains incorporated into the CAR [109,124,139]. Moreover, titration, purification and/or additional modification of CAR-mRNA may modulate CAR surface expression and consequently the antigen-specific effector functionality, while also minimizing induced cell death [110,111]. Panjwani and colleagues were the first to report findings from a preclinical trial in large animals, in which pet dogs with spontaneous lymphoma were treated with an anti-canine CD20 CAR [109]. Dogs serve as a very good model for human disease as they develop similar cancers with respect to the genetics, biology, treatments, and responses [109,140]. In a case study of one dog, these investigators reported achieving temporary stable disease after three doses of CAR-mRNA T cells. Interestingly, the dog developed anti-mouse antibodies after repeated exposure to the scFv of murine origin, similar to what has been observed in humans [141]. This highlights the fact that host compatibility is critical and less immunogenic CAR designs are needed.

It could be argued that in practice, CAR persistence is needed for favorable clinical outcomes and, therefore, CAR-mRNA electroporation does not represent a real therapeutic alternative. Incorporating multiple doses of CAR-mRNA T cells (or any other type of antigen-specific immune receptor) into the treatment schedule was expected to overcome the limited persistence of the cells [139]. Early reports varied in both CAR T cell doses and frequency of injections, but all achieved significant tumor regression and delayed disease progression [122,129,130]. The majority of subsequent studies opted for weekly administration of CAR-mRNA T cells, corresponding to the duration of transient expression of the receptor [109,112,113,123]. Regardless of the transient persistence of CAR-mRNA T cells, multiple preclinical studies have demonstrated significant improvements in overall survival of tumor-bearing mice after a single-dose of CAR-mRNA T cells [108,112,114,115,120,123,124]. Furthermore, it was found that complete ablation of previously administered CAR-T cells using cyclophosphamide combined with multiple decreasing IV doses was most effective in leukemia and disseminated neuroblastoma mouse models [115,116]. However, in a neuroblastoma model, only temporary control of disease was achieved with CAR-mRNA T cells, while the majority of controls treated with lentiviral CAR-T cells went into remission [115]. Two other studies also combined cyclophosphamide lymphodepletion with either a CD30 targeted CAR or FRβ-targeted CAR [119,132]. Neither was able to induce complete remission and the CAR-mRNA T-cell therapy was inferior to their respective lentiviral counterparts [132]. These outcomes may be explained by the minimal migration and infiltration of the CAR-mRNA T cells during their short life-span in combination with receptor internalization following T-cell stimulation [115,133]. Therefore, the antitumor effect of CAR-mRNA T cells may be

maximized by intratumoral administration, as seen with TCR-mRNA T cells [115,133]. Furthermore, this approach might reduce on-target off-tumor toxicities when targeting antigens that exhibit considerable expression on normal cells as well [133].

Similar to TCR-mRNA, most studies using CAR-mRNA have focused on the modification of activated or expanded conventional PBLs; however, γδ T cells are also a population of interest for CAR engineering [92,135]. Allogeneic CAR-T-cell therapy using γδ T cells isolated from healthy donors is possible due to their antitumor activity and MHC-independent antigen recognition. Comparable to CAR-modified CD8 T cells, MCSP-CAR mRNA-transfected γδ T cells are able to specifically lyse antigen-positive target cells, while secreting less proinflammatory cytokines, possibly reducing the risk of CRS [92]. In addition, antitumor cytotoxicity mediated through endogenous receptors was not affected by the introduction of CAR, as demonstrated by the strong killing capacity in vitro against the MHC-negative and antigen-deficient Daudi cell line. Several enhancements outside the CAR construct have also been studied in the context of CAR-T cells. As previously evaluated in T cells transfected with two TCRs, TETARs have been further explored by simultaneous electroporation of mRNAs encoding a gp100-specific TCR and MCSP-specific CAR [125]. When challenged in vitro with tumor cells carrying both antigens, T cells expressing both receptors produced more cytokines compared to that of a balanced mix of T cells with a single receptor. The combination of receptors also had a synergistic effect on cytolytic capacity. Interestingly, when TCR was introduced by lentiviral transduction and CAR by mRNA electroporation, the addition of CAR in TETARs had little beneficial killing effect compared to that of the TCR-only cells [126]. In an effort to combine the benefits of a TCR and CAR into a single molecule, Wälchli and colleagues created a so called TCR-CAR by fusing the extracellular domain of TCR chains to the intracellular portion of a second-generation CAR [138]. In contrast to the traditional concept of CARs, this setup allows targeting of peptides presented by the MHC complex, while maintaining CAR-like (co)stimulation of T cells. Furthermore, introduction of TCR-CAR mRNA into non-T cells is able to render them pMHC restricted. CAR-T-cell therapy can also benefit from electroporation of siRNA [83,139]. For instance, Simon *et al.* co-transfected CAR mRNA and siRNAs against the immune checkpoints PD-1 and CTLA-4 [127]. Downregulation of PD-1 and CTLA-4 peaks at two days post-electroporation and has a synergistic effect on antigen-specific cytotoxicity, but not on antigen-specific cytokine release. The double knock-down results of CAR-T cells are not significantly better than those of single PD-1 knock-down CAR-T cells. This indicates that CTLA-4 silencing has a minimal effect, possibly due to the lack of expression of its natural ligands by the tumor cells.

Nonetheless, this study provides evidence of a novel strategy for the enhancement of CAR-T-cell therapy.
Transient modification of cells with CARs can also be used in other applications. For instance, Liu *et al.* established a novel expansion method by introducing anti-CD3ε CAR mRNA into peripheral blood mononuclear cells (PBMCs) to generate OKT3-28BB T cells [108]. Compared to T cells expanded with CD3/CD28 Dynabeads, OKT3/IL-2 expansion generates a more differentiated (effector memory) T-cell population, with decreased migratory and proliferative capacity, but with increased direct tumor control. Additional co-electroporation of costimulatory molecule ligands CD86 and 4-1BB ligand (4-1BBL) in OKT3-28BB T cells results in a more naive phenotype similar to CD3/CD28 expanded T cells, while maintaining in vitro tumor killing capacity similar to more differentiated OKT3/IL-2 expanded T cells generated when virally transduced with an anti-mesothelin CAR (aMeso CAR) [108]. However, subsequent in vivo ovarian and leukemia mouse models showed OKT3-28BB-expanded aMeso CAR-T cells to be inferior to CD3/CD28 expanded aMeso CAR-T cells.

Messenger RNA-based CAR-T-cell therapies have not yet achieved preclinical efficiencies approaching that of viral CAR-T cells. Nonetheless, they may still significantly contribute to immunotherapy in several ways. Cheaper and less complex release testing and manufacturing of clinical grade mRNA compared to that of viral vectors facilitate quicker clinical translation for toxicity testing [116,123,142]. The combination of time-limited adverse events and short-term cytotoxicity has prompted investigators to suggest the use of T cells equipped with a potent, but toxic CAR as "induction therapy", to be followed by stably transduced CAR-T cells as "consolidation therapy" [122]. This hit-and-run strategy may also prove useful for inducing epitope spreading, which is associated with favorable clinical outcome [131] and bystander destruction of antigen-negative tumor cells, avoiding immune escape [112].

Clinical studies of RNA CAR-T cells are limited to only a few early-phase clinical trials, primarily in solid tumors (clinicaltrials.gov Identifier: NCT01355965, NCT01897415, NCT03060356, NCT03608618, NCT01837602), but also in hematological malignancies (clinicaltrials.gov Identifier: NCT02277522, NCT02624258, NCT02623582, NCT03448978) (**Table 3**). Beatty and colleagues were the first to report on the safe and effective use of aMeso CAR-mRNA T cells without prior lymphodepletion [143,144]. Consistent with the transient nature of the cell product, CAR transcripts are detectable for up to three days and toxicities are limited and temporary. Notably, one patient from the same study went in anaphylactic shock after administration of the third dose of aMeso CAR-T cells, which was determined to be caused by a humoral response against

the murine scFv of the CAR and led to an adjustment of the CAR-T-cell administration protocol [141]. This patient achieved a partial response after only three injections, but progressed after six months [143]. Importantly, CAR-mRNA T cells are found able to traffic to and infiltrate the tumor, and induce epitope-spreading [143,144]. Similarly, intratumoral injection of anti-cMet CAR-T cells into breast tumors demonstrated extravasation into the bloodstream without any signs of off-tumor toxicity [145]. Another phase I trial by the same team will investigate the safety of IV injection of these CAR-T cells. Svoboda *et al.* reported on five patients with classical Hodgkin lymphoma treated with six injections of CD19-targeted CAR-T cells [146]. Two patients obtained a clinical response after one month, which are thought to be linked to the persistence of the CAR-T cells after injection. These responses were only transient, but could be restored by additional treatment such as anti-PD-1 therapy or autologous HSCT [146]. Notably, these early-phase clinical trials are limited to only a few repeated injections of CAR-mRNA T cells. Future investigations should include more extensive administration schedules, which will require a considerable amount of autologous T cells. Considerations regarding a clinical expansion protocol specifically for CAR-mRNA T cells were recently investigated [128,147,148].

**Table 4. Electroporation settings for T cells with chimeric antigen receptors**

| Cell type | Antigen-CAR type | EP system (Company) | Type of pulse | Voltage (V) | Capacitance (µF) | Time (ms) | EP gap (mm) | EP buffer (µL) | Amount of mRNA (µg) | Cell number (x10$^6$ cells) | Cell concentration (x10$^6$ cells/mL) | % CAR expression after EP (time point) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Expanded γδ and CD8 T cells | aMCSP-28z | Gene Pulser Xcell (Bio-Rad) | SW | 500 | n.a. | 5 | 4 | n.d. | 15 | n.a. | 1 | n.d. | [92] |
| PBMC | aCD3-28BBz | ECM830 (BTX) | SW | n.d. | n.a. | n.d. | 2 | 100 | n.d. | n.a. | 100-300 | >90% | [108] |
| Expanded T cells | ahCD19-z, acCD20-z | Electro Square Porator (n.d.) | SW | 500 | n.a. | 0.7 | 2 | 200 | 20 | 20 | n.a. | >90% | [109] |
| Expanded CD25-depleted T cells | aCD19-CD28/OX40z | ECM830 (BTX) | SW | 500 | n.a. | 2 | 4 | 300-700 | 0.1-150 µg/mL | n.a. | 10 | 94.1% (18-20h) | [110] |
| Stimulated T cells | aCD19-BBz | Nucleofector T-cell transfection kit (Lonza) / ECM830 (BTX) | n.d. (T23) / SW | n.d. | n.d. | n.d. | n.d. | 100 | 10 | 5 | n.a. | n.d. | [111] |
| Expanded T cells | aCD19-BBz, aCD19-28z | ECM830 (BTX) | SW | n.d. | n.a. | n.d. | 2 | 100 | 1 / 5 / 10 | n.a. | 100-300 | 94% (18h) | [112] |
| Expanded T cells | aFRa-27z, aCD19-27z | ECM830 (BTX) | SW | 500 | n.a. | 0.7 | 2 | 100 | 10 | n.a. | 100 | >95% | [113] |
| Stimulated T cells | aCD19-BBz | ECM830 (BTX) | SW | n.d. | n.a. | n.d. | 2 | 100 | 10 | n.a. | 100-300 | 99% | [114] |
| Expanded T cells | aCD19-BBz, aGD2-BBz | ECM830 (BTX) | SW | n.d. | n.a. | n.d. | 2 | 100 | 10 | n.a. | 100-300 | >95% | [115] |
| Stimulated T cells | aCD19-BBz, aMeso-BBz | ECM830 (BTX) | SW | n.d. | n.a. | n.d. | 2 | 100 | 10 | n.a. | 100-300 | >99% | [116] |
| Expanded T cells | aCD19-z | ECM830 (BTX) | SW | n.d. | n.a. | n.d. | 2 | 100 | 10 | 5 | n.a. | n.d. | [117] |
| Expanded T cells | aCD19-BBz | ECM830 (BTX) | SW | n.d. | n.d. | n.d. | 2 | 100 | 100 | n.a. | 100 | n.d. | [118] |
| Stimulated T cells | aCD33-BBz | ECM830 (BTX) | SW | n.d. | n.a. | n.d. | 2 | 100 | 10 | n.a. | 100-300 | >90% | [119] |
| Expanded T cells | aCD37-BBz | ECM830 (BTX) | SW | 500 | n.a. | 2 | 4 | n.d. | 100 µg/mL | n.a. | 62.5 | >90% (18h) | [120] |
| Expanded CD8 T cells | aBCMA-28z | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 72% (4h) | [121] |
| Stimulated T cells | aMeso-z, aMeso-28z, aMeso-BBz | ECM830 (BTX) / Maxcyte (Maxcyte) | n.d. | n.d. | n.d. | n.d. | 2 / OC-400 Chamber | 100 / 200 | 10 / 20 | n.a. | 100-300 | >95% | [122] |
| PBL or PBMC | aMeso-BBz | GT System (Maxcyte) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 200 µg/mL | n.a. | 500 | >35% of CD3$^+$ | [123] |
| Expanded CD4 or CD8 T cells | aMCSP-z, aMCSP-28z | Gene Pulser Xcell (Bio-Rad) | SW | 500 | n.a. | 5 | 4 | 100-600 | 150 µg/mL | n.a. | 140 | >95% (4h) | [124] |
| CD8 T cells | aMCSP-28z | Gene Pulser Xcell (Bio-Rad) | SW | 500 | n.a. | 5 | 4 | n.d. | 10 | n.d. | n.d. | 85% (8h) | [125] |
| Expanded T cells | aMCSP-28z | Gene Pulser Xcell (Bio-Rad) | SW | 500 | n.a. | 5 | 4 | n.d. | n.d. | n.d. | n.d. | ~80% | [126] |
| CD4 or CD8 T cells | aMCSP-28z, CEA-28z | Gene Pulser Xcell (Bio-Rad) | SW | 500 | n.a. | 5 | 4 | n.d. | n.d. | n.d. | n.d.- | n.d. | [127] |
| Expanded T cells | aMCSP-28z | Gene Pulser Xcell (Bio-Rad) | SW | 500 | n.a. | 5 | 4 | n.d. | 150 µg/mL | n.a. | 150 | 88% (8h) | [128] |
| Stimulated PBL | aHER2-28z | ECM830 (BTX) | SW | 400 | n.a. | 0.5 | 2 | 100 | 10 | 1 | n.a. | 90-99% (16h) | [129] |
| Stimulated PBL | aHER2-28z | ECM830 (BTX) | SW | 400 | n.a. | 0.5 | 2 | 100 | 10 | 1 | n.a. | >98% | [130] |
| CD4 and CD8 T cells | aHER2, aCEA | Gene Pulser Xcell (Bio-Rad) | SW | 500 | n.a. | 5 | 4 | 100-600 | 150 µg/mL | n.a. | 140 | ErbB2: 83.0% / 89.7% (4h)<br>CEA: 82.1% / 81.4 % (4h) | [131] |
| Expanded T cells | aFRb-z, aFRb-28z | ECM830 (BTX) | SW | 500 | n.a. | 0.7 | n.d. | n.d. | 10 | 10 | n.a. | High | [132] |
| Expanded T cells | aEGFR-28z | Amaxa 4D Nucleofector (Lonza) | DQ-115 | n.d. | n.d. | n.d. | n.d. | 200 | 3 | 20 | n.a. | CD8$^+$ T cells: ~60% (24h)<br>CD4$^+$ T cells: ~30% (24h) | [133] |
| Expanded CD4 or CD8 T cells | NKG2D-28z | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 100 | 10 | ≤ 6 | n.a. | High | [134] |
| Expanded γδ T cells | aNKG2D-z | NEPA21 (Nepa Gene) | n.d. | 240 | n.a. | 4 | 2 | n.d. | n.d. | n.d. | n.d. | 96% | [135] |
| Expanded T cells | aNKG2D-z, aNKG2D-27z, aNKG2D-28z, aNKG2D-28BBz | NEPA21 (Nepa Gene) | n.d. | 240 | n.a. | 4 | 2 | 100 | 5 | n.a. | 10 | >92% (24h) | [136] |
| Expanded T cells | aEpCAM-28BBz | NEPA21 (Nepa Gene) | n.d. | 240 | n.a. | 4 | 2 | 200 | 20 | n.d. | n.d. | 68.5% | [137] |
| Expanded T cells | DMF5 TCR-28z, Radium-1 TCR-28z | ECM830 (BTX) | SW | 500 | n.a. | 2 | 4 | n.d. | 100 µg/mL | n.a. | 70 | High (8h) | [138] |

Abbreviations: aCEA, anti-carcinoembryonic antigen; aEpCAM, anti-epithelial cell adhesion molecule; aFRa, anti-folate receptor alpha; aHER2, anti-human epidermal growth factor receptor 2; aMCSP, anti-melanoma-associated chondroitin sulfate proteoglycan; aMeso, anti-mesothelin; CAR, chimeric antigen receptor; DMF5, MART-1$_{26\text{-}35}$ specific; EGFR, epidermal growth factor receptor; EP, electroporation; gp100, glycoprotein 100; h, hours; mRNA, messenger RNA; n.a., not applicable; n.d., no data; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; Radium-1, specific to frame-shift mutation in TGF beta Receptor 2; TCR, T cell receptor; -27z, CD27-CD3ζ signaling domain; -28BBz, CD28-4-1BB-CD3ζ signaling domain; -28z, CD28-CD3ζ signaling domain; -BBz, 4-1BB-CD3ζ signaling domain; -z, CD3ζ signaling domain.

## Adjuvant gene transfer

Taking advantage of the flexibility that mRNA electroporation offers for the simultaneous introduction of multiple genes into T cells, some studies have focused on adjuvants, alone or together with immune receptors to redirect T-cell specificity. Kunii *et al.* demonstrated that mRNA co-electroporation of a linker for activation of T cells (LAT) variant that is resistant to ubiquitination (2KR LAT) and a CAR or TCR can improve downstream signaling, thereby promoting increased CD8 T-cell cytotoxic capacity and Th1 CD4 T cell polarization [117]. A follow-up study using TCR-modified 2KR LAT transgenic mice revealed T cells with a more differentiated phenotype, but demonstrated no beneficial effect compared to that of wild-type LAT [149]. As a less differentiated phenotype correlates with reduced clinical effect [108], a temporary modification may not only be safer, but may also improve efficacy. To our knowledge, Lee *et al.* were the first to report on fourth generation CAR-T cells that were modified to express both IL-2 and an HER2-specific CAR (CAR/IL-2) [130]. When intratumorally injected into established subcutaneous ovarian cancer, CAR/IL-2 T cells are slightly better at controlling tumor growth than single modified CAR-mRNA or IL 2 mRNA T cells, which is mediated by the recruitment of NK cells to the tumor site. As T-cell homing deficits have been seen in multiple cases of cancer [150], T cells have been mRNA-(co-)transfected with chemokine receptors, such as the C-X-C motif chemokine receptor (CXCR) 2—also called IL-8 receptor—[151,152], C-C chemokine receptor type (CCR) 7 or CXCR4 [110,151,152] in effort to improve T-cell homing and migration towards the tumor site. IL-8 is secreted by multiple types of cancer, playing an important role in tumor escape. Unfortunately, many T cells do not express the receptor for IL-8 and are incapable of trafficking towards IL-8-secreting tumor cells. This approach may be helpful for malignancies, such as brain tumors, in which immune cell trafficking is limited. Gross and colleagues described mRNA-engineered T cells with a caTLR4, which circumvents the need of toll-like receptor (TLR) 4 ligand binding [153]. CaTLR4-engineered T cells show an enhanced antitumor profile with increased secretion of different cytokines (including IFN-γ), expression of T cell activation markers and the killing of melanoma cells. Interleukins such as IL 2, IL 12, and IL-15 have been proven to be beneficial in cancer immunotherapy. However, the injection of soluble cytokines or the stable expression of IL-12 in some cases led to unacceptable toxicities in clinical trials [154]. In vivo analysis of T cells mRNA electroporated with single-chain IL-12 alone or in combination with CD137 ligand revealed that intratumoral injection of the engineered cells enables rejection of local and distant tumors [155], while limiting the duration of potential adverse toxicities with its transient expression. T cells have also been transfected with cytokine-encoding mRNAs to induce

their membrane presentation and improve T-cell growth and functionality in comparison to their soluble counterparts. Co-transfection of caTLR4 and cytokine IL-2, IL-12, or IL-15 further stimulates T-cell functionality compared to that of caTLR4 alone, although each of the cytokines affects the functionality differently [156]. For instance, caTLR4 combined with IL-2 or IL-12 act synergistically to potentiate the secretion of effector factors, while the combination of caTLR4 and IL-15 increase the expression of activation markers. In a similar fashion, T cells have been RNA-engineered with constitutively active CD40 (caCD40), a member of the type 1 TNFR family mainly expressed by professional APCs [157]. As for caTLR4, activation of CD40 is mediated by engagement with CD40L. However, caCD40 expression can be achieved by homo-oligomerization of the CD40 signaling domain [157]. Simultaneous transfection with caCD40 and caTLR4 further improves T cell activity compared to that of either of the two receptors alone [157]. Combinations of these receptors and the cytokines mentioned above promote IFN-γ secretion and the upregulation of T-cell activation markers by mRNA-engineered T cells [158], confirming the benefit of adding multiple adjuvants. Importantly, the enhanced T-cell IFN-γ production, observed in the aforementioned studies [153,156-158], often correlates with clinical efficacy and tumor regression. However, it has yet to be demonstrated whether co-transfection of TCR-mRNA with these or other costimulatory molecules would have an impact on TCR-engineered T cells. T cells can also act as carriers for secreted anti-cancer molecules. For example, T cells have been RNA-transfected with a cytotoxic immunotoxin from a truncated version of *Pseudomonas* exotoxin A and used to attack the neovasculature of tumors [159].

# Messenger RNA electroporation of natural killer cells

NK cells are a component of the innate immune system and comprise 5–15% of peripheral blood lymphocytes [160]. They are mainly found in circulating blood and hematopoietic tissues, but after activation, they can migrate to the site of infection or tumor growth. NK cells can exert both cytotoxic and regulatory functions and are involved in tumor surveillance without depending on a specific antigen. Due to their properties, they have been extensively used in cancer immunotherapy. In effort to direct their specificity towards TAAs, NK cells have been primarily engineered with CARs using mRNA electroporation (**Table 5**). Prior to that, due to the low frequency of NK cells in peripheral blood, they are usually expanded ex vivo to reach sufficient numbers for adoptive transfer [161]. As examples, expansion of NK cells from bulk PBMCs or previously isolated NK cells can be achieved using autologous feeder cells in

combination with anti-CD16 antibody [162], in cell-free conditions via addition of exogenous proliferation, maturation, and survival-inducing common gamma chain cytokines, such as IL-15 and IL-21 [163], or using artificial APCs modified to express membrane-bound cytokines and 4-1BBL [164-167]. In particular, IL-15 induces NK cell maturation and provides support for NK-cell survival in peripheral lymphoid organs [168]. With a protocol using IL-15 to stimulate primary NK cells prior to electroporation with second-generation and third-generation anti-CD19 CAR-mRNAs, it has been demonstrated that differentiation status can play an important role in the success of CAR engineered NK cells [169]. Compared to non-stimulated cells, CAR expression is optimal after incubation of the cells for 3 days with IL-15. Moreover, the killing activity of engineered NK cells towards CD19-positive targets is specifically enhanced by the presence of the anti-CD19 CAR. Interestingly, CAR-modified NK cells are able to respond to the specific ligands independently of the NK inhibitory receptor NKG2A. However, they remained sensitive to inhibitory signals of killer immunoglobulin-like receptors.

Carlsten and colleagues described a current GMP-compliant transfection protocol for mRNA electroporation of primary ex vivo expanded NK cells [170]. As a proof-of-concept, NK cells were transfected with green fluorescent protein (GFP) or CD34 mRNA. Expression of GFP, which has a long half-life, is detectable for up to 3 weeks. Surprisingly, 95% of the NK cells are GFP positive up to 7-9 days. In contrast, surface expression of CD34 lasts up to 7 days. Importantly, after mRNA electroporation, there is no significant drop in viability, proliferation, or cytotoxic activity, nor are there observable changes in the NK cell surface receptors. In a study using another GMP-compliant transfection protocol, ex vivo expanded NK cells were compared to unstimulated peripheral blood NK cells [171,172]. Both expanded and unstimulated NK cells retained good viability accompanied by reporter gene expression of greater than 80% at 24 hours post transfection [171]. Specific cytotoxic activity against acute lymphoblastic leukemia and B-lineage chronic lymphocytic leukemia cells was achieved by introduction of a third-generation anti-CD19 CAR into both types of NK cells. However, CAR expression persisted for only up to three days, whereas other studies have reported longer CAR expression kinetics, of up to six days after transfection [166,169]. In an in vivo follow-up study, expanded and anti-CD19 CAR mRNA electroporated NK cells were injected intravenously or intraperitoneally into immunodeficient mice engrafted with CD19-positive human B-lineage acute lymphoblastic leukemia (B-ALL) cells [173]. CAR-modified NK cells administered by either route significantly reduced the leukemia burden compared to that of the control NK cells.

CD20 antigen has also been a target for CAR-engineered NK cells as a treatment against CD20-positive B-cell leukemia and non-Hodgkin lymphoma (NHL) cells [166]. CAR-electroporated expanded peripheral blood NK cells are not influenced by activating or inhibitory receptors, but exhibited enhanced in vitro toxicity and cytokine production. Moreover, tumor burden is significantly reduced in NOD scid gamma (NSG) mice xenografted with rituximab-sensitive or rituximab-resistant Burkitt lymphoma cells after three administrations of the CAR-engineered NK cells, which correlates with longer survival compared to that of untreated mice. However, most of the xenografted mice ultimately succumb to the disease and die. In a follow-up analysis [174], the investigators showed that a combination of anti-CD20 CAR-NK cells with the histone deacetylase inhibitor romidepsin further enhances the cytotoxic capacity of CAR-NK cells against Burkitt lymphoma cells, both in vitro and in vivo. The synergistic effect of the combinatorial treatment also prolongs the survival rate of the xenografted NSG mice, which provides the basis for evaluation of other combinatorial approaches.

Similar to T cells, NK cells have been mRNA-reprogrammed with homing chemokine receptors like CCR7 [170], CXCR4 [175], and CXCR1 [176]. CCR7, which is naturally only present in a small subset of NK cells, induces NK-cell migration towards secondary lymphoid tissues, such as lymph nodes, where hematological tumor cells may be found. However, CCR7 expression is lost upon expansion ex vivo. Introduction of CCR7 also significantly induces the in vitro migration of NK cells towards chemokine (C-C motif) ligand (CCL)19 [170]. As occurs with native expression of CCR7 in NK cells, exposure to increasing doses of CCL19 reduces CCR7 expression in the engineered NK cells, homing these cells to CCL19-rich sites. In addition, transfection of NK cells isolated from donors expressing the Fc gamma receptor IIIa (CD16)-158F/F homozygous variant with mRNA encoding the high-affinity antibody-binding receptor variant CD16-158V induces greater antibody-dependent cellular cytotoxicity-mediated activity against rituximab coated lymphoma cells [170]. A detailed protocol for the mRNA electroporation of primary NK cells, including tips for its optimization, has been reported [177]. With regard to CXCR4, NK cells modified with a gain-of-function variant of this receptor demonstrate improved migration to the bone marrow [175]. Co-electroporation of CXCR1-mRNA and CAR-mRNAs, the latter being composed of the extracellular domain of NKG2D and against tumor-associated NKG2D ligands, has been shown in a xenograft mouse model to redirect NK cell infiltration towards human tumor sites, without affecting CAR-mediated killing [176]. This demonstrates that the benefits of combining mRNAs encoding chemokine and TAA specific receptors observed in T cells also apply to NK cells. The strategy of using the extracellular domain of NKG2D in a CAR to substitute the scFv region in the CAR has recently been studied

in a clinical trial against colorectal cancer (NCT03415100; **Table 3**) [178]. The investigators first compared in a xenograft mouse model the efficacy of an NKG2D-CAR with a conventional CD3ζ intracellular domain (NKG2Dζ) to that of a CAR with DNAX activation protein of 12 kDa (DAP12) signaling domain (NKG2Dp). NKG2Dp was superior to NKG2Dζ in terms of antitumor activity against NKG2D ligand-positive tumor cells [178]. Subsequently, the NKG2Dp-CAR was used in patient-derived or haploidentical family donor-derived NK cells in the clinical trial, showing that multiple rounds of CAR-mRNA-electroporated NK cells have clinical benefit in controlling malignant ascites [178]. NK cells can also be transfected with other molecules such as iron oxide contrast agents. Although not used for gene transfer, these clinically applicable reagents can be useful for monitoring the distribution of CAR-engineered NK cells via magnetic resonance imaging [179]. This represents an advancement in non-invasive strategies to assess the efficacy and trafficking of gene-engineered NK cells to tumor sites in patients.

Cord blood (CB)-derived NK cells have also been tested for gene transfer via mRNA electroporation [180]. These cells share characteristics with peripheral blood NK cells in terms of cytotoxic activity and proliferation; however, as with their peripheral blood counterparts, CB-derived NK cells must be expanded ex vivo in order to obtain sufficient amounts for modification. In this case, expansion of CB-derived NK cells can be accomplished using umbilical cord mesenchymal stem cells as feeder cells. When electroporation of plasmid DNA and mRNA are compared, CB-derived NK cells show better transfection efficiency with GFP-encoding mRNA compared to that with plasmid DNA, while maintaining their cytotoxic characteristics. Interestingly, when two different electroporation systems were tested, the GenePulser II electroporator from Bio-Rad and the Nucleofector from Amaxa, which includes its proprietary transfection medium, the Amaxa system showed a 5-fold increase in GFP expression compared to the GenePulser II system. However, both systems yielded very low numbers of CB-derived NK cells after transfection with either sources of genetic material, with mean viabilities as low as 10% [180].

Due to the scarcity and low functionality of peripheral blood NK cells in patients, NK cell lines have been established from human clonal NK-cell lymphomas [181]. The NK 92 cell line, established by Gong and co-workers [182], is the most extensively used NK cell line. This cell line has been used in both preclinical and clinical studies for the evaluation of CARs due to its excellent antitumor cytotoxic properties [181]. A report by Boissel and colleagues [183] compared the electroporation efficiency of cDNA versus mRNA for an anti-CD19 CAR in NK-92 cells. The mRNA-electroporated NK-92 cells

express the receptor more efficiently than that of their cDNA-electroporated counterparts with minimal loss of cell viability. Moreover, NK-92 cells transfected with anti-CD19 CAR-mRNA are able to kill CD19-positive B-ALL cell lines that were previously NK-92 resistant, as well as primary B-cell chronic lymphocytic leukemia (B-CLL) cells. In a similar study, these investigators compared the transfection efficiency of lentiviral vectors to mRNA electroporation in NK-92 cells. They used two CARs targeting either CD19-positive and CD20-positive cells in B-CLL cells. The results revealed significantly higher expression levels of both CD19 and CD20 CAR in NK-92 cells electroporated with CD19-CAR and CD20-CAR mRNAs compared to those transformed by lentiviral transduction. The differences in CAR expression also correlate with enhanced killing of primary B-CLL cells [184].

**Table 5. Electroporation settings for natural killer cells**

| Cell type | Gene(s) | EP system (Company) | Type of pulse | Voltage (V) | Capacitance (µF) | Time (ms) | EP gap (mm) | EP buffer (µL) | Amount of mRn.a. (µg) | Cell number (x$10^6$ cells) | Cell concentration (x$10^6$ cells/mL) | % Protein expression (time point) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Expanded NK cells | aCD20-BBz CAR | Amaxa nucleofector II (Lonza) | Program U-001 | n.d. | n.d. | n.d. | n.d. | 100 | 80-100 µg/mL | 6-8 | n.a. | 94.1% (20h) | [166,167] |
| Stimulated NK cells | aCD19-28/OX40z CAR<br>aCD19-BBz CAR | ECM830 (BTX) | SW | 500 | n.d. | 2 | 4 | 400 | 100 µg/mL | n.d. | 10-50 | 80-90% (24h) | [169] |
| Expanded NK cells | CCR7, CD16, CD34 | MaxCyte GT (MaxCyte) | Program for NK cells proprietary to MaxCyte | n.d. | n.d. | n.d. | OC-100 | 100 | 1 µg/$10^6$ cells | n.d. | n.d. | ~90% (24h) | [170] |
| Expanded NK cells | $CXCR4^{R334X}$<br>$CXCR4^{WT}$ | MaxCyte GT (MaxCyte) | NK2-OC | n.d. | n.d. | n.d. | n.d. | n.d. | 4 µg/$10^6$ cells | n.d. | 1–2×$10^6$ cells/10 µL | Peak at 4-8h | [175] |
| Expanded NK cells | CXCR1<br>aNKG2DL-z CAR<br>aNKG2DL-28z CAR<br>aNKG2DL-28BBz CAR | NEPA21 (Nepa Gene) | n.d. | 240 | n.a. | 4 | 2 | 100 | 5 µg | 10 | n.d. | 95% (24h) | [176] |
| Expanded NK cells | CCR7 | MaxCyte GT (MaxCyte) | NK2-OC | n.d. | n.d. | n.d. | n.d. | n.d. | 0.5, 1, 2, 4, or 8 µg/$10^6$ cells | n.d. | 1–2×$10^6$ cells/10 µL | Peak at 8h | [177] |
| Unstimulated and expanded NK cells | aCD19-BBz CAR | MaxCyte GT (MaxCyte) | Unstimulated-NK#1<br>Expanded-NK#3 | n.d. | n.d. | n.d. | MaxCyte processing chamber | n.d. | 100 µg/ml | n.d. | 100-300 | ~60% (n.d.) | [171] |
| Unstimulated and expanded NK cells | aCD19-BBz CAR | MaxCyte GT (MaxCyte) | Static NK#2 | n.d. | n.d. | n.d. | n.d. | 50 | 100–200 mg/ml | 10-15 | 200-300 | Unstimulated: 40.3% (24h)<br>Expanded: 61.3% (24h) | [172] |
| Unstimulated and expanded NK cells | aCD19-BBz CAR | MaxCyte GT (MaxCyte) | Static NK#2 | n.d. | n.d. | n.d. | n.d. | 50 | 150–200 µg/mL | 10-15 | n.d. | Unstimulated: 18.1-59.2% (24h)<br>Expanded: 28.2-92.4% (24h) | [173] |
| Expanded NK cells | aCD20-BBz CAR | Amaxa nucleofector II (Lonza) | Program U-001 | n.d. | n.d. | n.d. | n.d. | 100 | 80-100 µg/mL | 6-8 | n.a. | 95% (24h) | [174] |
| Expanded NK cells from cord blood | GFP | Genepulser II (Bio-Rad) | n.d. | 250<br>300<br>300 | 300<br>150<br>300 | n.a. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.3%<br>10%<br>0.4%<br>(24h) | [180] |
| | | Amaxa nucleofector (Lonza) | Program U-01 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 52.5% (24h) | |
| NK-92 | GFP, αCD19-z CAR | Gene Pulser II (Bio-Rad) | n.d. | 300 | 150 | n.d. | 4 | 250 | 40 µg/mL (GFP)<br>120 µg/mL (CAR) | 2 | 8 | 76% GFP<br>47.2% CAR (24h) | [183] |
| NK-92 | GFP<br>aCD19 CAR<br>aCD20 CAR | Gene Pulser II (Bio-Rad) | n.d. | 300 | 150 | n.d. | n.d. | 250 | 40 µg/mL | 2 | n.d. | 55.8% (24h) | [184] |

Abbreviations: CAR, chimeric antigen receptor; CCR7, C-C chemokine receptor type 7; CXCR(number), C-X-C chemokine receptor type (number); EP, electroporation; GFP, green fluorescent protein; h, hours; n.a., not applicable; n.d., no data; NK, natural killer; NKG2D, natural killer group 2D; mRNA, messenger RNA; SW, square wave; -28z, CD28-CD3ζ signaling domain;-28BBz, CD28-4-1BB-CD3ζ signaling domain; -28/OX40z, CD28-OX40-CD3ζ signaling domain; -BBz, 4-1BB-CD3ζ signaling domain.

# Messenger RNA electroporation of B cells

B cells have also been evaluated over the past two decades as potential professional APCs for tumor immunology (**Table 6**). Similar to DCs, B cells express homing receptors CD62L, CCR7, and CXCR4, which facilitate migration to secondary lymphoid organs and chemotactically attract T cells [185]. In addition, ex vivo cultures of small amounts of starting material with CD40L and IL-4 allow for the production of large numbers of licensed B cells (CD40-B cells) that highly express MHC and costimulatory molecules [186]. Preclinical evaluation has repeatedly demonstrated that CD40-B cells can be efficiently electroporated with TAA mRNA. Similar to DCs, RNA-loaded activated B cells are capable of activating in an MHC-dependent manner T cells directed against tumor-specific antigens, such as MART-1, survivin, CEA, latent membrane protein 1, and WT1, even using patient-derived cells [186-192]. Despite their practical advantages, activated B cells have been shown to be inferior T cell stimulators compared to that of DCs [193,194]. Simultaneous transfection of antigen and immune stimulatory molecules, such as OX40L, 4-1BBL, IL-12p35 and IL-12p40, can bring CD40-B cells on par with mature DCs in terms of inducing antigen-specific T cell responses in vitro; however, this could not be replicated in vivo [194].

Currently, there are no in-human clinical trials underway for mRNA-electroporated B cells. However, Sorenmo and colleagues have reported the first preclinical large animal trial using RNA-loaded CD40-B cells to treat dogs with NHL [190]. They opted for whole tumor RNA instead of tumor-specific antigen RNA to allow for a polyclonal antitumor T-cell response. For vaccine production, they used their previously developed stably transfected K562-CD40L cell line, avoiding the need for difficult to obtain recombinant trimeric soluble CD40L or xenogeneic NIH3T3-tCD40L feeder cells [195]. After remission through induction chemotherapy, 19 dogs were vaccinated on opposed flanks with CD40-B cells loaded with full autologous tumor RNA or canine distemper virus hemagglutinin as an immunological control [190]. While no significant improvements in time to relapse or survival in first remission were noted after induction chemotherapy compared to the control group, four out of ten dogs that relapsed and received salvation chemotherapy benefited from the tumor RNA loaded CD40-B cell vaccination. In addition, there were no long-term treatment related toxicities. These results, combined with the continuous development of GMP-compatible B cell activation and expansion methods [196], further pave the way towards the first in-human clinical trials of mRNA/total RNA electroporated CD40-B cells.

**Table 6. Electroporation settings for B cells**

| Cell type | Gene(s) | EP system (Company) | Type of pulse | Voltage (V) | Capacitance (µF) | Time (ms) | Gap (mm) | Buffer (µL) | Amount of mRNA (µg) | Cell number (x10$^6$ cells) | Cell concentration (x10$^6$cells/mL) | % Protein expression after EP (time point) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CD40-B cells | GFP<br>Tumor RNA | Modified square-wave electroporator (Amaxa) | SW<br>(Pulse Program U08) | n.d. | n.d. | n.d. | n.d. | 100 | 2 | 2-2.5 | n.a. | >80% GFP (24h) | [186] |
| CD40-B cells | CDV-HA<br>EGFP | Modified square-wave electroporator (Amaxa) | SW<br>(Pulse Program U08) | n.d. | n.d. | n.d. | n.d. | 100 | 2 | 2-2.5 | n.a. | 86% EGFP (24h) | [195] |
| CD40-B cells | Survivin<br>GFP<br>Tumor RNA | Modified square-wave electroporator (Amaxa) | SW<br>(Pulse Program U08) | n.d. | n.d. | n.d. | n.d. | 100 | 2-5 (Survivin and GFP)<br>2 / 10 (Tumor Rn.a. | 2-2.5 | n.a. | n.d. | [188] |
| LPS-B cells<br>CD40-B cells | EGFP<br>CEA | Gene Pulser (BTX) | SW | 300 | n.a. | 0.5 | 2 | 200 | 20 | n.a. | 5 | ~50% EGFP (24h) | [189] |
| CD40-B cells | CDV<br>Tumor RNA | Nucleofector device (Amaxa) | Pulse program U08 | n.d. | n.d. | n.d. | n.d. | <250 | 2 (CDV)<br>10 (Tumor Rn.a.) | <5 | 20 | n.d. | [190] |
| CD40-B cells | deltaLMP1 | ECM830 (BTX) | SW | 350 | n.a. | 0.35 | 2 | 40 | 20 | n.a. | 25 | >70% (36h) | [191] |
| CD40-B cells | Tumor RNA | n.d. | n.d. | 300 | 150 | n.d. | n.d. | 100 | 2 | 2-2.5 | n.a. | n.d. | [192] |
| CpG-B cells<br>LPS-B cells<br>CD40-B cells | IL-12p35<br>IL-12p40<br>CD80<br>OX40L<br>4-1BBL<br>Actin<br>OVA | ECM830 (BTX) | SW | 340 | n.a. | 0.5 | 2 | 200 | 5 | 2 | n.a. | IL-12p70: ~17,5 ng/mL<br>CD80: 95%<br>OX40L: 95%<br>4-1BBL: 90%<br>(24h) | [194] |

Abbreviations: CDV, canine distemper virus; CDV-HA, canine distemper virus HA polypeptide; CEA, carcinoembryonic antigen; deltaLMP1, mutant latent membrane protein 1; EGFP, enhanced green fluorescent protein; EP, electroporation; GFP, green fluorescent protein; h, hours; IL-12, interleukin 12; LPS, lipopolysaccharide; mRNA, messenger RNA; n.a., not applicable; n.d., no data; OVA, ovalbumin; OX40L, OX40 ligand; SW, square wave; 4-1BBL, 4-1BB ligand.

# Concluding remarks

Gene transfer by mRNA electroporation can be used to express (multiple) desired proteins in immune cells. On the forefront of this technique are DCs, which can be RNA-electroporated for a wide range of purposes. Although most often DCs are electroporated with TAA-mRNA to redirect the immune system to a specific tumor antigen, they are also engineered to enhance their functionality. As such, they can now be induced to interact with a larger variety of immune effector cells and display a more activated phenotype. In contrast to lymphocyte engineering, DCs are almost exclusively mRNA-engineered by means of electroporation rather than transduction methods. The main reasons for this difference are the limited lifespan of DCs and their non-proliferating properties. This makes electroporation the most appropriate technique for DC engineering. Although this technique is well-established for transfecting DCs, leading to multiple clinical trials for different types of cancer, it has not fully reached its potential use in lymphocytes. The prevalent tactic for lymphocyte engineering is the administration of immune receptors, such as TCRs and CARs, capable of recognizing tumor antigens. In this case, researchers tend to apply more permanent approaches such as viral transduction or transposon systems, ensuring the cells will express the protein of interest throughout their lifespan. The fact that the balance remains in favor of stable methods of lymphocyte engineering is exemplified by the lack of clinical trials using TCR-mRNA lymphocytes, except for one trial that was promptly terminated. CAR-engineered lymphocytes have been slightly more favored, although mRNA-based CAR-T-cell therapies have not yet achieved (pre)clinical efficiencies seen with virally produced CAR T cells. However, mRNA technology has proven to be flexible, allowing for rapid validation of the many iterations required to optimize TAA-specific TCR discovery and CAR design. The flexibility that mRNA electroporation offers is also highlighted by the possibility of multiplexing different mRNAs as well as siRNAs for gene silencing or guide RNAs using the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system for targeted genome editing [197].

In contrast to viral vectors, knock-in CRISPR/Cas and transposon systems and plasmid DNA transfection, transient gene expression linked to the natural decay of the introduced mRNA provides an accurate system to control the synthesis of transgenic proteins. Similarly, tight control of transgenic cell dose in patients is possible through repeated administration of mRNA-electroporated cells. Even considering the costs related to repeated administrations, mRNA transfection is a less costly cell engineering platform due to the limited laboratory requirements and equipment needed, as well as

the less cumbersome regulatory aspects for its introduction into the clinic compared to that of other methods. In terms of safety, viral transduction and non-viral stable genetic modification (e.g., transposons) comes at a price as concerns arise due to the possibility of insertional mutagenesis caused by the integration of the genetic material into the genome of the modified cell. Although this risk in principle is theoretical and it has not been reported to date, the potential concern should not be dismissed. In this regard, mRNA electroporation offers a safer alternative. Studies with CAR-T cells have shown that, when the therapy causes severe adverse effects, virally transduced T cells may have a greater negative impact as these cells will persist and continue damaging the patient longer than those prepared with transient expression methods. To reduce this, additional safety mechanisms such as suicide genes should be implemented; however, the potential risk may remain. Although adverse effects may be limited over time by the transient nature of mRNA electroporation, these safety mechanisms have also been included in the mRNA sequence as an added safety control. Improved cell activation and trafficking towards the tumor site has also been achieved through the co-electroporation of other RNAs that encode cytokines or chemokine receptors. Overall, the benefits mRNA electroporation provides for the genetic engineering of immune cells justify its application in cancer therapies.

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> **A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.**
>
> — Marie Skłodowska Curie

# 4

# Efficient and non-genotoxic RNA-based engineering of human T cells using tumor-specific T-cell receptors with minimal TCR mispairing

This chapter has been published in:

Campillo-Davo D*, Fujiki F*, Van den Bergh JMJ, De Reu H, Smits EL, Goosens H, Sugiyama H, Lion E, Berneman ZN, Van Tendeloo VFI.
*Frontiers in Immunology* (2018);9:2503.

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# Abstract

Genetic engineering of T cells with tumor specific T-cell receptors (TCR) is a promising strategy to redirect their specificity against cancer cells in adoptive T cell therapy protocols. Most studies are exploiting integrating retro- or lentiviral vectors to permanently introduce the therapeutic TCR, which can pose serious safety issues when treatment-related toxicities would occur. Therefore, we developed a versatile, non-genotoxic transfection method for human unstimulated CD8+ T cells. We describe an optimized double sequential electroporation platform whereby Dicer-substrate small interfering RNAs (DsiRNA) are first introduced to suppress endogenous TCR $\alpha$ and $\beta$ expression, followed by electroporation with DsiRNA-resistant tumor-specific *TCR* mRNA. We demonstrate that double sequential electroporation of human primary unstimulated T cells with DsiRNA and *TCR* mRNA leads to unprecedented levels of transgene TCR expression due to a strongly reduced degree of TCR mispairing. Importantly, superior transgenic TCR expression boosts epitope-specific CD8+ T cell activation and killing activity. Altogether, DsiRNA and *TCR* mRNA double sequential electroporation is a rapid, non-integrating and highly efficient approach with an enhanced biosafety profile to engineer T cells with antigen-specific TCRs for use in early phase clinical trials.

# Introduction

Cancer is one of the leading causes of death in the world, according to the World Health Organization. Traditionally, the first lines of cancer treatment are chemotherapy, radiotherapy and/or surgery. However, the high incidence of relapse among cancer patients led to the development of new strategies exploring the use of our immune system as a refined and more specific tool to fight cancer [1]. In particular, among the different cancer immunotherapies available, adoptive cell transfer of T cells has been the focus of numerous advances in medicine. In fact, the potential of adoptive T-cell therapy has been demonstrated in both malignant and infectious diseases [2]. In cancer immunotherapy, many of these therapies focus on tumor associated antigens (TAAs) that are overexpressed in cancer cells and are only present in limited amounts in other healthy tissues [3]. Yet the negative selection of self-antigen reactive T cells translates into scarcity of circulating TAA-specific T cells, challenging their ex vivo isolation and demanding timely and large-scale ex vivo expansion [4]. To circumvent this limitation, T cell receptor (TCR) gene engineering of bulk T cells is increasingly becoming the method of choice to produce large amounts of redirected T cells [5]. However, the clinical efficacy of TCR-redirected T cells is still not satisfactory, and serious adverse effects have been observed in clinical trials [5]. First, gene transfer methods involving transduction by retro- or lentiviral vectors can integrate viral DNA into the host genome potentially leading to insertional mutagenesis that could disrupt genes important for cell function or promote tumorigenesis [6]. Second, in the event of unanticipated transgenic TCR specificities, permanent expression of transgenic TCR could produce long-lasting toxicities with severe consequences [5,6]. Third, strategies to improve the efficacy of the therapy, including modifications of transgenic TCR structure via introduction of murine domains to enhance preferential pairing or artificial enhancement of TCR affinity could result in undesired immunogenicity, are technically demanding and costly [7,8]. Fourth, concomitant expression of endogenous and transgenic TCR genes produces two sets of TCR alpha (TCR$\alpha$) and beta (TCR$\beta$) chains that can pair incorrectly [9], generating two mispaired TCR heterodimers that reduce transgenic TCR levels [10] and may lead to on-target and off-target toxicities in patients [11]. These data have prompted us to develop a safer, faster and more widely applicable method for TCR engineering of T cells. Based on our longstanding expertise with clinical tools using mRNA-modified dendritic cell (DC) vaccines in acute myeloid leukemia (AML) patients [12,13], we adapted our mRNA electroporation protocol to human resting $CD8^+$ T cells for rapid and efficient transient TCR expression [14–17]. Furthermore, we implemented an RNA interference step for substantial reduction of TCR mispairing, enhancing the safety profile of TCR-engineered T cells. Overall, we

present a double sequential electroporation of DsiRNA and codon-optimized *TCR* mRNA as a non-genotoxic, highly efficient and versatile non-viral platform with an enhanced biosafety profile to engineer T cells with TCRs for adoptive T cell immunotherapy.

# Results

## Cloning of WT1-specific *TCR* mRNA and validation in a 2D3 cell model

We established a cytotoxic T lymphocyte (CTL) clone reactive to $WT1_{126–134}$ peptide from an HLA-A∗02:01$^{+}$ patient with acute myeloid leukemia (AML) with a favorable clinical response in our Wilms' tumor protein 1 (WT1)-targeted DC vaccination trial (ClinicalTrials.gov NCT00834002) and with polyepitopeWT1-specific CTL responses [12] (**Figure 1A**). After isolation of TCR$\alpha$ and TCR$\beta$ genes, the wild-type TCR$\alpha$ and TCR$\beta$ sequences were linked with a P2A peptide sequence [18] and inserted into a plasmid vector for bicistronic and equimolar expression of both TCR chains ($WT1_{126}$ *TCR*-wt mRNA; **Figure 1B**). To enhance *TCR* mRNA translation, the TCR$\alpha$ and TCR$\beta$ sequences were codon-optimized and the order of the TCR genes was reversed [19], inserting the TCR$\beta$ before the P2A peptide sequence ($WT1_{126}$ *TCR*-co mRNA; **Figure 1B**). After in vitro *TCR* mRNA generation, we validated transgenic TCR expression in a 2D3 cell line originating from TCR$\alpha\beta$-deficient Jurkat 76 cells (**Figure S1**). High levels of $WT1_{126}$ TCR were detected in 2D3 cells 4 hours (h) after $WT1_{126}$ *TCR*-wt or $WT1_{126}$ *TCR*-co mRNA electroporation (56.3 ± 0.3% and 71.9 ± 1.5%, respectively; mean ± SEM of 3 replicates). $WT1_{126}$ TCR expression was higher after transfection with *TCR*-co mRNA as compared to *TCR*-wt mRNA at most time points post-electroporation, whilst transgenic TCR was lost 5 days after transfection with either mRNA (**Figure S1A**). To analyze the functional avidity of the cloned TCR, $WT1_{126}$ *TCR*-wt or $WT1_{126}$ *TCR*-co mRNA-electroporated 2D3 cells were cultured with T2 cells pulsed with titrated amounts of $WT1_{126–134}$ peptide. TCR functionality was confirmed by marked expression levels of enhanced green fluorescent protein (EGFP) reporter gene at high peptide concentrations for both *TCR*-wt and *TCR*-co mRNA-electroporated 2D3 cells, with identical TCR activation thresholds at a $WT1_{126–134}$ peptide concentration of $10^{-3}$ μM (**Figure S1B**).

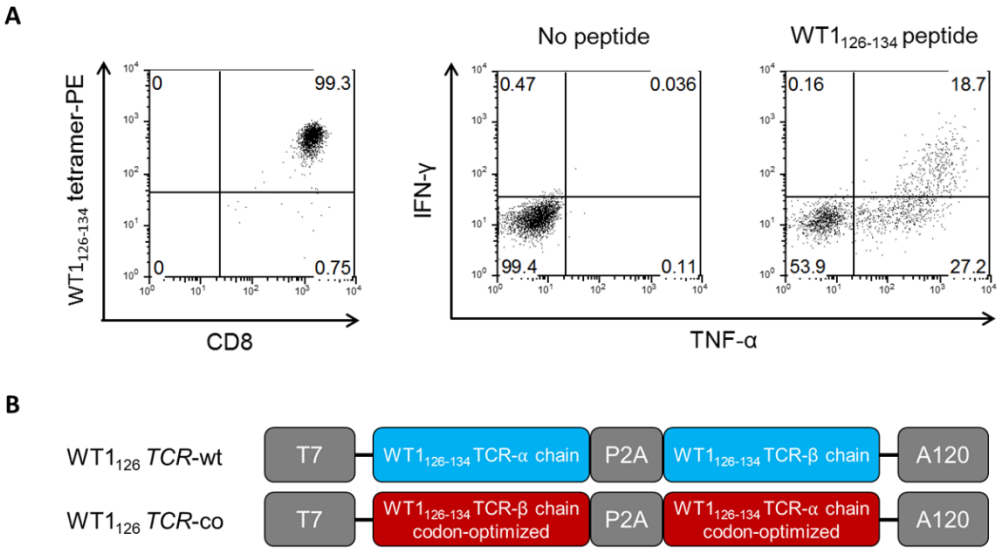


**Figure 1. Isolation and characterization of WT1$_{126}$-specific CTL clone.** (**A**) WT1$_{126–134}$/HLA-A*02:01 tetramer staining and WT1$_{126–134}$ peptide-specific interferon (IFN)-γ and tumor necrosis factor (TNF)-α production of the WT1$_{126–134}$-reactive CTL clone. (**B**) Schematic representation of pST1 plasmid vectors containing the WT1$_{126–134}$-specific wild-type (WT1$_{126}$ *TCR*-wt) and WT1$_{126–134}$-specific codon-optimized (WT1$_{126}$ *TCR*-co) TCR cassettes. WT1, Wilms' tumor 1; wt, wild-type; co, codon-optimized; T7, T7 promoter; P2A, picornaviral 2A-like sequence; A120, 120-mer poly(A) tail.

## Electroporation of DsiRNA targeting *TRAC* and *TRBC* transcripts inhibits endogenous TCR expression

To tackle the problem of mispairing in TCR-engineered primary T cells, we focused on using RNA interference to mediate downregulation of endogenous TCR transcripts combined with codon-optimized *TCR* mRNA transfection. In view of the superiority of Dicer-substrate small interfering RNAs (DsiRNA) vs. canonical small interfering RNA in silencing of target mRNA [20–22], we designed DsiRNA duplexes to specifically recognize the coding sequences of wild-type TCR alpha (*TRAC*) and TCR beta (*TRBC*) constant regions (**Figure 2**). Thus, wild-type, but not codon-optimized TCR sequences would be sensitive to DsiRNA-mediated knockdown. We first analyzed suppression efficiency of TCR-specific DsiRNA (DsiRNA) compared to mock electroporation (Mock) and DsiRNA specific for *EGFP* (DsiRNA$_{EGFP}$) in TCR$^+$ Jurkat E6-1 cells by RT-qPCR 24 h after electroporation (**Figure 2A**). There was a significant, more than 6-fold decrease in *TRAC* expression and more than 3-fold decrease in *TRBC* expression when

cells were electroporated with DsiRNA compared to mock electroporation ($P \leq 0.0001$). *TRAC* and *TRBC* expression levels after $DsiRNA_{EGFP}$ electroporation remained similar to the mock electroporation, confirming that efficient inhibition of TCR transcripts was only achieved by TCR-specific DsiRNA. We then evaluated the specific silencing effect of DsiRNA on the transgenic wild-type *TCR* mRNA sequence and the DsiRNA resistance of the codon-optimized *TCR* mRNA sequence in TCR$\alpha\beta$-deficient 2D3 cells. As shown in **Figure 2B**, simultaneous transfection with DsiRNA and $WT1_{126}$ *TCR*-wt mRNA led to a substantial decrease in transgenic TCR expression 24 h after electroporation as compared to the electroporation of the $WT1_{126}$ *TCR*-wt mRNA only (21.7 ± 3.8 and 51.2 ± 3.9%, respectively), whereas TCR levels remained stable after electroporation of $WT1_{126}$ *TCR*-co mRNA with or without DsiRNA (80.1 ± 2.4 and 80.3 ± 2.6%, respectively). This illustrates the specificity of the DsiRNA for wild-type TCR sequences and shows that codon-optimization protects transgenic *TCR* mRNA sequences from degradation by our designed DsiRNA. Next, we assessed the degree of DsiRNA-mediated knockdown of endogenous TCR in purified human resting $CD8^+$ T cells following simultaneous electroporation with DsiRNA and $WT1_{126}$ *TCR*-co mRNA or electroporation with either of them alone. Significant reduction in total TCR expression was observed 3 or 4 days after electroporation in those conditions where DsiRNA was added ($P \leq 0.05$; **Figure 2C**). TCR surface levels were measured up to 6 days post-transfection and declined to about 50% of total TCR levels in DsiRNA-treated cells (50.3 ± 6.6 %) compared to non-treated cells (95.4 ± 1.0%). $WT1_{126-134}$/HLA-A*02:01 tetramer staining of these cells showed a significantly higher $WT1_{126}$ TCR expression when DsiRNA was simultaneously electroporated with $WT1_{126}$ *TCR*-co mRNA (33.8±2.7%), resulting in a 17% increase in transgenic TCR expression 24 h after electroporation in comparison to electroporation of mRNA alone (28.8 ± 3.2% **Figure 2D**). A correlation analysis for tetramer positive and transgenic TCRa or b chain positive cells could be possible at the time that antibodies specific for the transgenic TCR$\alpha$ or TCR$\beta$ chains would be available.

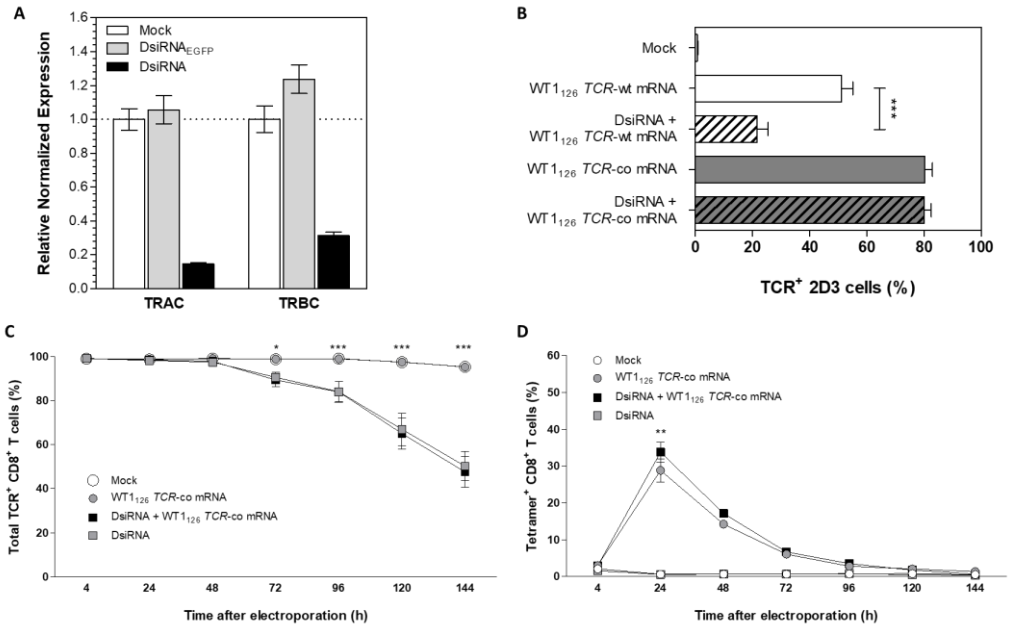


**Figure 2. Silencing effect of DsiRNA against *TRAC* and *TRBC* mRNAs upon simultaneous DsiRNA and *TCR* mRNA electroporation.** (**A**) Analysis of *TRAC* and *TRBC* gene expression using RT-qPCR in Jurkat E6-1 cells after single electroporation with a control DsiRNA against *EGFP* (DsiRNA$_{EGFP}$), with DsiRNA targeting *TRAC* and *TRBC* (DsiRNA) or no DsiRNA (mock). Expression levels were normalized to the reference genes importin-8 and ribosomal protein L13A and analyzed relative to mock electroporation. (**B**) TCR-deficient 2D3 cells were electroporated simultaneously with wild-type (-wt) or codon-optimized (-co) WT1$_{126}$ *TCR* mRNA and DsiRNA against *TRAC* and *TRBC* or electroporated with WT1$_{126}$ *TCR* mRNA only. TCR surface expression was analyzed 24 h after transfection (mean ± SEM of 3 replicate experiments). Primary unstimulated CD8$^+$ T cells were electroporated simultaneously with WT1$_{126}$ *TCR*-co mRNA and DsiRNA against *TRAC* and *TRBC* or with *TCR* mRNA only. The percentage of total TCR expression (**C**) and percentage of transgenic TCR expression (**D**) was measured in primary unstimulated CD8$^+$ T cells at different time points after electroporation ($n = 3$; mean ± SEM). *$P <$ 0.05; **$P < 0.01$; ***$P < 0.001$; *TRAC*, T-cell receptor alpha constant region; *TRBC*, T-cell receptor beta constant region; Mock, mock electroporation; DsiRNA$_{EGFP}$, Dicer-substrate small interfering RNA directed against *EGFP* gene; DsiRNA, Dicer-substrate small interfering RNAs directed against *TRAC* and *TRBC* genes; WT1, Wilms' tumor 1; wt, wild-type; co, codon-optimized.

## DsiRNA electroporation 24 h prior to TCR codon-optimized mRNA electroporation drastically increases transgenic TCR expression

To fully exploit the silencing potential of DsiRNA and to optimize transgenic TCR expression, we tested sequential electroporation of 2D3 cells with DsiRNA followed by WT1$_{126}$ *TCR* mRNA electroporation 6 or 24 h later and analyzed TCR surface expression levels 24 h after the second electroporation (**Figure 3A**). Superior and significant reduction of TCR levels was observed when DsiRNA transfection was performed 24 h prior to *TCR*-wt mRNA electroporation (4.9 ± 0.5%), as compared to a 6 h interval (18.4 ± 3.3%). Kinetics of TCR expression of double sequentially-electroporated 2D3 cells with 24 h between electroporations showed a sustained and marked downregulation of TCR expression 24 h after electroporation of WT1$_{126}$ *TCR*-wt mRNA when cells were pre-treated with DsiRNA (from 69.8 ± 4.9% to 4.9 ± 0.5% with a decrease of 93%, $P \leq 0.001$; **Figure 3B**). We analyzed the degree of silencing in Jurkat E6-1 cells 24 h after DsiRNA/mock double sequential electroporation (i.e., *TRAC* and *TRBC* levels analyzed 48 h after DsiRNA electroporation) by RT-qPCR (**Figure S2**). We observed a significant, more than 6-fold downregulation of *TRAC* mRNA levels and more than 2-fold downregulation of *TRBC* mRNA levels compared to double sequential mock electroporation ($P \leq 0.01$ for *TRAC* and $P \leq 0.05$ for *TRBC*), similar to the results obtained 24 h after one electroporation with DsiRNA only (**Figure 2A**). These results indicate that the silencing effect of the DsiRNA on the target endogenous TCR transcripts is still markedly present after a second electroporation and, more importantly, that the DsiRNA still exert their effect 48 h after DsiRNA electroporation. This guarantees that TCR mispairing is being prevented when the peak of transgenic TCR expression occurs after DsiRNA/TCR-co double sequential electroporation. To further investigate the degree of mispairing between two TCRs expressed concomitantly, we generated from another CTL clone of the same patient, a TCR reactive to the WT1$_{37–45}$ peptide (**Figure S3A**) and produced the codon-optimized mRNA construct (WT1$_{37}$ *TCR*-co mRNA; **Figure S3B**). Using the same optimized double sequential electroporation platform with 24 h between first and second electroporation, 2D3 cells were transfected with DsiRNA against *TRAC* and *TRBC* mRNAs or a control DsiRNA targeting *EGFP* mRNA prior to electroporation with WT1$_{37}$ *TCR*-co mRNA or a combination of WT1$_{37}$ *TCR*-co mRNA and WT1$_{126}$ TCR-wt and stained with WT1$_{37–45}$/HLA-A∗02:01 and WT1$_{126–134}$/HLA-A∗02:01 tetramers 24 h after mRNA electroporation. Of note, the WT1/HLA-A∗02:01 tetramers used to quantify WT1$_{37}$ or WT1$_{126}$ TCR expression cannot bind to mispaired TCRs. As shown in **Figure 3C**, 2D3 cells electroporated with WT1$_{37}$ *TCR*-co mRNA expressed high levels of WT1$_{37}$ TCR (93.0 ± 1.8%), whereas a significant

reduction of 33% ($P \leq 0.001$) was observed when WT1$_{126}$ *TCR*-wt mRNA was co-electroporated (62.1 ± 0.9%), indicative for the degree of mispairing when two TCRs are expressed in the same cell. Importantly, complete inhibition of mispairing between the two TCRs could be achieved upon pre-transfection with DsiRNA directed against *TRAC* and *TRBC* mRNAs (93.7 ± 0.6%), but not DsiRNA$_{EGFP}$ (60.9 ± 2.0%), leading to a full recovery of WT1$_{37}$ TCR expression ($P \leq 0.001$; **Figure 3C**, **upper**). Similarly, the percentage of WT1$_{126}$ TCR positive cells was nearly abolished in cells treated with DsiRNA directed against *TRAC* and *TRBC* (0.49 ± 0.02%), but not against *EGFP* mRNA (23.0 ± 1.3%; **Figure 3C, lower**), demonstrating the efficacy and specificity of DsiRNA for downregulation of *TCR*-wt mRNA.

## Double sequential electroporation of DsiRNA and TCR codon-optimized mRNA boosts transgenic TCR expression in primary CD8$^+$ T cells

Next, the optimized DsiRNA+*TCR* mRNA double sequential electroporation protocol was validated in human primary resting CD8$^+$ T cells from healthy donors (**Figure 4**). We observed a 2-fold increase in codon-optimized TCR expression using the double sequential electroporation (42.6 ± 4.9%; mean ± SEM of n = 15) vs. a single *TCR* mRNA electroporation (19.3 ± 2.2%; $P \leq 0.001$; **Figure 4A,B**). Transgenic TCR expression was maintained for at least 5 days after WT1$_{126}$ *TCR*-co mRNA electroporation, with superior TCR expression kinetics up until day 4 when T cells were pre-treated with DsiRNA (19.6 ± 2.5% for DsiRNA+WT1$_{126}$ *TCR*-co mRNA vs. 8.7±1.9% for WT1$_{126}$ *TCR*-co mRNA only at day 4; **Figure 4C**). Gene expression analysis of endogenous *TRAC* and *TRBC* transcripts revealed that DsiRNA targeting these sequences significantly downregulated the levels of *TRAC* and *TRBC* transcripts in resting CD8$^+$ T cells. Expression levels were decreased more than 3-fold compared to mock electroporation ($P \leq 0.01$).

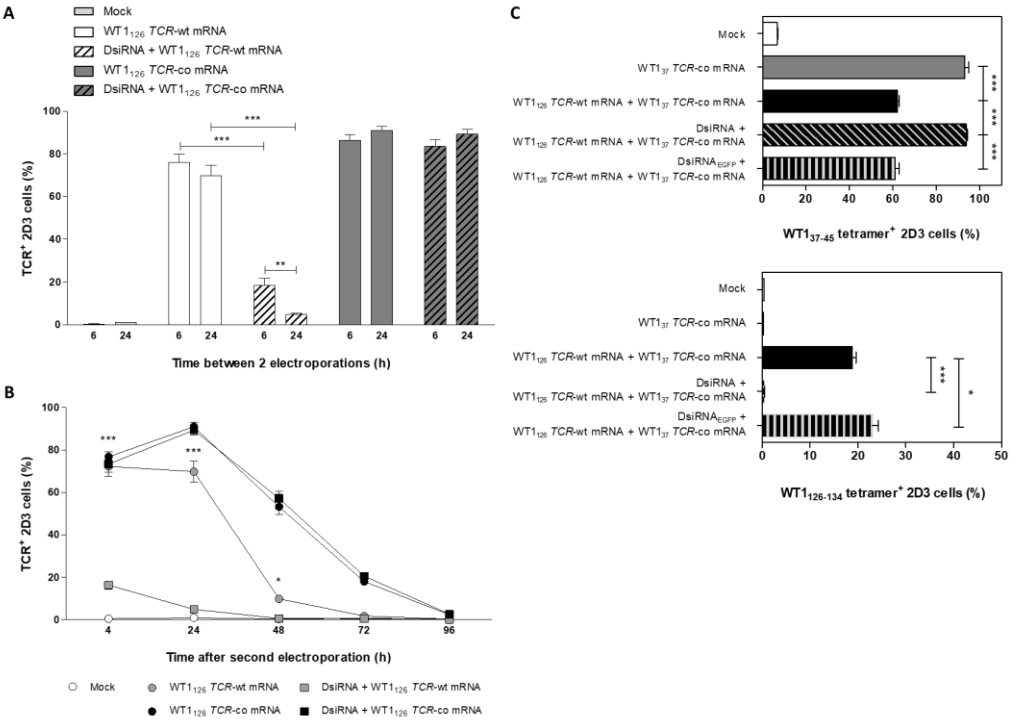


**Figure 3. Optimization of double sequential electroporation with DsiRNA and *TCR* mRNA in 2D3 cells.** (**A**) Influence of different time spans between first and second sequential electroporation on transgenic TCR expression in TCR$\alpha\beta$-deficient 2D3 cells. DsiRNA electroporation was performed 6 or 24 h prior to WT1$_{126}$ *TCR* mRNA electroporation. (**B**) Kinetics of transgenic TCR expression in double sequentially-electroporated 2D3 cells. DsiRNA electroporation was performed 24 h prior to WT1$_{126}$ *TCR* mRNA electroporation. (**C**) Effect of mispairing on transgenic TCR expression. 2D3 cells were electroporated with a DsiRNA specific for *EGFP* (DsiRNA$_{EGFP}$) or DsiRNA for wild-type *TRAC* and *TRBC* genes (DsiRNA) 24 h before electroporation with WT1$_{37}$ *TCR*-co mRNA or a combination of WT1$_{37}$ *TCR*-co and WT1$_{126}$ *TCR*-wt mRNAs. Transgenic TCR expression was analyzed 24 h after mRNA transfection with WT1$_{37–45}$/HLA-A*02:01 tetramers (upper panel) and WT1$_{126–134}$/HLA-A*02:01 tetramers (lower panel). All graphs show the results for 3 independent experiments (mean ± SEM). ***$P < 0.001$; Mock, mock electroporated; WT1, Wilms' tumor 1; wt, wild-type; co, codon-optimized; DsiRNA, Dicer-substrate small interfering RNAs directed against *TRAC* and *TRBC* genes; DsiRNA$_{EGFP}$, Dicer-substrate small interfering RNA directed against *EGFP* gene.

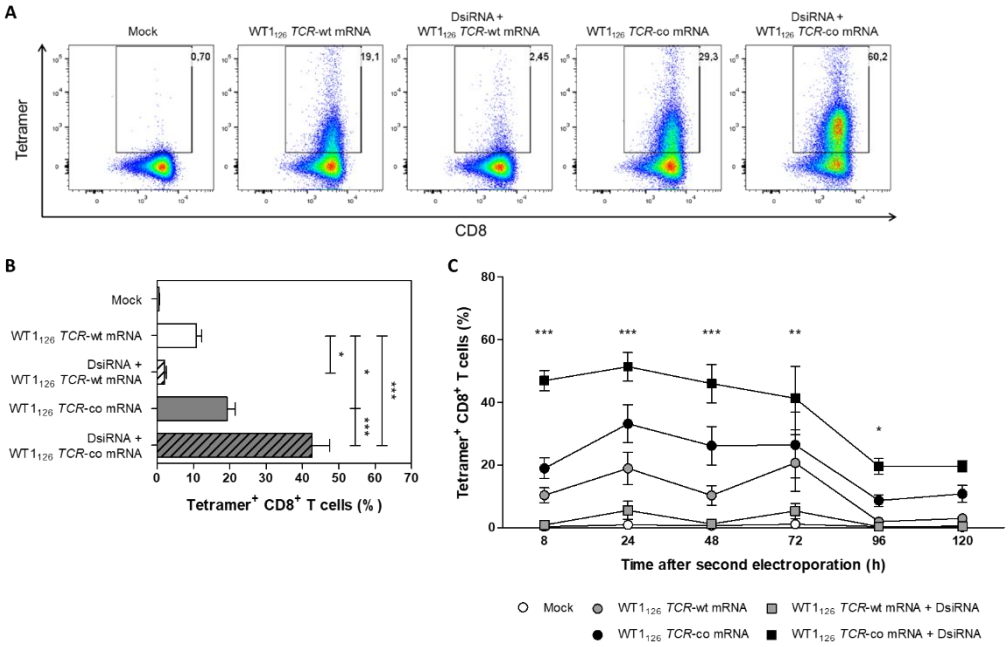


**Figure 4. Analysis of transgene WT1$_{126}$ TCR expression in human primary resting CD8$^+$ T cells after double sequential electroporation with DsiRNA transfection performed 24 h prior to WT1$_{126}$ *TCR* mRNA transfection.** (**A**) Representative flow cytometric analysis by WT1$_{126–134}$/HLA-A*02:01 tetramer staining 24 h after the second electroporation showing transgenic TCR expression from one out of 15 donors. The percentage of tetramer-positive CD8+ T cells is indicated in the upper right corner. (**B**) Transgenic TCR expression of double sequentially-electroporated resting CD8$^+$ T cells was evaluated 24 h after *TCR* mRNA electroporation by WT1$_{126–134}$/HLA-A*02:01 tetramer analysis ($n$ = 15, mean ± SEM). (**C**) Kinetics of transgenic TCR expression after second electroporation of resting CD8$^+$ T cells ($n$ = 3, mean ± SEM). *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001; Mock, mock electroporated; WT1, Wilms' tumor 1; wt, wild-type; co, codon-optimized; DsiRNA, Dicer-substrate small interference RNAs directed against *TRAC* and *TRBC* genes.

## Redirection of effector response of primary resting CD8⁺ T cells via DsiRNA/*TCR* mRNA double sequential electroporation promotes killing of target cells

We evaluated whether the improved TCR expression after double sequential electroporation correlated with enhanced redirected T-cell effector functions (**Figure 5**). First, to assess the functional avidity of the TCR for its cognate peptide, electroporated CD8+ T cells were assayed for interferon (IFN)-γ production upon recognition of epitope-carrying target cells. DsiRNA-mediated silencing of the endogenous *TCR* mRNA in *TCR* mRNA-electroporated CD8⁺ T cells led to a significantly improved recognition of $WT1_{126–134}$ peptide-pulsed target cells as compared to their non-silenced counterparts up to a $WT1_{126–134}$ peptide concentration of $10^{-2}$ μM ($P \leq 0.001$; **Figure 5A**). This activation threshold is equivalent to that observed in TCR-deficient 2D3 cells (**Figure S1B**). Similar results were obtained upon analysis of granzyme B secretion in supernatants of double sequentially-electroporated CD8⁺ T cells co-cultured with peptide-pulsed T2 cells **(Figure 5B)**. In this case, pre-treatment with DsiRNA of$WT1_{126}$ *TCR*-co mRNA electroporated T cells led to a 2.4-fold increase compared to non-treated cells (554.0 ± 232.5 pg/mL and 234.3 ± 82.0 pg/mL, respectively). Second, double sequentially-electroporated CD8⁺ T cells were analyzed for expression of activation markers CD69 and CD137 after co-culture with peptide-pulsed T2 cells (**Figure 5C,D**). DsiRNA-pre-treated and $WT1_{126}$ *TCR*-co mRNA-transfected CD8⁺ T cells exhibited significantly higher frequencies of CD69 (70.4 ± 3.2%) and CD137 (29.3 ± 2.3%) positivity in an antigen-specific manner, as compared to cells that were electroporated with *TCR*-co mRNA only (62.3 ± 3.0% CD69+ and 17.8 ± 1.5 % CD137⁺ CD8⁺ T cells), reaching a difference of 64% for CD137. Frequencies of both CD69⁺ and CD137⁺ CD8⁺ T cells was always significantly lower when these cells were electroporated with $WT1_{126}$ *TCR*-wt mRNA either pre-treated with DsiRNA or not (42.4 ± 3.5% vs. 57.4 ± 4.6 % for CD69 and 3.3 ± 0.4 % vs. 13.1 ± 1.4 for CD137; **Figure 5C,D**). Finally, we evaluated the cytotoxic capacity of transfected resting CD8+ T cells (**Figure 6**). Antigen-specific cytotoxicity by $WT1_{126}$ *TCR*-co mRNA-electroporated CD8⁺ T cells was superior in DsiRNA pre-treated (52.4 ± 3.8%) as compared to non-pre-treated CD8⁺ T cells (38.8 ± 2.1%), whereas it was virtually reduced to mock levels in DsiRNA-pretreated $WT1_{126}$ *TCR*-wt mRNA-electroporated T cells (28.3 ± 1.6%; **Figure 6A,B**). There was no significant difference in mean levels of cytotoxicity after wild-type or codon-optimized mRNA transfection without DsiRNA pre-treatment (35.9 ± 1.9 and 38.8 ± 2.1%, respectively).

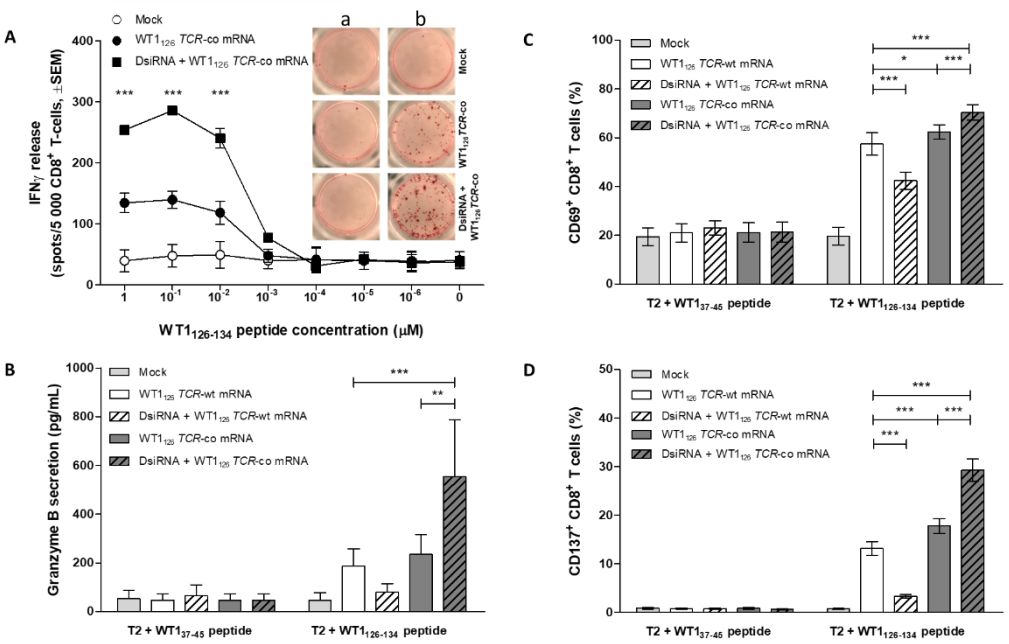


**Figure 5. Effect of DsiRNA-mediated silencing of endogenous *TCR* on WT1$_{126}$ TCR avidity and antigen-specific activation in resting CD8$^+$ T cells after double sequential electroporation with DsiRNA transfection performed 24 h prior to WT1$_{126}$ *TCR* mRNA transfection.** (**A**) Release of IFN-γ was measured by IFN-γ ELISpot after co-culture of double sequentially-electroporated CD8$^+$ T cells and T2 cells that were pulsed with decreasing concentrations of WT1$_{126–134}$ peptide ($n$ = 2, mean ± SEM). Within the graph, representative wells of co-cultures with non-peptide-pulsed T2 cells (a) or peptide-pulsed T2 cells (b, 1μM peptide). (**B–D**) Primary unstimulated CD8$^+$ T cells were double sequentially-electroporated with WT1$_{126}$ *TCR* mRNA after DsiRNA or mock (no RNA) electroporation. Transfected CD8$^+$ T cells were co-cultured with peptide-pulsed T2 cells in an effector:target ratio of 4:1. After 24 h, cells were pelleted by centrifugation and supernatants were collected. (**B**) Secretion of granzyme B was analyzed in supernatants using a human granzyme B ELISA kit ($n$ = 4, mean ± SEM). Flow cytometric analysis of antigen-specific T cell activation was analyzed by activation-induced upregulation of surface markers CD69 (**C**) and CD137 (**D**) in CD8$^+$ T cells ($n$ = 5, mean ± SEM). *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001; IFN-γ, interferon-γ; Mock, mock electroporated; WT1, Wilms' tumor 1; co, codon-optimized; DsiRNA, Dicer-substrate small interference RNAs directed against *TRAC* and *TRBC* genes.

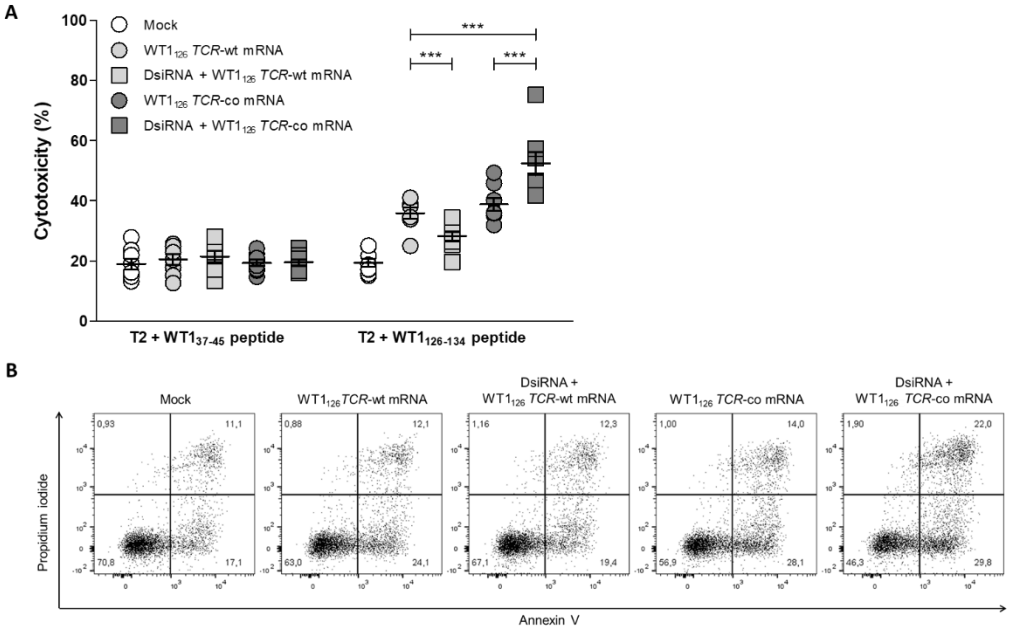


**Figure 6. Antigen-specific cytotoxicity of primary resting CD8⁺ T cells is boosted after double sequential electroporation with DsiRNA and WT1126 *TCR*-co mRNA.** (**A**) Cytotoxic activity of double sequentially-electroporated CD8⁺ T cells after 6 h of co-culture with peptide-pulsed T2 cells (E:T ratio = 20:1, $n$ = 8, mean ± SEM). WT1$_{37–45}$ peptide-pulsed T2 cells served as negative control target. (**B**) Representative example of WT1$_{126–134}$ peptide-pulsed T2 cell cytotoxicity mediated by double sequentially-electroporated CD8⁺ T cells after 6 h of co-culture. The percentage of cells is indicated in each quadrant. ***$P$ < 0.001; Mock, mock electroporated; WT1, Wilms' tumor 1; wt, wild-type; co, codon-optimized; DsiRNA, Dicer-substrate small interfering RNAs directed against *TRAC* and *TRBC* genes.

# Discussion

In recent years, different strategies to improve TCR gene transfer and functionality in T cells have been developed. Advances in this field include retro- and lentiviral transduction protocols to achieve stable and long-term TCR expression, modification of TCR affinity, incorporation of cysteine bonds or murinization of constant regions to enhance TCR pairing and conjugation of TCRs with co-stimulatory signals [23]. However, clinical safety issues, complex TCR manipulations and high costs associated with these methods are an obstacle for widespread clinical use. Here, we describe a double sequential electroporation procedure with DsiRNA and codon-optimized *TCR* mRNA for rapid TCR engineering of T cells. This non-viral and non-genotoxic approach results in robust transient expression of transgenic TCR and superior T-cell effector function of primary resting CD8+ T cells while preventing TCR mispairing by DsiRNA-mediated silencing of endogenous TCR. Regardless of the origin of wild-type TCR sequences (endogenous or $WT1_{126}$ *TCR*-wt mRNA) and of T cells (Jurkat cell lines or primary CD8+ T cells), electroporation of DsiRNA reproducibly led to a reduction in TCR expression from these wild-type sequences. In contrast, DsiRNA transfection enhanced the surface expression of transgenic TCR after electroporation with codon-optimized *TCR* mRNA in the presence of a wild-type and/or endogenous *TCR* mRNA. This confirms the specificity of the DsiRNA for wild-type TCR sequences and the reduction of TCR mispairing. Using this strategy, production of TCR-engineered T cells is greatly simplified and broadly applicable because codon optimization is a commonly available tool and because the particular design of the DsiRNA will allow the suppression of any endogenous TCR. This method should avoid the need for more complex TCR modifications in order to improve transgenic TCR pairing. In general, mRNA electroporation is one of the methods of choice for non-viral transfection of immune cells, including dendritic cells [14] and T cells [15], and can be adopted for TCR engineering of primary unstimulated T cells as demonstrated in this and other studies [24,25]. Thus, resting T cells can be transfected and antigen-activated without the need for pre-activation culture protocols. This is an advantage for clinical T cell therapy purposes, as it considerably cuts production time and costs. Since simultaneous electroporation of DsiRNA and codon-optimized *TCR* mRNA produced a low percentage increase in TCR levels we aimed to improve expression of transgenic TCR by transfecting DsiRNA prior to codon-optimized *TCR* mRNA electroporation. During the optimization of the double sequential electroporation, best results were obtained with a 24 h interval between DsiRNA and codon-optimized *TCR* mRNA electroporation, pointing to possible overlapping kinetics of transgenic TCR expression and DsiRNA-mediated silencing [26] of endogenous TCR. Therefore, 24 h double

sequential electroporation provides a time window for DsiRNA assembly with RNA-induced silencing complex (RISC), RISC activation and downregulation of endogenous *TCR* mRNA before introducing a new *TCR* mRNA. Importantly, the DsiRNA-mediated silencing of the endogenous TCR is markedly present at least until the point when transgenic TCR expression from codon-optimized *TCR* mRNA is at its highest levels, ensuring that TCR mispairing is avoided when the T cells display their maximum functionality. With regards to other silencing strategies, levels of transgenic TCR expression after treatment with DsiRNA were comparable, if not higher, to those obtained by retroviral transduction of constructs containing short hairpin RNA (shRNA) or clusters of primary microRNAs (pri-miRNAs) and siRNA-resistant antigen-specific TCR [27,28]. In a preclinical study using these shRNA-containing retroviral vectors, Ochi and collaborators [29] reported that transduced CTLs from leukemia patients showed high antileukemic responses against autologous tumor cells in vitro as well as in vivo in a mouse xenograft model, providing evidence that silencing of endogenous TCR is a powerful tool for T cell-based cancer immunotherapy. Other non-viral approaches have also been exploited for the transfection of T cells, such as the electroporation of a DNA plasmid integrating the Sleeping Beauty transposon/transposase system [30,31]. However, as occurs with integrating viral vectors, the risk of insertional mutagenesis is an important element to consider for its clinical application. Compared to these strategies to express different forms of RNA interference or to introduce a transgenic TCR, we show here that double sequential electroporation with DsiRNA and *TCR* mRNA is an efficient non-integrating system that rapidly redirects and boosts T-cell effector function. This highlights the potential efficacy of this immunotherapy for clinical trials. Electroporation provides a time window in which transgenic TCR is present and engineered CTLs will recognize the antigen of interest, followed by natural degradation of transfected DsiRNA and mRNA and restitution to their previous TCR phenotype. With our method, the introduction of DsiRNA increases transgenic TCR expression, yet the duration of transgenic TCR expression remains the same with or without DsiRNA. We showed the kinetics of the surface expression of the antigen-specific TCRs on viable cells after double sequential electroporation. Since we worked with unactivated T cells directly from PBMC after CD8+ T cell isolation, the viability and life span of these cells will be limited unless growth factors to promote T cell survival are added to the culture medium. In our experiments, there was no pre-activation of T cells nor addition of cytokines. Therefore, TCR expression on viable cells was measured up to 5–6 days after transfection, time in which T-cell viability was naturally declining due to the absence of survival signals. By doing so, we prevented the introduction of any confounding factors that might had influenced the kinetics of the TCR expression. Consequently, because of the transient

nature of the electroporated DsiRNA and mRNA, one single administration may not be enough for clinical effect in large-scale clinical trials. To ensure the effectiveness of our approach repeated administrations of TCR-engineered T cells would be required in order to avoid T-cell expansion protocols. Then, the isolated T cells can be cryopreserved in different aliquots (TCR-engineered or not) for later use, outrivaling other methods by its rapid production for administration to patients. Time-limited expression of transgenic TCR also favors the possibility of testing the safety of a particular transgenic TCR and the potential presence of "off-target" specificities from the introduced TCR in phase I studies prior to trials with more stable and expensive expression systems and/or complete disruption of endogenous TCR sequences [32]. In the case of the specificity of the DsiRNA, it is worth mentioning that the DsiRNA used in this study were analyzed for their specificity against the wild-type or codon-optimized *TRAC* and *TRBC* transcripts. In the event of any potential off-target effects eliciting the downregulation of other non-targeted mRNAs, the transient nature of the electroporated DsiRNA will prevent the development of long-lasting adverse effects. As for any other immunotherapy, CTL dosage and frequency of injections will have to be tested to ascertain the efficacy of one or more administrations. To this end, the field of chimeric antigen receptors (CAR)-modified T cells has also been exploiting mRNA electroporation in the last years [33–38] with different results. With regards to dosage and efficacy, Barrett *et al.* [34] showed that human mRNA-electroporated CD19-specific CAR-engineered CTLs had potent in vitro antileukemic killing activity against $CD19^+$ cell lines and reduced disease burden within 1 day after injection of a single dose in a mouse model xenografted with human CD19+ leukemia cells. In another study from the same group [38], mice injected with primary leukemia cells were given multiple doses of anti-CD19 CAR T cells engineered either via electroporation or lentiviral transduction. Repeated injections of mRNA-electroporated CAR-engineered CTLs combined with lymphodepletion achieved similar results when compared to stable lentiviral transduction, emphasizing the applicability and efficiency of RNA-engineered T cells for the clinic. In fact, clinical trials have been conducted to study the efficacy and safety of mRNA-transfected CAR T cells for the treatment of cancer [39–41], underscoring the importance of transient systems to test possible toxicities prior to more stable approaches. On this subject, although infusion of mRNA-electroporated CAR T cells was well tolerated in general and serious adverse effects were not or possibly not related to the study drug, one patient showed anaphylaxis with production of IgE antibodies against murine antibody-derived antigen binding domain of the mesothelin-specific CAR [39]. Finally, combining TCR engineering with blockade of immune checkpoint proteins, such as programmed cell death protein-1 (PD-1) [42], is an appealing strategy to redirect CTL specificity while reducing PD-1-induced anergy

[43,44]. For instance, Iwamura *et al.* [45] showed that antigen-stimulated T cells expressed programmed death-ligand (PD-L) 1 and 2 and that electroporation of siRNA to downregulate expression of PD-L1/2 combined with retroviral transduction of a melanoma-specific TCR resulted in increased effector function against MAGE-A4$^{+}$ cells. Thus, the combination of DsiRNAs specific for immune checkpoint inhibitors and for the endogenous TCR chains in a double sequential electroporation system could be further analyzed to maximize the success of cancer treatments.

# Conclusion

We generated a novel non-viral and non-genotoxic platform for efficient T-cell receptor engineering for the development of a safer, faster and cost-effective adoptive T-cell therapy. Electroporation of T lymphocytes with DsiRNA prior to electroporation of codon-optimized *TCR* mRNA leads to robust expression of introduced TCR while inhibiting TCR mispairing and results in superior functionality of TCR-engineered cells. In our view, these results warrant further in vivo validation of this promising non-integrating, efficient and affordable system to safely TCR engineer T cells for clinical trials.

# Materials and methods

## Study design

The hypothesis of this study was that sequential electroporation of wild type TCR-specific DsiRNA and codon-optimized *TCR* mRNA would improve transgenic TCR expression by silencing of endogenous TCR transcripts in vitro. We tested transfection of DsiRNA and *TCR* mRNA using cell lines and primary samples from anonymous healthy donors provided by the Blood Service of the Flemish Red Cross (Mechelen, Belgium), following the approval of the Ethics Committee of the Antwerp University Hospital and the University of Antwerp (Antwerp, Belgium) under reference number 16/35/357. Information regarding number of replicates can be found in the figure legends. Validation of the specificity and efficacy of DsiRNA and optimization of double sequential electroporation were performed using cell lines that endogenously express TCR or by electroporation of wild-type *TCR* mRNA. Epitope-specific T cell effector function was analyzed by co-culture of cells with a tumor cell line in the presence of relevant or irrelevant peptides.

## T-cell isolation and cell lines

Peripheral blood mononuclear cells (PBMCs) from anonymous healthy donors were separated from whole blood using Ficoll density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare). Cytotoxic CD8+ T cells were positively selected using human CD8 magnetic microbeads (Miltenyi Biotec), following manufacturer's instructions. Isolated $CD8^+$ T cells were then used in electroporation experiments and were considered to be in a resting phase since no pre-activating treatment was applied. Purity of isolated $CD8^+$ T cells was analyzed by staining with anti-human CD3-PerCP, CD4-PE and CD8-FITC or matched isotype control monoclonal antibodies (mAbs; BD Biosciences). Samples were measured on a FACScan flow cytometer (BD Biosciences). The human acute T cell leukemia cell lines Jurkat Clone E6-1 (ATCC, TIB-152) and 2D3 [46] were maintained in Roswell Park Memorial Institute 1640 (RPMI) culture medium (Gibco Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen). 2D3 cells were generated from TCR$\alpha\beta$-deficient Jurkat 76 cells by transduction with human CD8 alpha-E2A-CD8 beta construct (both Jurkat 76 cells and CD8-encoding plasmid were kind gifts of Prof. Hans Stauss, Institute of Immunity and Transplantation, University College London, London, UK) and with a plasmid vector containing the enhanced green fluorescent protein (EGFP) gene under the control of a nuclear factor of activated T-cell (NFAT) promoter (NFAT-EGFP plasmid kindly provided by Prof. Takashi Saito, Riken Research Center for Allergy and Immunology, Yokohama, Japan). HLA-A∗02:01-positive T2 cells, a human lymphoblastoid cell line with transporter associated with antigen presentation (TAP) deficiency that can be loaded with exogenous MHC class I-restricted peptides, were kindly provided by Dr. Pierre Van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium) and were maintained in Iscove's Modified Dulbecco's Medium (IMDM; Gibco Invitrogen) supplemented with 10% FBS. Cell lines were maintained in logarithmic growth phase at 37°C in a humidified atmosphere supplemented with 5% CO2.

## Cloning of $WT1_{37–45}$- and $WT1_{126–134}$-Specific TCR Genes and Vector Construction

$WT1_{37–45}$ and$WT1_{126–134}$-specific CTL clones were established from an AML patient (UPN08) (12) by single-cell sorting of $WT1_{37–45}$/HLA-A∗02:01 or $WT1_{126–134}$/HLA-A∗02:01 tetramer-positive CTLs. Briefly, frozen PBMCs were thawed and stained with 7-AAD (eBioscience), WT1/HLA-A∗02:01 PE-labeled tetramers (Medical & Biological Laboratories Co.), anti-human CD3-Pacific Blue (clone UCHT1) and CD8-APC-Cy7 (clone SK1) mAbs (BD Biosciences) and single-cell sorting was performed using FACSAria (BD Biosciences). The sorted cells were expanded by co-

culture with irradiated allogeneic PBMCs in the presence of interleukin (IL)-2 (100 IU/ml; Shionogi & Co., Ltd.) and phytohemagglutinin (PHA; Remel Inc., 2μg/ml) in a 96-well round-bottom plate. Expanded CTL clones were screened for $WT1_{37–45}$ or $WT1_{126–134}$ specificity by tetramer staining or intracellular cytokine assay. WT1-specific TCR_ and TCR_ genes from established clones were isolated by a 5′-RACE PCR method and identified by the International Immunogenetics Information System (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanTcR) as described previously [47]. The cloned wild type (wt) TCR$\alpha$ and TCR$\beta$ genes were linked with the 2A sequence from porcine teschovirus-1 (P2A) (18) and cloned into the Spe I-Xho I site of pST1 plasmid [48,49] ($WT1_{126}$ TCR-wt, **Figure 1B**). The pST1 $WT1_{126}$ TCR-co vector was derived from the pST1 $WT1_{126}$ TCR-wt vector by codon-optimization of the $WT1_{126}$ TCR-wt sequence and insertion of TCR$\beta$ before the 2A peptide sequence (28) ($WT1_{126}$ TCR-co, **Figure 1B**). For the $WT1_{37–45}$-specific TCR, only the pST1 $WT1_{37}$ TCR-co vector containing the codon-optimized TCR was generated ($WT1_{37}$ TCR-co, **Figure S3**).

### In vitro mRNA transcription

SoloPack Golden supercompetent E. coli cells were transformed with pST1 DNA plasmids according to manufacturer's instructions. Transformed E. coli cells were cultured in LBkanamycin agar plates and incubated overnight at 37°C and amplified in LB-kanamycin cultures at 37°C under constant motion. Plasmid DNA isolation and purification from bacterial cells were performed using the Nucleobond Xtra Midi EF and Nucleobond finalizer kits (Macherey-Nagel). Next, plasmid DNAs were digested with Sap-I restriction enzyme (Thermo Fisher Scientific) for 16 h at 37°C. Capped mRNA transcripts were synthesized from linearized plasmids and purified by DNase digestion and LiCl precipitation using a mMessage mMachine T7 in vitro transcription kit (Life Technologies) following manufacturer's recommendations.

### Single electroporation

Before electroporation, $10 \times 10^6$ viable 2D3 or human primary unstimulated $CD8^+$ T cells were washed twice in cold serum-free Opti-MEM I medium (Gibco Invitrogen), resuspended in 200 μL of the same medium and transferred to a 4.0-mm electroporation cuvette (Cell Projects). Next, one microgram of in vitro transcribed mRNA per $10^6$ cells and/or a 100 μM pool of two DsiRNA against the wild-type sequences of the T-cell receptor constant alpha and beta regions (*TRAC* and *TRBC*) in a 1:1 ratio, or a control DsiRNA against *EGFP* (Integrated DNA Technologies) were added to the cuvette. Electroporations were performed in a Gene Pulser Xcell™ device (Bio-Rad Laboratories) using Square Wave protocol (500V, 5ms, 0 gap, 1 pulse). As a negative control, cells were electroporated under the same conditions without the addition of any RNA ("Mock"). Immediately after electroporation, cells were transferred to 5mL of RPMI medium supplemented with 10% FBS (2D3 cells) or AIM-V medium (Gibco Invitrogen) with 10% human AB serum (Gibco Invitrogen) ($CD8^+$ T cells) and incubated for a minimum of 20 min at 37°C and 5% CO2 prior to analysis. For further analysis, cells were centrifuged and resuspended in RPMI supplemented with 5% FBS (Jurkat E6-1 or 2D3 cells) or AIM-V medium with 5% human AB serum (primary $CD8^+$ T cells).

### Double sequential electroporation

Similar to single electroporation of DsiRNA, $10 \times 10^6$ viable Jurkat E6-1, 2D3 or human primary unstimulated $CD8^+$ T cells were electroporated with 100 μM pool of two DsiRNA against the wild-type sequences of the T-cell receptor constant alpha and beta regions (*TRAC* and *TRBC*) in a 1:1 ratio, with a control DsiRNA against *EGFP* or mock electroporated (no addition of RNA) using the same settings applied for single electroporations. Immediately after electroporation, cells were transferred to 5mL of RPMI medium supplemented with 10% FBS (Jurkat E6-1 or 2D3 cells) or AIM-V medium (Gibco Invitrogen) with 10% human AB serum (Gibco Invitrogen) ($CD8^+$

T cells) and incubated for a minimum of 20 min at 37°C and 5% CO2. After incubation, cells were transferred to 6-well plates (Greiner Bio-one) and incubated at 37◦C and 5% CO2. Twenty-four hours after first electroporation, cells were harvested and analyzed for cell concentration and viability. Then, samples were washed twice with cold serum-free Opti- MEM I medium (Gibco Invitrogen), resuspended in 200 µL of the same medium and transferred to a 4.0-mm electroporation cuvette (Cell Projects). Next, none (mock) or 1 µg of in vitro transcribed mRNA per $10^6$ viable cells was added to the cuvette. Cells were electroporated using the abovementioned settings. For the optimization of the double sequential electroporation, 2D3 cells were also incubated for 6 h after first electroporation and prior to the second electroporation. Yield 24 h after the second electroporation ranged from approximately 60–70% cells from the total primary $CD8^+$ T cells before electroporation, with an average viability of 87% after the second electroporation. For further analysis, cells were centrifuged and resuspended in RPMI supplemented with 5% FBS (Jurkat E6-1 or 2D3 cells) or AIM-V medium with 5% human AB serum (primary $CD8^+$ T cells).

### Analysis of transgenic TCR surface expression

2D3 cells were harvested after electroporation and stained with the following mAbs: anti-human anti-pan TCRαβ-PE (clone BW242/412; Miltenyi Biotec), CD3-PerCP (clone SK7), CD8- FITC (clone SK1) or isotype control mAbs (BD Biosciences) for 30 min at 4◦C. After washing, samples were resuspended in 200 µL of FACS buffer (FACSFlow sheath fluid, BD Biosciences; 0.1% bovine serum albumin (BSA), Sigma-Aldrich; 0.05% sodium azide, Merck) and measured on a FACScan flow cytometer (BD Biosciences). Alternatively, 2D3 cells were incubated with $WT1_{37–45}$/HLA-A∗02:01 tetramer-APC and $WT1_{126–134}$/HLAA∗ 02:01 tetramer-PE (monomers kindly provided by Prof. D. A. Price, Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff, UK) for 30 min at 37°C, washed and stained with anti-human CD8-Pacific Blue (clone 3B5; Life Technologies), CD3-PerCP-Cy5.5 (clone UCHT1) mAbs (BD Biosciences), and LIVE/DEAD fixable aqua dead cell stain kit (Thermo Fisher Scientific) for 30 min at 4°C. After washing, cells were resuspended in 200 µL of FACS buffer for flow cytometric analysis using a FACSAria II flow cytometer (BD Biosciences). Human primary resting $CD8^+$ T cells were harvested after electroporation at different time points and stained with $WT1_{126–134}$/HLA-A∗02:01 tetramer-PE for 30 min at 37°C. Next, cells were washed in FACS buffer and stained with anti-human CD3-PerCP (clone SK7), CD8-FITC (clone SK1) mAbs (BD Biosciences), and LIVE/DEAD fixable aqua dead cell stain kit (Thermo Fisher Scientific) for 30 min at 4°C. After washing, cells were resuspended in 200 µL of FACS buffer for flow cytometric analysis using a FACSAria II flow cytometer (BD Biosciences).

### RT-qPCR analysis

Twenty-four hours after one or two electroporations, total RNA was extracted from Jurkat E6-1 cells or human primary resting $CD8^+$ T cells using RNeasy Micro kit (QIAGEN), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthetized by reverse transcription from total RNA samples using iScript cDNA synthesis kits (Bio-Rad) and diluted in water to a final concentration of 5 ng/µL. Real-time PCR reactions were performed in duplicate or quadriplicate on a CFX96TM real-time PCR detection system(Bio-Rad) using SsoAdvanced TM Universal SYBRR Green Supermix (Bio-Rad) and PrimePCRTM primers (Bio-Rad) to detect and quantify the relative abundance of T-cell receptor alpha constant region mRNA (*TRAC*; forward primer: 5′-CTGTCTGCCTATTCACCGATT-3′, reverse primer: 5′-GTCAGATTTGTTGCTCCAGG-3′) and T-cell receptor beta constant region mRNA (*TRBC*; forward primer: 5′-GGTGAATGGGAAGGAGGTG-3′, reverse primer: 5′-GTATCTGGAGTCATTGAGGGC-3′) transcripts. Importin-8 (*IPO8*, Hs.505136) and ribosomal

protein L13A (*RPL13A*, Hs.523185) were chosen as reference genes [50]. Results were analyzed using CFX Manager (v3.1, Bio-Rad).

### Avidity testing of peptide-specific TCR

2D3 cells were used to analyze the avidity for the cognate peptide and functionality of the TCR after cloning. Briefly, T2 cells were pulsed with $WT1_{126–134}$ peptide (JPT Peptide Technologies) at decreasing concentrations of a 10-fold serial dilution for 90 min at room temperature under constant motion. Electroporated 2D3 cells were cultured with peptide-pulsed T2 cells at an effector target ratio of 2:1 for 5 h. After incubation, cultures were stained with anti-human CD3-PerCP (clone SK7) and CD8-PE mAbs (clone SK1; BD Biosciences) for 30 min at 4°C, washed and resuspended in FACS buffer. Recognition of peptide-pulsed T2 cells was analyzed by TCR activation-mediated EGFP expression using a FACScan flow cytometer (BD Biosciences).

### IFN-γ ELISpot

Antigen recognition of TCR-specific peptide-pulsed T2 cells by electroporated human primary resting $CD8^+$ T cells was analyzed using human IFN-γ ELISpot basic kit (Mabtech) following manufacturer's recommendations. T2 cells were pulsed with different concentrations of a 10-fold serial dilution of $WT1_{126–134}$ peptide for 90 min at room temperature under constant motion. For the co-cultures, $5 \times 10^3$ electroporated $CD8^+$ T cells per well were added to $3 \times 10^4$ peptide-pulsed T2 cells per well in 0.45 µm hydrophobic Immobilon-P PVDF membrane 96-well plates (Merck Millipore). Plates were incubated overnight at 37°C and 5% CO2, developed and assessed on an AID ELISpot reader system (AID Autoimmun Diagnostika). Spot-forming cells (SFC) were analyzed using AID ELISpot Software version 4.0.

### Cytotoxicity assay

The killing capacity of electroporated human primary resting $CD8^+$ T cells against T2 cells was determined using a flow cytometry-based protocol as described previously with minor modifications [51]. Briefly, prior to co-culture tumor cells were stained with PKH67 green fluorescent cell linker dye (Sigma- Aldrich) according to the manufacturer's protocol. PKH67+ T2 cells were incubated with$WT1_{37–45}$ or$WT1_{126–134}$ peptide (JPT Peptide Technologies) in AIM-V medium (Gibco Invitrogen) for 90 min at room temperature under constant motion. Next, T2 cells were cultured alone or with electroporated human primary resting $CD8^+$ T cells for 6 h at an effector-target ratio of 20:1. After co-culture, samples were stained with propidium iodide (PI) and APC-labeled annexin V (BD Biosciences). Samples were analyzed using a FACSAria II flow cytometer (BD Biosciences). Cytotoxicity was calculated based on the survival of $PKH67^+$ T2 cells using the following equation:

$$\% \text{ Cytotoxicity } = 100 - \left[\left(\frac{\%\ annexin\ V^-\ PI^-\ T2\ cells\ co\text{-}cultured\ with\ CD8^+\ T\ cells}{\%\ annexin\ V^-\ PI^-\ T2\ cells\ cultured\ without\ CD8^+\ T\ cells}\right) \times 100\right]$$

### Flow cytometric analysis of activation markers

For the analysis of TCR specificity, $1 \times 10^6$ T2 cells were peptide pulsed with 10µg/mL of $WT1_{37–45}$ or $WT1_{126–134}$ peptide (JPT Peptide Technologies) in 1 mL of AIM-V medium (Gibco Invitrogen) for 90 min at room temperature under constant motion. Next, T2 cells were washed and resuspended in AIM-V medium with 5% human AB serum and added to electroporated human primary resting $CD8^+$ T cells at an effector-target ratio of 4:1 and incubated for 20 h at 37°C and 5% CO2. After incubation, supernatants were collected for analysis of cytokine secretion and cells were stained with anti-human CD8-Pacific Blue (clone 3B5; Life Technologies), CD3-PerCP-Cy5.5 (clone UCHT1), CD14- FITC (clone M'P9), CD19-FITC (clone 4G7), CD69-APC-Cy7 (clone FN50),

CD137-PE (clone 4B4-1) mAbs (BD Biosciences), and LIVE/DEAD fixable aqua dead cell stain kit (Thermo Fisher Scientific) for 30 min at 4°C. Cells were washed and analyzed using a FACSAria II flow cytometer (BD Biosciences).

### Cytokine secretion assays

Secretion of IFN-γ and granzyme B by electroporated human primary resting $CD8^+$ T cells was determined by enzyme-linked immunosorbent assay kits (ELISA; respectively, Peprotech, Affimetrix and R&D Systems) following manufacturer's instructions in supernatants of co-cultures used for the analysis of activation markers. All ELISA plates were measured using a Victor 3 multilabel plate reader (Perkin Elmer).

### Statistical analysis

Flow cytometry data were analyzed using FlowJo software (v10.2, TreeStar Inc). Prism software (v5, GraphPad) was used for graphing and statistical calculations. Data were analyzed using repeated measures one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparisons between different electroporation conditions. Results were considered statistically significant when P-value was less than 0.05.

# Supplementary material

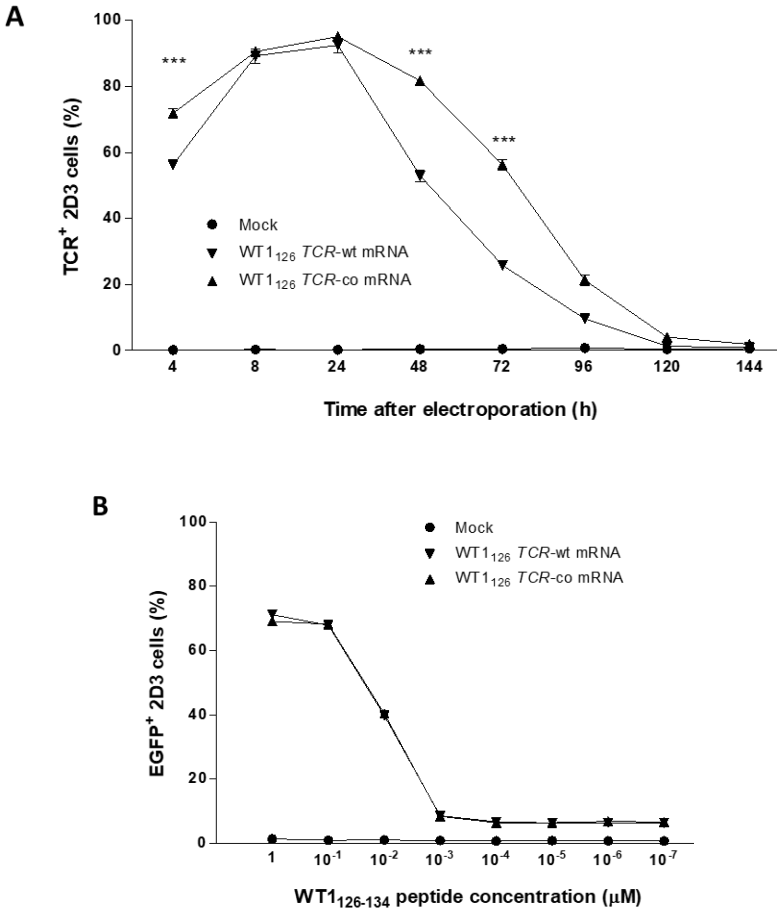


**Figure S1. Validation of WT1$_{126}$ *TCR* mRNA in 2D3 cells.** (**A**) Percentage over time of transgenic TCR expression after electroporation of TCRαβ-deficient 2D3 cells, as measured by anti-pan TCRαβ mAb staining (3 replicates, mean ± SEM). (**B**) Avidity of WT1$_{126}$ TCR was assessed by TCR activation-mediated EGFP expression in 2D3 cells after co culture with WT1$_{126\text{-}134}$ peptide-pulsed T2 cells. ***$P < 0.001$; Mock, mock electroporation; WT1, Wilms' tumor 1; wt, wild-type; co, codon-optimized.

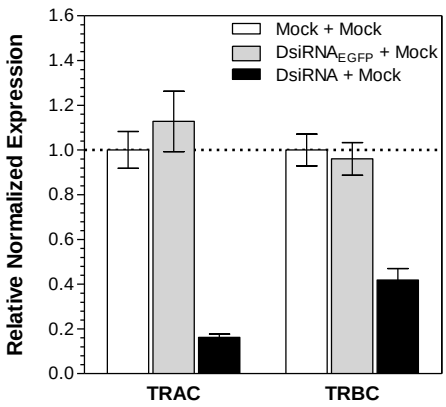


**Figure S2. Specific downregulation of *TRAC* and *TRBC* mRNAs 48h after DsiRNA electroporation.** RT-qPCR was used to determine the levels of *TRAC* and *TRBC* mRNAs in TCR$^+$ Jurkat E6-1 cells 48h after DsiRNA/mock double sequential electroporation. The cells were electroporated first with TCR-specific or EGFP-specific DsiRNA or mock electroporated; 24 h later they all underwent a second mock electroporation. Expression levels were normalized to the reference genes importin-8 and ribosomal protein L13A and analyzed relative to mock/mock double sequential electroporation. *TRAC*, T-cell receptor alpha constant region; *TRBC*, T-cell receptor beta constant region; Mock + Mock, double sequential mock electroporation; DsiRNA$_{EGFP}$ + Mock, electroporation with Dicer-substrate small interfering RNA directed against *EGFP* mRNA followed by mock electroporation; DsiRNA + Mock, electroporation with Dicer-substrate small interfering RNAs directed against *TRAC* and *TRBC* mRNAs followed by mock electroporation.

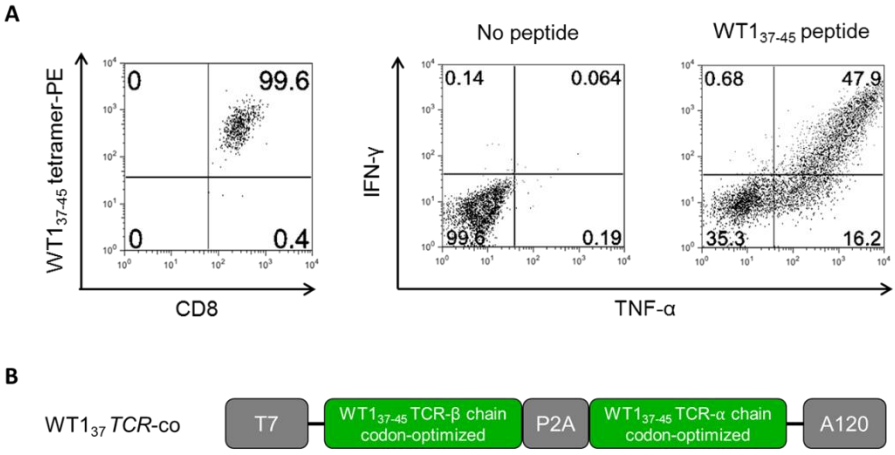


**Figure S3. Isolation and characterization of WT1$_{37-45}$-specific CTL clone.** (**A**) WT1$_{37-45}$/HLA-A*02:01 tetramer staining and WT1$_{37-45}$ peptide-specific IFN-γ and TNF-α production of the WT1$_{37-45}$-reactive CTL clone. The percentage of cells is indicated in each quadrant. (**B**) Schematic representation of pST1 plasmid vector containing the WT1$_{37-45}$-specific codon-optimized (WT1$_{37}$ TCR-co) TCR cassette. WT1, Wilms' tumor 1; wt, wild-type; co, codon optimized; T7, T7 promoter; P2A, picornaviral 2A-like sequence; A120, 120-mer poly(A) tail.

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**Science, for me, gives a partial explanation for life. In so far as it goes, it is based on fact, experience and experiment.**

— Rosalind Franklin

# 5

# Rapid assessment of functional avidity of tumor-specific T-cell receptors using an antigen-presenting tumor cell line electroporated with full-length tumor antigen mRNA

This chapter has been published in:

Campillo-Davo D, Versteven M, Roex G, De Reu H, van der Heijden S, Anguille S, Berneman ZN, Van Tendeloo VFI, Lion E. *Cancers* (2020);12(2):256.

# Abstract

The functional avidity of T-cell receptor (TCR)-engineered T cells towards their cognate epitope plays a crucial role in successfully targeting and killing tumor cells expressing the tumor-associated antigen (TAA). When evaluating in vitro functional T-cell avidity, an important aspect that is often neglected is the antigen-presenting cell (APC) used in the assay. Cell-based models for antigen-presentation, such as tumor cell lines, represent a valid alternative to autologous APCs due to their availability, off-the-shelf capabilities, and the broad range of possibilities for modification via DNA or messenger RNA (mRNA) transfection. To find a valuable model APC for in vitro validation of TAA Wilms' tumor 1 (WT1)-specific TCRs, we tested four different WT1 peptide-pulsed HLA-A2+ tumor cell lines commonly used in T-cell stimulation assays. We found the multiple myeloma cell line U266 to be a suitable model APC to evaluate differences in mean functional avidity (EC50) values of transgenic TCRs following transfection in Jurkat 2D3 cells. Next, to assess the dose-dependent antigen-specific responsiveness of WT1-TCR-engineered 2D3 T cells to endogenously processed epitopes, we electroporated U266 cells with different amounts of full-length antigen *WT1* mRNA. Finally, we analyzed the functional avidity of WT1-TCR-transfected primary CD8 T cells towards *WT1* mRNA-electroporated U266 cells. In this study, we demonstrate that both the APC and the antigen loading method (peptide pulsing versus full-length mRNA transfection) to analyze T-cell functional avidity have a significant impact on the EC50 values of a given TCR. For rapid assessment of the functional avidity of a cloned TCR towards its endogenously processed MHC I-restricted epitope, we showcase that the TAA mRNA-transfected U266 cell line is a suitable and versatile model APC.

# Introduction

T-cell receptor (TCR) gene therapy is a promising strategy in cancer immunotherapy, capitalizing on the use of TCR-engineered T cells targeting tumor-associated antigens (TAAs) expressed by cancer cells [1]. An essential element for the success of this type of therapy is the ability of TCR-engineered T cells to recognize the TAA, even at low epitope densities [2,3]. The threshold of activation of a T cell, defined as functional avidity, is a measurement of its effector response towards a particular surface density of the epitope [4]. Usually, it is evaluated in vitro by analyzing the response of T cells in peptide titration experiments. In this type of experiments, target cells are pulsed with decreasing concentrations of major histocompatibility complex (MHC)-binding peptides. The mean functional avidity, usually described by EC50, represents the peptide dose at which half-maximal activation of the T-cell population is reached. This value depends on the affinity and avidity of the TCR for its cognate peptide-MHC (pMHC) ligand and, therefore, it varies between different T-cell clones or TCR-engineered T cells. Generally, higher functional avidities—i.e., lower EC50 values—are linked to the recognition of lower epitope densities on the surface of antigen-presenting cells (APCs), and, thus, to better responses towards those cells [5,6]. Hence, the analysis of antigen-specific T-cell responses is vital at a clinical and research level to obtain the best TCRs for adoptive T-cell therapies [7,8].

Measurement of T-cell functional avidity, however, can be challenging due to the vast array of analytical methods and the use of different types of cells presenting the antigen. Assays for the measurement of in vitro antigen-specific T-cell functional activity include direct cytotoxicity analysis by chromium ($^{51}Cr$) release [9] or flow cytometry-based killing assays [10], detection of intracellular expression of cytokines such as interferon-gamma (IFN-γ) or interleukin-2 (IL-2) [11,12], IFN-γ or granzyme B enzyme-linked immunospot (ELISpot) assays [13] and enzyme-linked immunosorbent (ELISA) assays [14], mobilization of CD107a [15,16], and upregulation of activation markers, e.g., CD69 or CD137 [17]. In some models using TCR-deficient Jurkat cells, TCR activation is measured by the TCR-triggered expression of the green fluorescent protein (GFP) [18] or a combination of fluorescent proteins for the analysis of different transcription factors associated with TCR signaling [19]. Despite the multiple ways to analyze T-cell functional avidity, little is known about the impact that an APC may have on the result. This is important because T-cell activity may vary depending on the epitope density displayed by the APC, but also on the ability of an APC to promote T-cell activation. Among the multiple possibilities, cells of autologous origin, such as peripheral blood mononuclear cells (PBMCs), monocyte-derived dendritic cells (DCs) and B-

lymphoblastoid cell lines represent the most common APCs in T-cell activation assays. Non-autologous cell-based models of APCs, including tumor cell lines such as T2 or K562 cells, are an alternative to the costly and laborious production of autologous APCs [20]. They represent an off-the-shelf approach that can be easily maintained, readily available, and modified as per request. Moreover, model APCs can be engineered with plasmid vectors or messenger RNAs (mRNAs) that encode the tumor antigens of interest. In particular, electroporation of antigen-encoding mRNA is a rapid and efficient method to induce neo-expression of the antigen in APCs. This technique allows the induction of multi-epitope T-cell responses, for example, in cancer patients following therapeutic vaccination with antigen-loaded DCs, such as the Wilms' tumor 1 (WT1) protein [7,21]. WT1 is a transcription factor overexpressed in leukemia and many solid tumors, but also present in healthy tissues [22]. Unfortunately, as an auto-antigen, T cells targeting self-TAAs such as WT1 with high avidity are scarce due to the negative selection that occurs in the thymus [23].

In the context of WT1-targeted adoptive TCR-engineered T-cell immunotherapies, methods to correctly assess the functional avidity of T cells engineered with WT1-specific TCRs are crucial for their success. In this study, we aimed to develop a reliable APC model for the evaluation of endogenously processed WT1 peptides and the avidity of WT1-specific TCRs. We analyzed the use of the HLA*A2:01-positive multiple myeloma cell line U266 as a tool for the rapid assessment of HLA-A2-restricted WT1-specific T-cell responses following electroporation with full-length *WT1* mRNA, in comparison with WT1 peptide loading. To the best of our knowledge, this is the first study comparing exogenous peptide-loading and full-length antigen mRNA electroporation of target cells to study the functional avidity of epitope-specific TCR-redirected T cells.

# Results

## Quantitation of WT1-presenting potential model APC

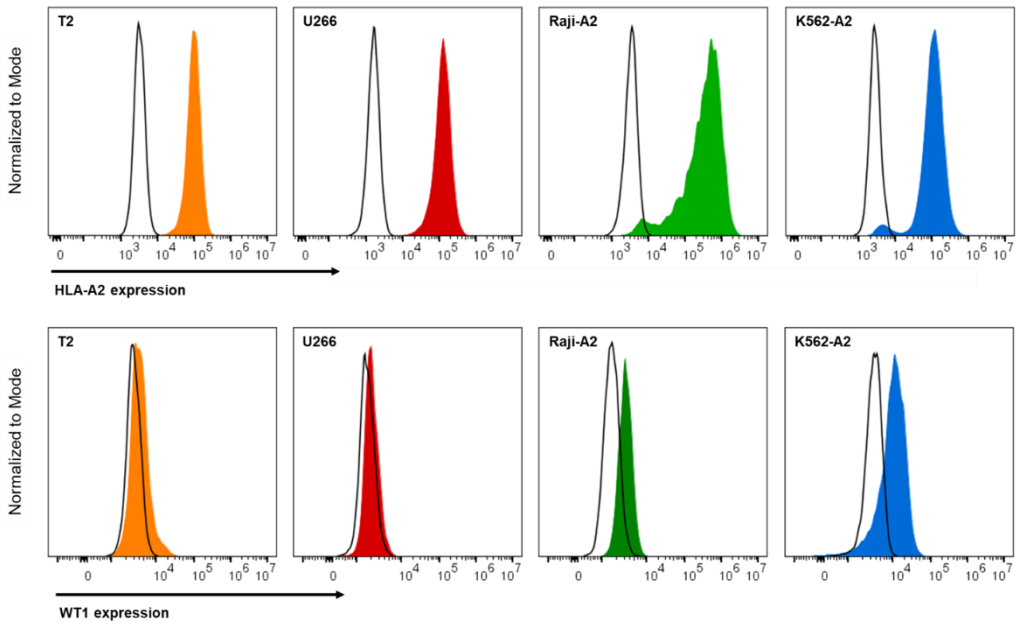
To evaluate the capacity of different cell lines to be used as model APCs for presentation of WT1-derived epitopes by HLA-A2, the expression of surface HLA-A2 and natural intracellular WT1 proteins of four potential cell lines was quantified: T2 [24], U266 [25], K562-A2 [26] and Raji-A2 [27] cells (**Figure 1**). All cell lines expressed HLA-A2, with percentages ranging from 95% to 99% of HLA-A2-positive cells (**Figure 1, upper panel**). With regards to the number of HLA-A2 molecules per cell, denoted as delta median fluorescence intensity (dMFI), T2 cells expressed the lowest levels of HLA-A2 molecules. On the contrary, Raji-A2 showed the highest levels of expression, whereas

U266 and K562-A2 cells showed similar intermediate levels. Confirming literature, K562-A2 was the only cell line that clearly expressed WT1 (68.14% $WT1^+$), whereas T2 and Raji-A2 cells expressed moderate amounts of the antigen (15.79% and 33.4% $WT1^+$, respectively) and U266 cells the lowest amounts (4.71% $WT1^+$) (**Figure 1, lower panel**).

## Functional avidity of WT1-specific T cells drastically differs depending on the APC used

To analyze the WT1 peptide-presenting capacity of the four model APC candidates, we used an in-house developed T-cell model assay, based on TCR-deficient $CD8^+$ Jurkat 2D3 cells that are electroporated with TCR$\alpha\beta$-encoding mRNAs and express enhanced green fluorescent protein (EGFP) via nuclear factor of activated T cells (NFAT) upon antigen-specific TCR triggering [28,29]. Transgenic TCR expression for two HLA-A2-restricted TCRs directed against two epitopes of the WT1 protein, $WT1_{37–45}$ and $WT1_{126–134}$ (WT1.37 and WT1.126 TCR, respectively), was maximal for both TCRs 24 h after electroporation (92.75 ± 1.5% WT1.37 $TCR^+$ and 94.48 ± 0.67% WT1.126 $TCR^+$ 2D3 cells; **Figure S1A**). Pulsed with decreasing concentrations of $WT1_{37–45}$ or $WT1_{126–134}$ peptides, the four model APCs were cultured with their respective WT1-*TCR* mRNA-electroporated 2D3 cells (**Figure 2**). The peak values of EGFP expression in 2D3 cells, corresponding to maximal T-cell activation, were detected with the highest peptide concentration for all cell lines (**Figure 2A,B**). The intensity of the T-cell response differed for both WT1-specific TCRs and depended on the APC type. When cultured with peptide-pulsed T2 cells, the highest percentages of $EGFP^+$ 2D3 cells were reached as compared to U266 cells, Raji-A2, and K562-A2 cells, the latter promoting the poorest T-cell activation against both WT1 peptides. T2 cells, together with Raji-A2, displayed higher background levels of non-specific activation for both WT1.37 and WT1.126 TCR-electroporated 2D3 cells. Compared to the response observed with non-pulsed model APCs, the threshold of activation with T2 cells was reached at $10^{-9}$ M for WT1.37 peptide ($p = 0.0002$; **Figure 2A**) and $10^{-7}$ M for WT1.126 ($p = 0.0001$; **Figure 2B**). In the case of U266, significant differences were detected at $10^{-7}$ M for both peptides ($p = 0.0007$ and 0.0456, respectively). As for Raji-A2 cells, the threshold of activation was reached at $10^{-8}$ M for WT1.37 peptide ($p = 0.0017$) and $10^{-6}$ M for WT1.126 ($p = 0.0015$). WT1.37 and WT1.126 $TCR^+$ 2D3 cells were only able to significantly respond to K562-A2 cells pulsed with a concentration of $10^{-5}$ M for both WT1 peptides ($p = 0.0284$, and $p = 0.0012$, respectively). Uniformly comparing all cell lines, percentages of EGFP expression were normalized for the calculation of EC50 values (**Figure 2C,D**). In the same line, the EC50 values strongly varied between cell lines. Again, T2 cells were capable of promoting the best T-cell response for both WT1-specific TCRs (EC50: 1.06 nM for WT1.37 TCR and 44.29 nM for WT1.126 TCR). On the opposite side, peptide-pulsed K562-A2 cells

induced T-cell responses at higher concentrations (EC50: 247.3 nM for WT1.37 TCR and 1060 nM for WT1.126 TCR). In the middle range, U266 and Raji-A2 cells promoted half-maximal responses at similar concentrations for the WT1.37 TCR (EC50 U266: 19.6 nM; EC50 Raji-A2: 10.08 nM), and WT1.126 TCR (EC50 U266: 148.8 nM; EC50 Raji-A2: 272 nM). These results show that for the same T-cell population expressing an antigen-specific TCR, the APC chosen for the peptide titration experiments has a pivotal role in the thresholds of T-cell activation.




| | % HLA-A2⁺ cells | dMFI HLA-A2 | % WT1⁺ cells |
|---|---|---|---|
| **T2** | 99.66 | 92434 | 15.79 |
| **U266** | 99.64 | 120091 | 4.71 |
| **Raji-A2** | 96.25 | 377216 | 33.4 |
| **K562-A2** | 95.59 | 105297 | 68.14 |

**Figure 1. HLA-A2 and WT1 expression on four model antigen-presenting cell (APC) lines.** Histograms (relative to mode) show the surface expression of HLA-A2 (upper panel) and the intracellular expression of WT1 (lower panel) of T2 (orange), U266 (red), Raji-A2 (green), and K562-A2 (blue) cell lines. HLA-A2 or WT1 expression (filled histograms) and isotype control (black line). The table shows HLA-A2 delta median fluorescence intensity (dMFI) values and percentage of HLA-A2 positive cells minus isotype staining (upper histograms) or percentages of WT1 positive cells minus isotype staining (lower histograms) for each cell line. HLA-A2, human leukocyte antigen A*02:01; WT1, Wilms' tumor 1 protein.

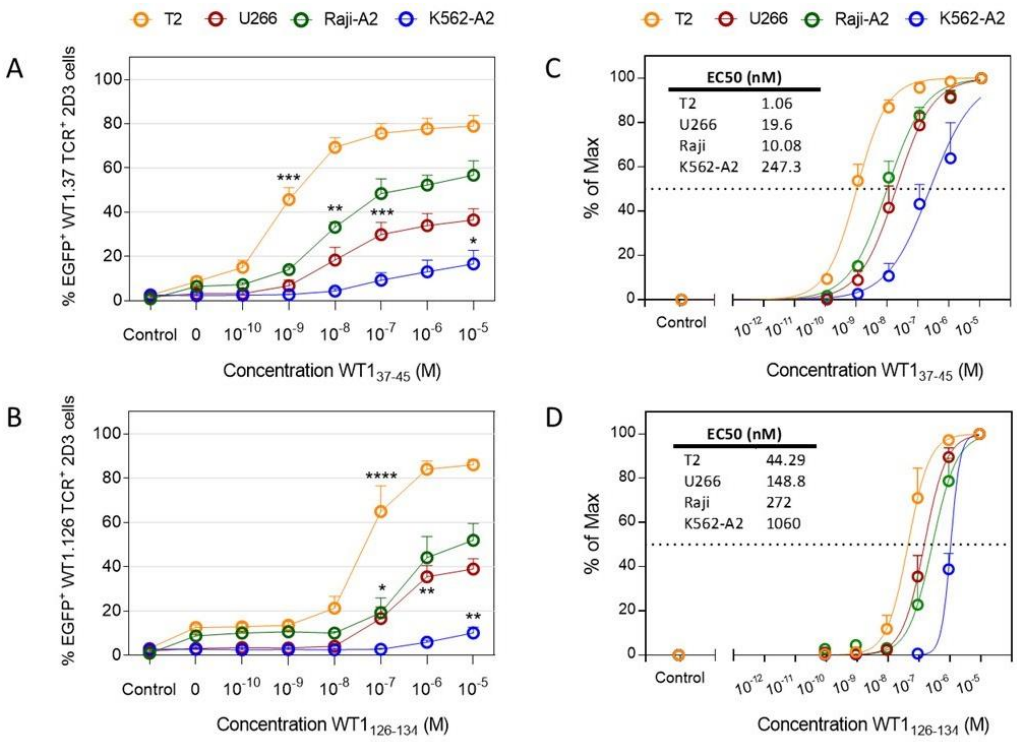



**Figure 2. Epitope-specific T-cell activation by four model APC lines.** Epitope-specific TCR activation was measured by expression of EGFP after WT1$_{37–45}$ (**A,C**) or WT1$_{126–134}$ (**B,D**) peptide-specific TCR-transfected 2D3 cells were cultured for 18–22 h with model APCs T2, U266, Raji-A2 or K562-A2 cells that were pulsed with decreasing concentrations of WT1 peptide. Control depicts unstimulated 2D3 cells only. Graphs show the results of three to five independent replicates, showing (**A,B**) mean % (± SEM) of EGFP positive cells and (**C,D**) % of maximal EGFP expression (± SEM). (**A,B**) Data were analyzed using one-way ANOVA followed by Dunnett's post hoc test (comparing to non-peptide pulsed cells). EC50, the concentration of WT1 peptide at which 50% of the maximal EGFP expression is reached. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

## Mimicking endogenous WT1 expression

Developing a model that can mimic the endogenous processing of WT1 in tumor cells in a controlled manner, among the four cell lines analyzed, T2 and K562-A2 cells are not model candidates because the former are unable to present internally-processed peptides and the latter are intrinsically highly positive for WT1. U266 and Raji-A2 cells generated similar EC50 values; however, they differed in natural WT1 expression levels. We selected the U266 cell line as the candidate model for further analysis due to the lower percentage of WT1 positive cells. Therefore, U266 cells, which naturally

express HLA-A2 and minimal levels of WT1, were electroporated with increasing amounts of *WT1* mRNA as the best model for presentation of internally-processed WT1 peptides (**Figure 3**). The increment in mRNA load resulted in an increase in the percentage of cells expressing the protein (**Figure 3A**), reaching the highest value of $WT1^+$ U266 cells (76.5 ± 3.66%) upon electroporation of 20 μg of *WT1* mRNA. A significant difference between the 5 μg and 20 μg mRNA condition in % of WT1-expressing cells was observed ($p = 0.0078$), demonstrating a dose-response dependency. Likewise, WT1 protein expression per cell increased with increasing mRNA concentrations after electroporation (**Figure 3B**). Next, we assessed the antigen-presenting capacity of the *WT1* mRNA-electroporated U266 cells in combination with WT1-*TCR* mRNA-electroporated 2D3 cells. EGFP expression by WT1.37 $TCR^+$ 2D3 cells (**Figure 3C, triangles**) was significantly higher than mock electroporation (0 μg *WT1* mRNA) when using 10 μg (13.56 ± 2.15%; $p = 0.0348$) and 20 μg (18.48 ± 3.28%; $p = 0.0025$) *WT1* mRNA, but not with 5 μg (9.04 ± 2.28%; $p = 0.3245$). This indicates that WT1.37 epitope density on U266 cells after electroporation with 5 μg of *WT1* mRNA/5 × $10^6$ cells per electroporation is not enough to surpass the threshold for WT1.37 TCR activation. On the other hand, WT1.126 $TCR^+$ 2D3 cells (**Figure 3C, circles**) were not able to respond significantly to any of the amounts of *WT1* mRNA used. Analyzing the amount of mRNA at which 50% of maximal EGFP response was obtained, WT1.37 TCR-engineered T cells showed an EC50 value at 6.54 μg *WT1* mRNA-electroporated U266 (**Figure 3D**). This information could support indicating the minimum dosage of mRNA that should be used in APCs for the evaluation of specific T-cell clones or TCR-engineered T cells.

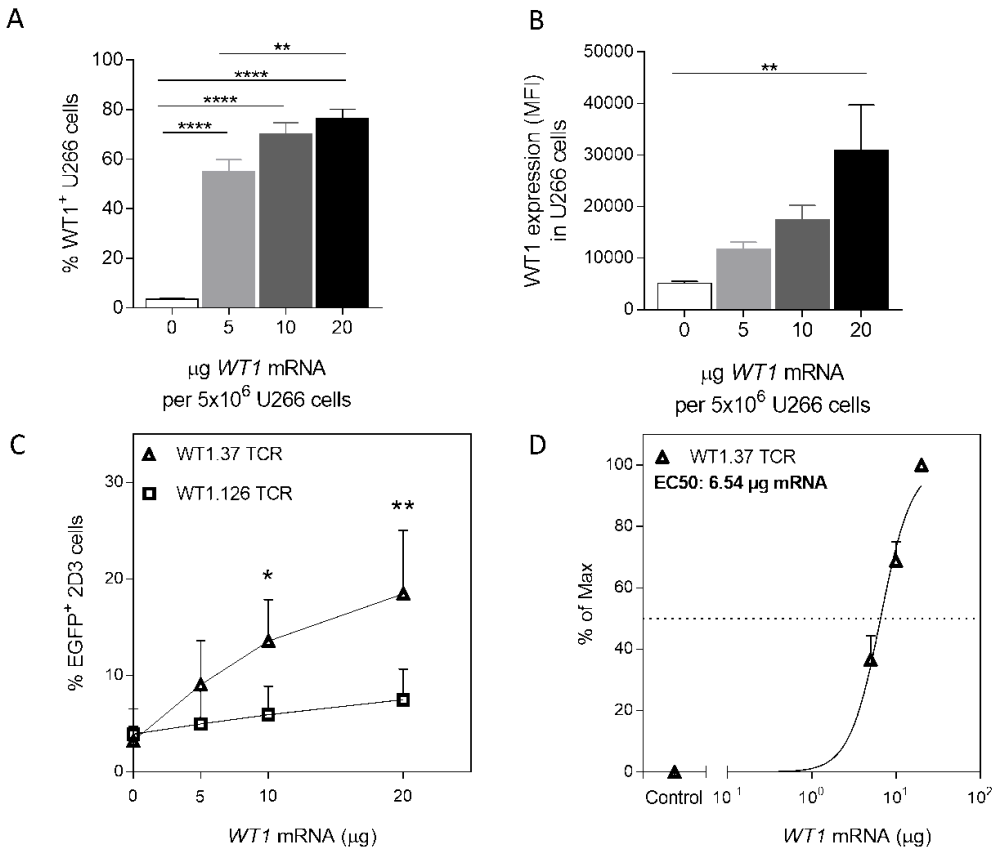



**Figure 3. Epitope-specific TCR-engineered 2D3 cells can recognize full-length antigen WT1 mRNA-electroporated U266 cells in a dose-dependent manner.** (**A**,**B**) Intracellular expression of WT1 is shown for U266 cells 24 h after electroporation with increasing amounts of WT1 mRNA per 5 × $10^6$ U266 cells. (**C**,**D**) 2D3 cells were electroporated with $WT1_{37–45}$- or $WT1_{126–134}$-specific *TCR* mRNAs. Specific activation was detected by NFAT-promoted EGFP expression in 2D3 cells after 18–22 h co-culture with U266 cells electroporated with increasing amounts of *WT1* mRNA. Graphs show the mean percentage of $WT1^+$ U266 cells ± SEM (**A**), the median fluorescence intensity (MFI) of U266 for WT1 expression ± SEM (**B**), the percentage of maximal EGFP expression ± SEM (**C**) and EC50, the amount of *WT1* mRNA at which 50% of the maximal EGFP expression is reached (**D**) of 3–4 independent replicates. (**A**,**B**) One-way ANOVA followed by Tukey's post hoc test. (**C**) One-way ANOVA followed by Dunnett's post hoc test (comparing to mock-electroporated cells). *, $P < 0.05$; **, $P < 0.01$; and ****, $P < 0.0001$.

## WT1 mRNA-electroporated U266 cells activate WT1-specific TCR-redirected primary human CD8 T cells in a dose-dependent manner

Further evaluating the antigen-presenting capacity of U266 cells, the functional avidity of unstimulated primary human CD8 T cells was analyzed in the context of WT1.37 and WT1.126 peptides using our in-house developed double sequential electroporation (DSE) T cell assay [28]. In brief, purified CD8 T cells were subjected to DsiRNA-*TCR* mRNA to downregulate the expression of endogenous TCR, before codon-optimized WT1-specific *TCR* mRNA electroporation. For both WT1.37 and WT1.126 TCRs, high TCR expression was achieved 24 h after *TCR* mRNA electroporation (66.9 ± 5.345% WT1.37/HLA-A2 tetramer$^+$ and 72.4 ± 3.88% WT1.126/HLA-A2 tetramer$^+$ for eight donors; **Figure S1B**). These WT1-TCR-engineered CD8 T cells were co-cultured with peptide-pulsed or *WT1* mRNA-electroporated U266 and analyzed for WT1-specific CD8 T-cell activation and functional avidity by upregulation of CD69 and CD137 activation markers (**Figure 4**). For the WT1.37 peptide (**Figure 4A**), significant differences compared to the non-peptide pulsed U266 cells were still detected at a peptide concentration of $10^{-8}$ M (8.61 ± 1.07% CD69/CD137$^+$; $p = 0.0313$), whereas the signal was lost at $10^{-9}$ M (4.4 ± 0.44% CD69/CD137$^+$; $p = 0.9931$). In analogy with 2D3 cells, primary CD8 T cells electroporated with WT1.126 TCR were less sensitive to lower concentrations of the cognate peptide, compared to WT1.37 TCR$^+$ CD8 T cells. U266 cells pulsed with a WT1.126 peptide concentration of minimal $10^{-7}$ M elicited significant primary T-cell activation (11.59 ± 1.64% CD69/CD137$^+$; $p = 0.0010$). EC50 values of functional avidity for WT1.37 (32.02 nM) and WT1.126 (135.3 nM) TCR-engineered primary CD8 T cells (**Figure 4B**) were comparable to those obtained for 2D3 cells (19.6 nM and 148.8 nM, respectively). These findings confirm the usefulness of U266 cells in peptide-pulsing assays for the assessment of the functional avidity of primary TCR-redirected T cells. With regard to the *WT1* mRNA-electroporated U266 cells (**Figure 4C**), only WT1.37 TCR-engineered primary CD8 T cells significantly responded to 10 µg (8.92 ± 1.71% CD69/CD137$^+$; $p = 0.0383$) and 20 µg of *WT1* mRNA (9.96 ± 1.82% CD69/CD137$^+$; $p = 0.0119$). No significant differences with WT1.126 TCR-engineered CD8 T cells towards U266 cells electroporated with increasing amounts of electroporated *WT1* mRNA were observed. In the case of WT1.37 TCR$^+$ CD8 T cells, 6.11 µg of *WT1* mRNA would be needed to reach upregulation of CD69 and CD137 in half of the maximal percentage of cells (**Figure 4D**), which is in line with the results using the 2D3 cell line (6.54 µg for WT1.37 TCR$^+$; **Figure 3D**). Taken together, these findings show that evaluation of T-cell functional avidity with WT1 peptide-pulsed or *WT1* mRNA-electroporated U266 cells remains constant for the TCRs analyzed regardless of

the source of T cells, and that this system can help to distinguish TCRs that will respond to epitope densities of naturally processed WT1 protein. Hence, the application of U266 cells as a suitable APC model for WT1 antigen-specific T-cell assays.

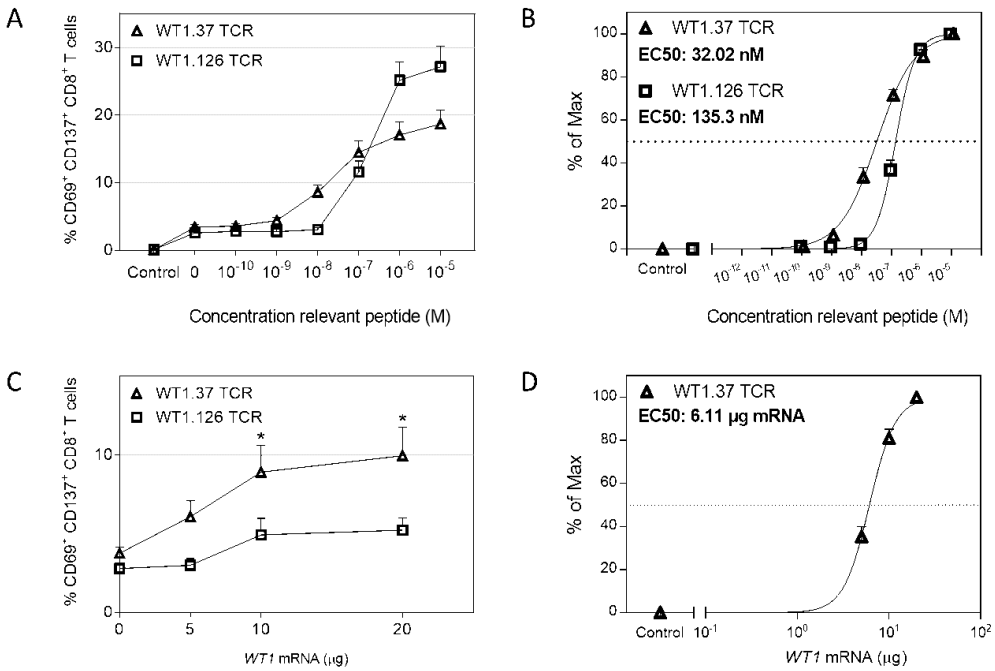



**Figure 4. Analysis of functional avidity of WT1 epitope-specific primary CD8 T cells using WT1 peptide-pulsed and *WT1* mRNA-electroporated U266 cells.** Surface expression of both CD69 and CD137 activation markers was measured on WT1.37 (triangles) and WT1.126 (squares) peptide-specific DSE-engineered primary CD8 T cells 24 h after co-culture with U266 cells that were either pulsed with decreasing (**A**,**B**) concentrations of $WT1_{37–45}$ or $WT1_{126–134}$ peptide, or *WT1* mRNA-electroporated (**C**,**D**). T cells only condition was used as a control. Graphs show mean ± SEM of % CD69/CD137 double positive CD8 T cells (A,C) or % of maximal CD69/CD137 expression ± SEM (**B**,**D**) for 6–8 donors. EC50, the concentration of WT1 peptides or amounts of electroporated *WT1* mRNA at which 50% of the maximal upregulation of CD69 and CD137 activation markers is reached. (**A**,**C**) One-way ANOVA followed by Dunnett's post hoc test (comparing to non-peptide pulsed or mock-electroporated cells). *, $P < 0.05$.

# Discussion

Cell-based model APCs represent a valid alternative to autologous APCs and commonly used methods for analyzing antigen-specific T-cell activation status, for promoting ex-vivo T-cell expansion, and for the immunomonitoring of T-cell responses in the course of a viral infection or against cancer antigens in clinical trials [30]. To better understand the effect of the model APCs in the measurement of functional avidity of T cells, we compared four different model APC tumor cell lines (T2, U266, Raji-A2, and K562-A2). We showed a differential response in functional avidity of WT1-specific TCR-engineered T cells against different peptide-pulsed model APC tumor lines. This information is vital for an accurate calculation of T-cell responses when selecting T-cell clones or TCR-engineered T cells for cancer immunotherapy. Two of these cell lines, T2 and K562-A2, are routinely used in T-cell assays. In particular, T2 cells are widely used in peptide-MHC class I binding assays [31] due to their deficiency in transporter associated with antigen presentation (TAP). This complex is involved in the translocation of proteasome-processed peptides from the cytosol into the lumen of the ER [32]. The TAP deficiency in T2 cells results in MHC instability and reduction of nearly 70% of HLA-A2 surface expression [33] that would explain the lower dMFI for HLA-A2 in these cells. The absence of TAP proteins also prevents the internal loading of TAP-dependent peptides onto the MHC molecules, making the HLA-A2 proteins available for the addition of exogenous peptides. Since endogenously processed and exogenously added peptides in peptide pulsing assays compete for the HLA-A2 molecules available [34], it is not surprising that TAP-deficient T2 cells outperformed Raji-A2, U266 cells, and K562-A2. However, manifested by the very low threshold of functional avidity when using peptide-pulsed T2 cells, they may reflect a non-physiological model that does not represent the actual T-cell functionality. This fact could lead to an overestimation of the T-cell functional avidity and to the selection of T-cell clones or TCRs that are of lower avidity towards more natural peptide-presenting target cells, particularly when screening for high avidity T-cell clones able to recognize tumor cells endogenously expressing, processing and presenting relevant tumor antigens.

A comparison between T2, K562-A2 and autologous B-LCL cells in a flow cytometry-based assay of T-cell killing capacity, showed that T cells cultured with peptide-pulsed T2 cells elicited a better response than those cultured with K562-A2 or B-LCL [35]. These results also indicate that T2 cells present a supraphysiological epitope density after incubation with exogenously added peptides. Interestingly, K562-A2 cells failed to properly activate T cells in our system. As reported by Britten *et al.*, this cell line is a

suitable model for interferon (IFN)-γ ELISpot assays [26]. Britten and colleagues transduced K562-A2 cells with tyrosinase for its endogenous expression or exogenously pulsed them with tyrosinase-derived peptides. Therefore, T-cell responses to the natural expression of the ligand were not evaluated. Moreover, K562 cells naturally express WT1; thus, it does not represent a convenient model APC for the customization of *WT1* mRNA intracellular levels. In this regard, Raji-A2 cells also did express the WT1 protein, albeit at very low levels. This fact, together with the dramatic overexpression of HLA-A2, tips the balance in favor of WT1-negative, naturally HLA-A2 expressing U266 cells.

In our study, the discrepancy in T-cell responses observed with the different cell lines highlights the importance of the APC when assessing functional avidity, but also the influence of the source of the studied epitope. In many types of malignancies, tumor cells downregulate the expression of MHC proteins [36] or have deficiencies in their antigen processing pathways [37], which negatively impacts the presentation and density of peptides on their surface. Since the expression of a precise pMHC complex on the surface of the model APC depends on its capability to internally process full antigens, the sole addition of exogenous synthetic peptides for T-cell assays may provide an incomplete and potentially misleading scenario for the analysis of T-cell functional avidity. We show that U266 cells can be efficiently electroporated with full-length antigen *WT1* mRNA. The electroporation of higher amounts of mRNA was correlated with an increase in WT1 expression. This represents a flexible system in which different amounts of mRNA can be tested prior to clinical trials with full-length tumor antigen mRNA-electroporated DCs. Moreover, *WT1* mRNA-electroporated U266 cells could be a useful alternative cell-based antigen presentation model to DCs [38,39], K562 cells [39] or PBMCs [40] for the oligo-clonal detection of WT1-specific T cell populations and immunomonitoring of T-cell responses in full-antigen mRNA-electroporation DC vaccination trials. The generation of autologous APCs for T-cell assays is not always possible and often entails a lengthy process required for every donor. This could be overcome by the use of U266 cells as model APCs. Moreover, epitope-specific T cell responses induced by *WT1* mRNA-electroporated U266 cells can be compared to a peptide-titration curve using the same cell line. Another advantage of *WT1* mRNA-electroporated U266 is the possibility of off-the-shelf production by freezing the cells after electroporation.

Our study also confirms the suitability of 2D3 cells for the analysis of TCR avidity, thanks to their expression of human CD8 co-receptor, the absence of a native TCRαβ, the simplicity to engineer them with an antigen-specific TCR, and the expression of

EGFP upon TCR triggering. The lack of endogenous TCR eliminates the possibility of TCR mispairing between endogenous and transgenic TCRs [41]. Therefore, EGFP expression can be directly correlated with the degree of introduced TCR triggering, i.e., the capacity of different APCs to present a peptide and to activate T cells. These findings are in accordance with previous reports showing that tetramers allow the quantification of antigen-specific T cells, but do not always provide accurate data on the functionality of T cells [42–45]. Regarding primary human CD8 T cells, activation markers enable the identification of all responder T cells after TCR triggering. One of the most common activation markers in flow cytometric analysis is CD137. Combined with CD69, CD137 is a powerful and sensitive tool to measure epitope-specific T cells regardless of the T-cell state of differentiation or subset [46].

# Conclusion

Our study demonstrates the relevance of comparing the APCs used in T-cell assays and the influence they may have when evaluating T-cell functional avidity. Here, we provide a versatile model to evaluate HLA-A2-restricted WT1 epitope-specific responses by TCR-engineered T cells based on the combination of a tumor cell-based APC with a rapid engineering method such as mRNA electroporation. This model could be valuable for the screening and selection of WT1-specific high-avidity TCRs intended for TCR-engineered therapies without the need for primary APCs. It can potentially be used to analyze other TAA-specific T cells, in particular, for those T cells with low circulating levels that are reactive against tumor-associated autoantigens in the style of WT1. Eventually, this platform could provide the basis for the development of an immunomonitoring tool to evaluate TAA-specific T-cell activity in clinical trials using TAA mRNA-electroporated DC vaccines for cancer immunotherapy.

# Materials and methods

### Cell lines and primary cells

The TCRαβ-deficient, CD8αβ and NFAT-EGFP stably-transfected T cell acute leukemia 2D3 cell line [28,29] was kindly provided by Prof. Haruo Sugiyama (Osaka University Graduate School of Medicine, Osaka, Japan) and maintained in Roswell Park Memorial Institute 1640 (RPMI) culture medium (Life Technologies, Merelbeke, Belgium) supplemented with 10% FBS. The HLA-A*02:01-positive WT1-negative human transporter associated with antigen presentation (TAP)-deficient lymphoblastoid T2 cell line was kindly provided by Dr. Pierre Van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium). U266 is an HLA-A*02:01-positive, WT1-negative multiple myeloma cell line and was a kind gift from Dr. Wilfred T.V. Germeraad (GROW School for Oncology & Developmental Biology, Maastricht University, Maastricht, The Netherlands). The HLA-A*02:01-transduced Burkitt's lymphoma Raji-derived Raji-A2 cell line was kindly provided by Dr. Mirjam Heemskerk (Leiden University Medical Center, Leiden, The Netherlands). The HLA-A*02:01-transduced human chronic myelogenous leukemia K562-derived K562-A2 cell line was a kind gift from Dr. Cedrik Britten (R&D Oncology, GlaxoSmithKline, Stevenage, UK). T2, U266, Raji-A2, and K562-A2 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies,) supplemented with 10% FBS. All cell lines were maintained in a logarithmic growth phase at 37 °C in a humidified atmosphere supplemented with 5% $CO_2$.

Blood samples of healthy anonymous donors were purchased from the Blood Service of the Flemish Red Cross (Mechelen, Belgium) following the approval by the Ethics Committee of the Antwerp University Hospital and the University of Antwerp (reference number 16/35/357). Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Diegem, Belgium), and CD8 T cells were selected using human CD8 magnetic microbeads for magnetic-activated cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotec, Leiden, The Netherlands). The purity of CD8 T cells after MACS isolation was analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Suarlée, Belgium) after staining with FITC-labeled anti-CD8, PE-conjugated anti-CD4 and PerCP-conjugated anti-CD3 monoclonal antibodies (mAbs; Becton-Dickinson (BD) Biosciences, Erembodegem, Belgium). After MACS isolation, CD8 T cells were centrifuged and resuspended in cryopreservation medium consisting of fetal bovine serum (FBS; Life Technologies) supplemented with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Diegem, Belgium). Aliquots of 20–35 × $10^6$ cells/mL were transferred to Mr. Frosty freezing containers (Thermo Fisher Scientific, Erembodegem, Belgium) filled with isopropyl alcohol (Yvsolab, Turnhout, Belgium) and kept in a −80°C freezer for at least seven days up to three weeks. Aliquots were thawed in pre-warmed AIM-V (Life Technologies) supplemented with 10% human AB serum (hAB; Life Technologies) and rested for at least one hour in a humidified 5% $CO_2$ incubator at 37°C.

### In vitro transcription of mRNA

The cloning of WT1-specific *TCR* genes, generation of the pST1 DNA plasmids containing the TCR constructs and generation of WT1-specific *TCR* mRNA by in vitro transcription (IVT) were performed as previously described [28,29]. Clinical-grade codon-optimized Sig-DC-LAMP *WT1* mRNA encoding isoform D of WT1 [21] was purchased from eTheRNA immunotherapies (Niel, Belgium).

### Electroporation

Electroporation of 2D3 cells with WT1-specific *TCR* mRNA was performed as previously described [28]. Double sequential electroporation (DSE) of human primary CD8 T cells was performed following [28], with minor modifications. Briefly, 10 or 20 × $10^6$ thawed viable human primary CD8 T cells were resuspended in 200 or 400 µL of serum-free Opti-MEM medium (Life Technologies) after thawing and transferred to a 4 mm-gap electroporation cuvette (Cell Projects, Harrietsham, UK). Next, cells were electroporated with 16 or 32 µL of a pool containing 100 µM of *TRAC*- and *TRBC*-specific DsiRNAs (Integrated DNA Technologies) in a ratio of 1:1. After electroporation, cells were transferred to pre-warmed AIM-V medium supplemented with 10% hAB, rested at 37°C in a humidified atmosphere supplemented with 5% $CO_2$ for at least 20 min, centrifuged (300× *g*, 3 min), transferred to 6-well plates and then incubated for 24 h. Second electroporation with in vitro transcribed mRNA was performed following the same protocol, using 1 µg of mRNA per $10^6$ cells. For the electroporation of U266 cells, 5 × $10^6$ viable cells were washed once with Opti-MEM I medium (Life Technologies), resuspended in 200 µL of the same medium, and then transferred to 4 mm-gap cuvettes (Cell Projects). Next, 5, 10 or 20 µg of clinical-grade IVT *WT1* mRNA was added to the cells before electroporation. Cells were electroporated in a Gene Pulser Xcell™ device (Bio-Rad Laboratories, Temse, Belgium) using the Time constant protocol (300 V, 8 ms, one pulse). After electroporation, all cells were transferred to pre-warmed recovery medium (RPMI supplemented with 10% FBS for 2D3 cells; AIM-V medium supplemented with 10% hAB for human primary T cells, and IMDM supplemented with 10% FBS for U266 cells) and rested for at least 20 min in a humidified 5% $CO_2$ incubator at 37°C. Before co-culture, cells were washed, resuspended in fresh medium and incubated for 4 h. When necessary, cells were electroporated without mRNA (mock) as a negative control.

### Flow cytometry

HLA-A*02:01 positivity of T2, U266, Raji-A2, K562-A2 cells was analyzed by direct staining using a PE-conjugated anti-human HLA-A*02 antibody (clone BB7-2; BioLegend, London, UK). HLA-A*02:01 expression on PBMC samples was detected by incubation with the supernatant of the hybridoma BB7.2 cell line (producer of anti-HLA-A*02 antibody, ATCC) for 15 min at room temperature. Then, cells were washed with FACS buffer (FACSFlow sheath fluid (BD Biosciences), 0.1% bovine serum albumin (BSA) (Sigma-Aldrich), 0.05% sodium azide (Merck, Overijse, Belgium), labeled with FITC-conjugated polyclonal rabbit anti-mouse immunoglobulins (Dako, Heverlee, Belgium) for 15 min at room temperature protected from light. WT1 expression was analyzed in samples from T2, U266, Raji-A2, and K562-A2 cell lines or electroporated U266 cells 24 h after *WT1* mRNA electroporation by intracellular staining. Cells were harvested for fixation and permeabilization using the Foxp3/Transcription factor staining buffer set (eBioscience, Life Technologies) according to the manufacturer's instructions. Next, cells were labeled with unconjugated mouse anti-human WT1 monoclonal antibody (clone 6F-H2, Dako)—which recognizes an epitope within residues 1-181 of all isoforms of the full-length WT1 protein—followed by PE-conjugated polyclonal rabbit anti-mouse immunoglobulins (Dako). As a control, samples were only incubated with PE-conjugated polyclonal rabbit anti-mouse immunoglobulins. WT1-specific TCR surface expression was evaluated 24 h after *TCR* mRNA electroporation in 2D3 and primary CD8 T cells. For 2D3 cells, samples were labeled with FITC-conjugated anti-CD8 (BD Biosciences) and PE-conjugated anti-pan TCR$\alpha\beta$ (Miltenyi Biotec) or isotype control mAb (BD Biosciences) for 15 min at room temperature. For primary CD8 T cells, samples were labeled with PE-conjugated $WT1_{37–45}$ or $WT1_{126–134}$ peptide/HLA-A*02:01 tetramers for 30 min at 37°C [28]. Then, cells were washed and labeled with FITC-conjugated anti-CD8 and PerCP-conjugated anti-CD3 mAbs (BD Biosciences) for 15 min at room temperature. All samples were washed previous to analysis on a CytoFLEX cytometer (Beckman Coulter).

### Peptide pulsing of tumor cells

Viable T2, U266, Raji-A2, and K562-A2 cells were harvested, washed once in serum-free IMDM medium, and resuspended using the same medium at a final concentration of $10^6$ cells/mL. Cells were split in tubes and pulsed with $WT1_{37–45}$ (VLDFAPPGA) or $WT1_{126–134}$ peptide (RMFPNAPYL) (JPT Peptide Technologies, Berlin, Germany) at decreasing concentrations of a ten-fold serial dilution from a concentration of 10 μg/mL for 60 min at room temperature under constant motion. After incubation, cells were washed and resuspended in IMDM supplemented with 10% FBS at a concentration of $5 \times 10^5$ cells/mL.

### Co-cultures

Electroporated 2D3 or DSE primary CD8 T cells were co-cultured with peptide-pulsed T2, U266, Raji-A2 and K562-A2 cells or electroporated U266 cells in triplicate in 96-well round-bottom plates at an effector:target (E:T) ratio of 2:1 (2D3 cells) or 4:1 (primary CD8 T cells). 2D3 cells or primary CD8 T cells cultured alone served as negative controls. Co-cultures were incubated for 18–22 h at 37°C in a humidified atmosphere supplemented with 5% $CO_2$.

### Analysis of epitope-specific T-cell activation

After co-culture, cells were harvested and analyzed for epitope-specific expression of the enhanced green fluorescent protein (EGFP; 2D3 cells) or expression of the activation markers CD137 and CD69 (primary CD8 T cells). Samples from 2D3 cell co-cultures were washed, incubated with PE-conjugated anti-CD8 for 15 min at room temperature. Then, samples were rewashed and stained with the nucleic acid dye 7-aminoactinomycin D (7-AAD; BD Biosciences) for 10 min at room temperature for the exclusion of nonviable cells before analysis on a CytoFLEX cytometer (Beckman Coulter). Samples from primary CD8 T cell co-cultures were washed and stained with anti-human PE-conjugated anti-CD137, PerCP-Cy5.5-conjugated anti-CD3, APC-Cy7-conjugated anti-CD69 (BD Biosciences) and Pacific Blue-conjugated anti-CD8 (Life Technologies) monoclonal antibodies and LIVE/DEAD fixable aqua dead cell stain kit (Thermo Fisher Scientific) for 15 min at room temperature. After incubation, cells were washed and analyzed using a FACSAria II cytometer (BD Biosciences).

### Statistical analysis

Data from flow cytometers were analyzed using FlowJo v10.2 software (TreeStar Inc, Ashland, OR, USA). Prism v5 software (GraphPad, San Diego, CA, USA) was used for graphing, statistical calculations, and calculation of EC50 values. Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's post hoc test where applicable for multiple comparisons. Results were considered to be statistically significant when *p*-value was less than 0.05. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ and **** indicates $P < 0.0001$.

# Supplementary material

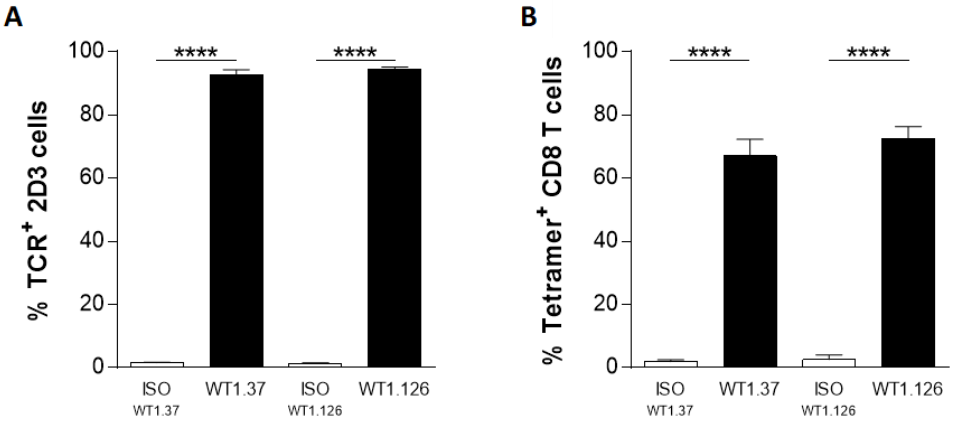



**Figure S1. WT1-specific TCR expression on 2D3 and primary CD8 T cells.** Surface expression of WT1-specific TCR in 2D3 cells (**A**) or primary CD8 T cells (**B**) was analyzed 24 h after electroporation with either WT137-45- (WT1.37) or WT1126-134-specific (WT1.126) codon-optimized *TCR* mRNA by anti-human TCR$\alpha\beta$ antibody staining (**A**) or by HLA:A*02:01/WT1$_{37\text{-}45}$ (WT1.37) or HLA:A*02:01/WT1$_{126\text{-}134}$ (WT1.126) tetramer staining (**B**). Mean ± SEM of 10 independent replicates (**A**) and of 8 donors (**B**) is shown. WT1, Wilms' tumor 1; ISO, isotype. ****, $P < 0.0001$.

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**There is strength in numbers, but organizing those numbers is one of the great challenges.**

— John C. Mather

# 6

# RNA-based co-transfer of human CD8αβ with WT1-specific TCRαβ redirects antileukemic activity of CD4 and γδ T cells towards MHC class I-restricted WT1 epitopes and boosts CD8 T-cell responses in combination with *CD3* mRNA

This chapter is under review in:

Campillo-Davo D, Flumens D, Roex G, Maarten Versteven1, Van Acker HH, Fujiki F, Sugiyama H, Berneman ZN, Van Tendeloo VFI, Anguille S, Lion E.
*Frontiers in Immunology* (2021).

# Abstract

Genetic transfer of T-cell receptors (TCRs) and chimeric antigen receptors (CARs) has revolutionized adoptive T-cell cancer therapies. In particular, TCR-T-cell therapies are based on redirecting T-cell specificity towards intracellular tumor antigens, and have mainly focused on engineering conventional cytotoxic CD8 T cells. However, there is growing interest in using other T-cell subsets, such as CD4 and γδ T cells. In this study, we evaluated whether CD4 and γδ T cells could be redirected towards leukemia-associated antigen Wilms' tumor 1 (WT1) using a major histocompatibility complex (MHC) class I-restricted WT1-specific TCR introduced via RNA-based engineering. We also studied whether co-transfection of *TCR* mRNA in combination with *CD8*αβ mRNA in CD4 and γδ T cells or with *CD8*αβ and *CD3*γδεζ mRNAs in CD8 T cells improves antigen-specific T-cell functional avidity and killing. We transfected resting and expanded primary human CD4 T cells and expanded primary human CD8 T cells following our in-house-developed protocol, in which electroporation with Dicer-substrate silencing RNA (DsiRNA) suppresses de novo expression of native TCR, followed by DsiRNA-resistant transgenic *TCR* mRNA transfection. This method allows minimal mispairing between native and introduced TCR chains. Expanded primary human γδ T cells were not electroporated with DsiRNA due to the absence of TCRαβ. High frequencies of transgenic MHC class I-restricted WT1-specific TCR-positive cells were obtained in expanded CD4 and γδ T cells. Importantly, TCR and CD8-engineered CD4 T cells and γδ T cells recognized and killed leukemic cells WT1-specifically as compared to TCR-engineered cells. Co-electroporation of CD8 T cells with WT1-specific *TCR*, *CD8* and *CD3* mRNAs also enhanced CD8 T-cell activation and antigen-specific killing as compared to either TCR-engineered or TCR- and CD8-engineered cells. In summary, RNA electroporation is a fast and efficient method to engineer primary human CD8, CD4 and γδ T cells for redirecting T-cell specificity. Transgenic CD8 expression in CD4 and γδ T cells and upregulation of CD8 and CD3 expression in CD8 T cells enable antigen recognition when T cells are engineered with TCRs of low/intermediate avidity. This research shows the potential of upregulating TCR co-receptors to improve T-cell functional avidity against leukemia-associated antigens in adoptive TCR-T-cell therapies.

# Introduction

Genetic engineering of T cells for adoptive cell transfer has marked a turning point in personalized immunotherapy, especially in the treatment of cancer. This strategy focuses on specifically targeting tumor-associated antigens (TAAs) by modifying T cells with nucleic acids that encode immune receptors such as chimeric antigen receptors (CARs) and T-cell receptors (TCRs) to improve T cell's ability to detect and eradicate tumor cells. T cells naturally recognize internally-processed proteins in the form of short peptides presented by molecules of the major histocompatibility complex (MHC) as peptide-MHC (pMHC) complexes by means of their TCR. Each TCR is specific for a pMHC, virtually allowing an infinity of pMHC combinations that can be exploited for TCR-T-cell therapy. To redirect their specificity towards cancer cells, alpha beta ($\alpha\beta$) T cells are genetically modified with TAA-specific TCRs. The majority of studies using adoptive TCR-engineered T-cell therapies for hematological malignancies have focused on engineering conventional CD8 T cells [1]. Despite the success and benefits of conventional CD8 T cells, other T cell populations, such as helper CD4 T cells and gamma delta ($\gamma\delta$) T cells, have gained attention as alternative and interesting subsets for genetic engineering in adoptive T-cell cancer therapies. $\gamma\delta$ T cells are a subset of unconventional T cells that express TCR$\gamma\delta$ instead of TCR$\alpha\beta$ and compose up to 10% of peripheral T cells [2]. Despite the low frequency in peripheral blood, these cells can be easily expanded ex vivo [3]. $\gamma\delta$ T cells have excellent natural antitumor properties that can be exploited as a cellular immunotherapy [4]. Moreover, as opposed to TCR gene transfer in classical $\alpha\beta$ T cells, mispairing between intrinsic TCR$\gamma\delta$ chains and transgenic TCR$\alpha\beta$ chains does not occur in redirected $\gamma\delta$ T cells [4], thus circumventing the safety issues associated with mispaired TCR$\alpha\beta$ combinations formed from a transgenic and native TCR in TCR-engineered $\alpha\beta$ T cells. One of the key differences between CD4 and $\gamma\delta$ T cells and conventional cytotoxic CD8 T cells is the expression of CD8 co-receptor. CD8 is an important factor in TCR-mediated antigen-specific recognition as it interacts with MHC class I molecules [5]. CD8$\alpha\beta$ heterodimer, mostly found in conventional peripheral TCR$\alpha\beta$ CD8 T cells, interacts with MHC class I molecules via CD8$\alpha$, and acts as TCR co-receptor for MHC class I restriction via CD8$\beta$, whereas CD8$\alpha\alpha$ homodimer is not correlated with MHC class I restriction [6,7]. Although a small population of $\gamma\delta$ T cells may express CD8 co-receptor in peripheral blood, most of the CD8-positive $\gamma\delta$ T cells are intraepithelial $\gamma\delta$ T cells expressing the CD8$\alpha\alpha$ homodimer [8,9]. Moreover, CD8 is a key factor in modulating TCR avidity and functional avidity, which is a measure of how well a T-cell expressing a certain TCR responds to different concentrations of cognate epitope presented by MHC molecules on an antigen-presenting cell [5,10-13]. Contrary to cytotoxic CD8 T cells, whose TCR

recognizes 8- to 10-mer peptide epitopes presented through MHC class I molecules, TCRs from helper CD4 T cells typically recognize longer (14- to 21-mer) peptide antigens bound to MHC class II molecules. However, viral-based redirection of CD4 T-cell specificity against melanoma epitopes presented by MHC class I molecules can be achieved through transduction of high-affinity TCRs with reactivities in the nanomolar range [14,15]. Above that threshold, activation of CD4 T cells engineered with MHC class I-restricted TCRs against viral epitopes is dependent on the co-expression of CD8 co-receptor [16-18]. Similar to CD4 T cells, viral-based TCR$\alpha\beta$-engineering of $\gamma\delta$ T cells, also in combination of CD8 co-receptor, has been reported to target viral and minor histocompatibility antigens [19]. These cells were able to respond against leukemic cells in an antigen-specific manner, especially in the presence of CD8 co-receptor. Many TAAs targeted using TCR-T cells, such as the key acute myeloid leukemia (AML) antigen Wilms' tumor 1 (WT1), are self-antigens that are overexpressed in leukemic cells, but are also present in normal tissues [20]. Due to mechanisms of negative selection in the thymus, T cells that are highly reactive against self-antigens are eliminated [5,21]. Those self-reactive T cells that remain prominently express TCRs of low or intermediate affinity, which translates into T-cell clones with low-intermediate functional avidity and inferior antitumor activity [5,21]. Therefore, TCRs isolated from these T-cell clones are usually weak binders of self-TAAs. TCR affinity may be artificially enhanced by affinity maturation processes. However, this type of approach has been linked to harmful cross-reactivities due to the supraphysiological affinities achieved by affinity maturation. Since TCR avidity is directly linked to the expression levels of TCR co-receptors, in this study, we aimed to enhance the avidity of intermediate-affinity MHC class I-restricted WT1-specific TCRs, and thus of tumor recognition, by inducing the expression of CD8 co-receptor in CD4 and $\gamma\delta$ T cells and upregulating the expression of CD8 and CD3 co-receptors in CD8 T cells via non-integrative RNA-based electroporation.

# Results

## Effective DsiRNA-mediated downregulation of *TRAC* and *TRBC* sequences in CD4 T cells

We recently showed that Dicer-substrate silencing RNAs (DsiRNAs) against T-cell receptor alpha constant (*TRAC*) and T-cell receptor beta constant (*TRBC*) regions of wild-type TCR$\alpha\beta$ chains could significantly reduce the expression of native TCRs, leading to improved transgenic TCR levels in TCR-engineered T cells [22]. Therefore, we first studied the potential of DsiRNA-mediated downregulation of native wild-type

TCR sequences in CD4 T cells. Similar to CD8 T cells, significant 3-fold downregulation of *TRAC* and *TRBC* relative normalized expression was achieved in resting CD4 T cells 24 h after DsiRNA electroporation compared to mock (no DsiRNA) electroporation ($P \leq 0.0001$; **Figure 1**). Similar *TRAC* and *TRBC* downregulation levels were also observed in expanded CD4 T cells (**Figure S1**).

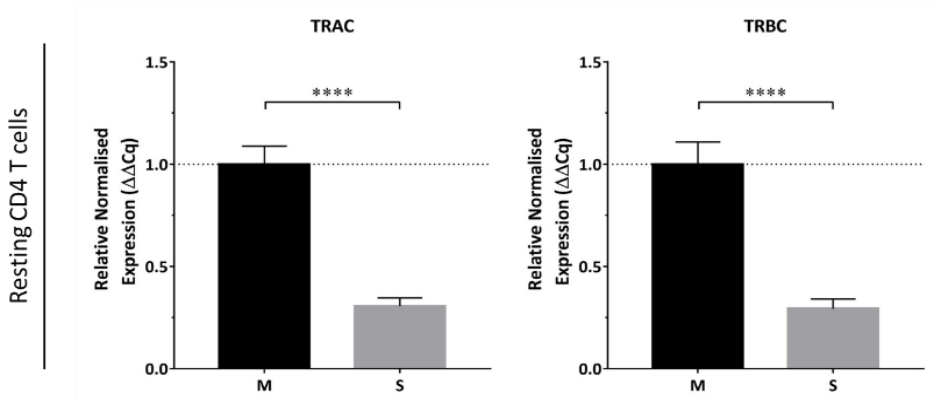



**Figure 1. RT-qPCR analysis of DsiRNA-mediated native TCR silencing capacity in resting CD4 T cells.** DsiRNA targeting T-cell receptor alpha constant (*TRAC*) and T-cell receptor beta constant (*TRBC*) regions of the native *TCR* sequences significantly downregulate *TRAC* and *TRBC* expression in resting CD4 T cells 24 h after DsiRNA electroporation. Graphs represent average relative normalized expression (ΔΔCq) ± standard error of the mean (SEM) for 7 independent donors (****, $P \leq 0.0001$). M, mock; S, DsiRNAs against *TRAC* and *TRBC* sequences.

## Efficient co-transfection of *CD8* and MHC class I-restricted *TCR* mRNA in CD8-negative primary CD4 and γδ T cells

Next, we evaluated the feasibility of using RNA-based methods for redirecting the specificity of primary human CD4 T cells and γδ T cells with a CD8 T-cell-derived HLA-A*02:01-restricted $WT1_{37\text{-}45}$-specific (T37) TCR. The in-house developed double sequential electroporation (DSE) protocol, by which DsiRNAs-mediated suppression of *TRAC* and *TRBC* is followed by T37 *TCR* mRNA electroporation alone (ST37) or in combination with *CD8* mRNA (ST37+CD8), was used for CD4 T cells [22]. To ensure that native TCR-targeting DsiRNAs and *CD8* mRNA co-electroporation did not have a deleterious effect in CD4 T cells, we first analyzed T37 *TCR* mRNA intracellular levels in resting CD4 T cells 24 h after DSE treatment (**Figure S2**). We observed a significant increase in T37 *TCR* mRNA levels in both ST37 and ST37+CD8 conditions compared to double sequential mock (MM) electroporation ($P < 0.01$), confirming that codon-optimized T37 *TCR* mRNA transfection is not targeted by DsiRNA-mediated silencing. Moreover, co-electroporation of *CD8* mRNA together with T37 *TCR* mRNA did not

significantly hinder *TCR* mRNA transfection efficiency. For γδ T cells, a single electroporation protocol either with T37 *TCR* mRNA or *CD8* mRNA alone (T37 and CD8 conditions, respectively) or both mRNAs combined (T37+CD8 condition) was performed instead of the DSE protocol. Contrary to CD4 T cells, γδ T cells do not express a native TCRαβ, not requiring the transfection of DsiRNAs against *TRAC* and *TRBC* to prevent TCRαβ mispairing [23,24]. However, the low numbers of circulating γδ T cells require expansion to obtain sufficient cells for transfection (**Figure S3**). Expansion of γδ T cells from PBMC using zoledronic acid, IL-2, and IL-15 rendered a highly pure γδ T-cell population two weeks after initiation of the culture (**Figure S3A and S3B**), mostly composed of TCR Vδ2-positive cells (**Figure S3C**), as reported previously by our group [3]. Thus, after expansion, γδ T cells were subjected to a single electroporation with *CD8* mRNA or T37 *TCR* mRNA alone, co-electroporation of both mRNAs together or a mock electroporation. Surface expression of T37 TCR was detected in resting and expanded CD4 T cells and in γδ T cells (**Figure 2**). $WT1_{37\text{-}45}$/HLA-A*02:01 tetramer staining was possible in ST37+CD8-electroporated cells in both resting and expanded CD4 T cells (26.7 ± 15.1 % and 68.4 ± 13.2 %, respectively). However, in ST37 conditions, T37 TCR was only detected in expanded CD4 T cells (21.9 ± 11.3 %), albeit at significantly lower levels than ST37+CD8-electroporated cells. Detection of T37 TCR in ST37-electroporated CD4 T cells was observed using a monoclonal antibody against the TCR Vβ21.3 variant of the T37 TCR in resting (47.8 ± 20.3 %) and expanded CD4 T cells (39.6 ± 17.8 %) (**Figure S4**). No differences were found in transgenic TCR expression levels between ST37 and ST37+CD8 conditions (resting CD4 T cells: 51.3 ± 25.4%; expanded CD4 T cells: 33.7% ± 12.3 %) (**Figure S4**). CD8 expression was confirmed in ST37+CD8-electroporated in resting (60.7 ± 30.2 %) and expanded (93.5 ± 2.1 %) CD4 T cells, whereas no CD8 expression was detected in ST37 conditions (**Figure 2**). Since γδ T cells express TCRγδ, an anti-human pan-TCRαβ antibody was used to analyze T37 TCR surface levels. T37 TCR was expressed at very high levels in T37 (83.5 ± 11.2 %) and T37+CD8-electroporated (78.1 ± 14.8 %) γδ T cells (**Figure 2**). No significant differences were detected in T37 TCR expression with and without *CD8* mRNA co-electroporation. Concerning CD8 expression, a small population of γδ T cells expresses this TCR co-receptor (mock: 8.6 ± 5.2 %; T37: 9.6 ± 6.7 %). However, as seen in CD4 T cells, CD8 levels were greatly enhanced by *CD8* mRNA transfection (CD8: 91.9 ± 4.2 %; T37+CD8: 89.4 ± 6.1 %).

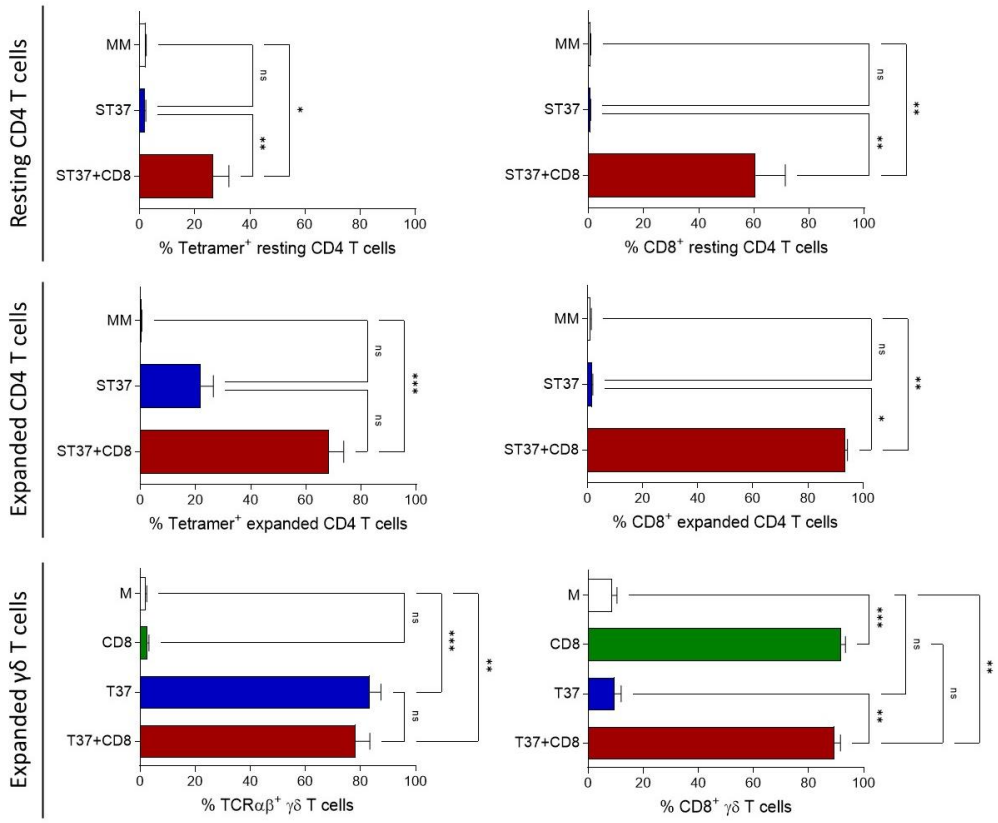
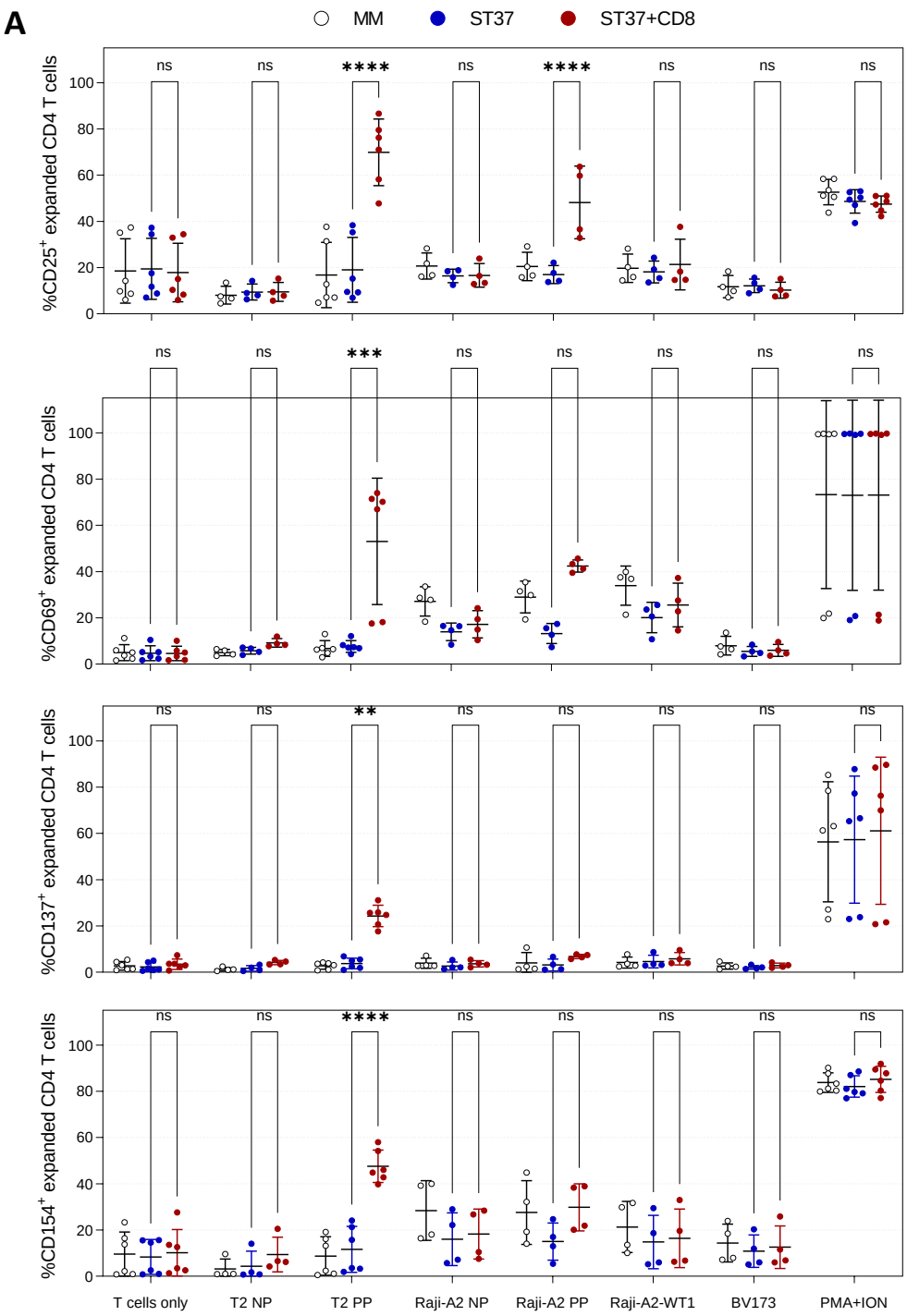



**Figure 2. Transgenic TCR and CD8 expression in mRNA-electroporated primary CD4 and γδ T cells**. T37 TCR (**left panel**) and CD8 (**right panel**) expression was measured 24 h after electroporation with WT1$_{37\text{-}45}$-specific *TCR* mRNA with or without *CD8* mRNA. Graphs represent mean ± standard deviation (SD) values for 6-8 independent donors that were analyzed using Kruskal-Wallis test followed by corrected Dunn's multiple comparison test (ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). M, mock; MM, double sequential mock electroporation; S, DsiRNA against *TRAC* and *TRBC*; T37, WT1$_{37\text{-}45}$ *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with *TCR* mRNA; CD8, *CD8* mRNA only.

## De novo transgenic expression of CD8 in primary CD8-negative T cells via *CD8* mRNA electroporation leads to MHC class I-restricted antigen-specific recognition of tumor cells

To determine whether TCR-redirected CD4 and γδ T cells are capable of recognizing tumor cells in an antigen-specific manner, T37 TCR-engineered resting and expanded CD4 T cells as well as expanded γδ T cells were subjected to different antigen-specific functional assays against tumor cells (**Figure 3-6**). Since expanded CD4 T cells expressed higher levels of T37 TCR than resting CD4 T cells, functional assays were performed in

expanded CD4 T cells only (**Figure 3 and 4**). Similar to CD8 T cells, CD4 T cells upregulate defined activation markers independent of the functional specialization of the cell upon recognition of their target antigen [25]. Therefore, we compared the antigen-specific upregulation of CD25 (interleukin-2 receptor alpha), CD69, CD137 (4-1BB), and CD154 (CD40 ligand) in expanded CD4 T cells (**Figure 3A**). We observed a significant increase ($P \leq 0.0001$) in CD25 expression in ST37+CD8 expanded CD4 T cells against HLA-A*02-positive $WT1_{37\text{-}45}$ peptide-pulsed T2 cells (69.9 ± 14.4 %) and HLA-A*02-positive $WT1_{37\text{-}45}$ peptide-pulsed Raji (Raji-A2) cells (48.2 ± 15.7 %) compared to ST37-electroporated cells (19.1 ± 14.0 % and 17.0 ± 3.9 %, respectively for T2 and Raji-A2 cells). A significantly higher expression of CD69, CD137, and CD154 was only found in co-cultures with $WT1_{37\text{-}45}$ peptide-pulsed T2 cells in ST37+CD8 CD4 T cells (53.1 ± 27.3 %, 24.3 ± 4.6 %, and 47.6 ± 7.0 %, respectively) compared to ST37 CD4 T cells (7.7 ± 2.5 %, 3.8 ± 2.3 %, and 11.6 ± 10.0 %, respectively). ST37 as well as ST37+CD8 CD4 T cells failed to express any of the evaluated activation markers in response to intracellularly processed WT1 in co-cultures with HLA-A*02-positive WT1-transduced Raji cells (Raji-A2-WT1) or with HLA-A*02-positive BV173 cells which naturally express WT1. CD127 (interleukin-7 receptor alpha) is an activation marker downregulated in CD4 T cells after antigen engagement [26,27]. We observed significant downregulation of CD127 in ST37+CD8 CD4 T cells in response to $WT1_{37\text{-}45}$ peptide-pulsed T2 and Raji-A2 cells (36.4 ± 9.7 % and 54.4 ± 14.4 %, respectively) versus ST37 CD4 T cells (76.7 ± 21.7 % and 80.4 ± 7.6 %, respectively) (**Figure 3B**). To confirm that T37 TCR-redirected CD4 T cells were functional in targeting WT1-presenting tumor cells, we analyzed secretion of granzyme B in supernatants of co-cultures of T37 TCR-redirected expanded CD4 T cells and HLA-A*02-positive tumor cell lines that were either pulsed with $WT1_{37\text{-}45}$ peptide or left unpulsed (**Figure 4**). ST37+CD8-engineered CD4 T cells, but not ST37 CD4 T cells secreted granzyme B in the presence of $WT1_{37\text{-}45}$ peptide-pulsed T2 (ST37+CD8: 2.8 ± 0.9 ng/mL versus ST37: 0.1 ± 0.03 ng/mL) and $WT1_{37\text{-}45}$ peptide-pulsed Raji-A2 cells (ST37+CD8: 2.3 ± 1.1 ng/mL versus ST37: 0.1 ± 0.1 ng/mL). However, as observed in the analysis of activation markers, ST37+CD8 CD4 T cells failed to respond to tumor cells that intracellularly processed WT1.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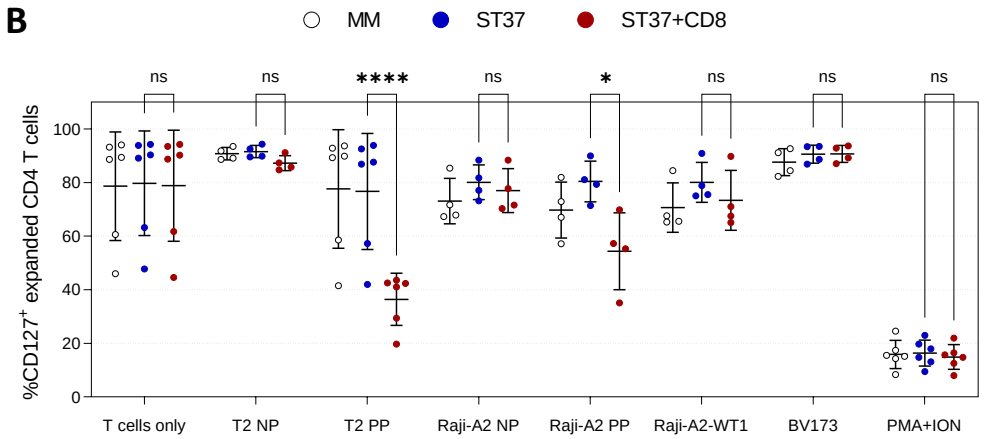



**Figure 3. Expression of activation markers in response to WT1-specific recognition in expanded primary CD4 T cells.** Upregulation of CD25, CD69, CD137 and CD154 expression (**A**) and downregulation of CD127 expression (**B**) in T37 TCR-redirected CD4 T cells were measured 24 h after co-culture with tumor cell lines that were pulsed (PP) with WT1$_{37\text{-}45}$ peptide or left unpulsed (NP). As a negative control, T cells were cultured in the absence of target cells (T cells only). As a positive control, T cells were cultured in the presence of phorbol 12-myristate 13-acetate and ionomycin (PMA+ION). Graphs represent mean ± SD values for 4-6 independent donors and were analyzed using two-way ANOVA followed by Tukey's multiple comparison test (ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$). MM, double sequential mock electroporation; S, DsiRNA against *TRAC* and *TRBC*; T37, WT1$_{37\text{-}45}$ *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with *TCR* mRNA.

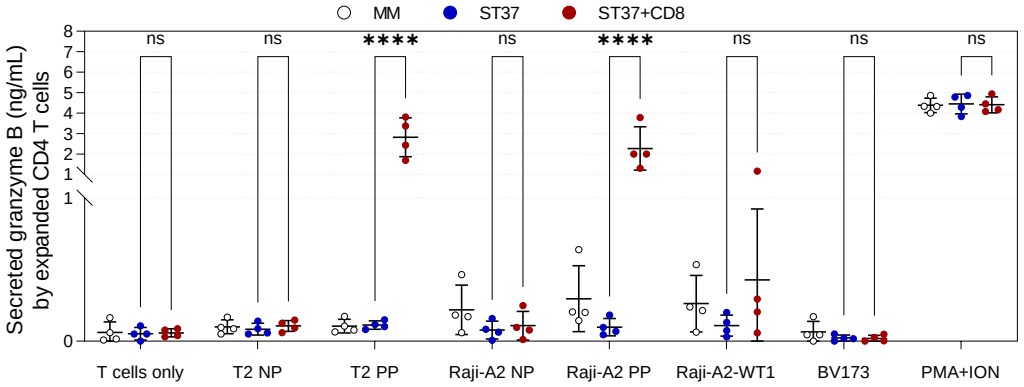



**Figure 4. Secretion of granzyme B in expanded primary T37 TCR-redirected CD4 T cells in response to WT1-specific recognition.** Supernatants from 24 h co-cultures of T37 TCR-redirected T cells and tumor cell lines that were pulsed with WT1$_{37\text{-}45}$ peptide (PP) or left unpulsed (NP), were harvested and analyzed for granzyme B presence using ELISA. As a negative control, T cells were cultured in the absence of target cells (T cells only). As a positive control, T cells were cultured in the presence of phorbol 12-myristate 13-acetate and ionomycin (PMA+ION). Graphs represent mean ± SD values for 4 independent donors and were analyzed using two-way ANOVA followed by Tukey's multiple comparison test (ns, not significant; ****, $P \leq 0.0001$). MM, double sequential mock electroporation; S, DsiRNA against *TRAC* and *TRBC*; T37, WT1$_{37\text{-}45}$ *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with *TCR* mRNA.

Next, we investigated whether the differences in expression of activation markers observed in ST37+CD8-engineered expanded CD4 T cells was limited to CD8-positive populations within ST37+CD8 CD4 T cells (**Figure S5 and S6**). First, a similar level of CD8 expression was measured in all ST37+CD8 CD4 T cell co-cultures, with an average value of 83.9 ± 11.8 % (pooled standard deviation), a maximum value of 88.5 ± 2.6 %, for ST37+CD8 CD4 T cells co-cultured with BV173 cells, and a minimum value of 77.3 ± 17.8 %, for ST37+CD8 CD4 T cells cultured in the presence of phorbol 12-myristate 13-acetate (PMA) and ionomycin (PMA+ION condition; **Figure S5**). Then, we divided ST37+CD8 expanded CD4 T cells into CD8-positive and CD8-negative subpopulations (**Figure S6**). As expected, antigen-specific upregulation of activation markers CD25, CD69, CD137 and CD154 and downregulation of marker CD127 was observed in CD8-positive ST37+CD8-redirected expanded CD4 T cells (**Figure S6A and S6C**, respectively). Interestingly, no statistically significant differences were observed in the upregulation of activation markers within the different co-cultures of CD8-negative ST37+CD8-redirected expanded CD4 T cells (**Figure S6B**). However, CD127 was significantly reduced in both CD8-positive and CD8-negative subsets within ST37+CD8-redirected expanded CD4 T cells co-cultured with $WT1_{37\text{-}45}$ peptide-pulsed T2 cells (35.3 ± 9.8 % and 43.2 ± 14.6 %, respectively) compared to those co-cultured with unpulsed T2 cells (87.3 ± 3.2 % and 86.0 ± 1.4 %, respectively; **Figure S6D**).

Regarding γδ T cells, **Figure 5** shows antigen-specific γδ T-cell functionality after redirection with $WT1_{37\text{-}45}$-specific *TCR* mRNA, *CD8* mRNA, or co-electroporation with both mRNAs. Compared to T37 γδ T cells, T37+CD8-redirected γδ T cells were significantly able to specifically kill HLA-A*02-positive $WT1_{37\text{-}45}$ peptide-pulsed T2 (56.1 ± 28.5 %), U266 (53.0 ± 24.3 %), K562-A2 (42,0 ± 20.3 %), and THP1 (35.3 ± 31.8 %) tumor cells in an antigen-specific manner (**Figure 5A**). However, no killing activity was detected against tumor cells that expressed WT1 either naturally (K562-A2 and THP1) or artificially after viral transduction (Raji-A2-WT1) or transfection with full-length *WT1*-encoding mRNA (U266 EP). Notably, although some T37+CD8-engineered γδ T-cell donors secreted granzyme B, a surrogate of antigen-specific killing activity, in response to $WT1_{37\text{-}45}$ peptide-pulsed tumor cells, no significant differences were found among the different mRNA-engineered γδ T-cell conditions (**Figure 5B**).

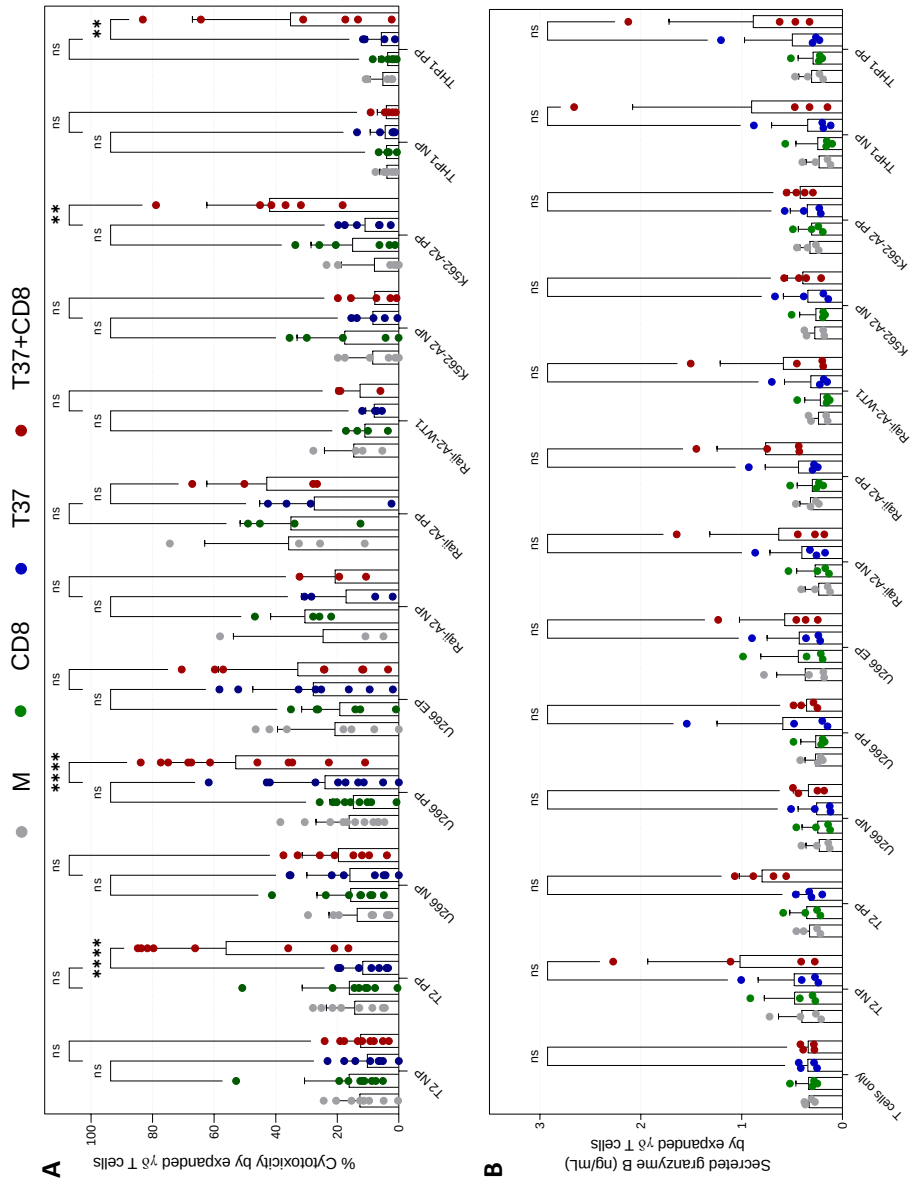



**Figure 5. Antigen-specific activity of T37 TCR-redirected γδ T cells against WT1$_{37\text{-}45}$ epitope presented by tumor cells.** (**A**) WT1-specific cytotoxic activity of T37 TCR-redirected γδ T cells was measured 4 h after co-culture with tumor cell lines that were pulsed with WT1$_{37\text{-}45}$ peptide (PP), left unpulsed (NP) or electroporated with full-length *WT1* antigen-encoding mRNA (EP). (**B**) Granzyme B secretion was measured by ELISA in supernatants from 4 h co-cultures. Mock (M) electroporation appears in grey, *CD8* mRNA only (CD8) condition in green, WT1$_{37\text{-}45}$ *TCR* mRNA only (T37) condition in blue, and WT1$_{37\text{-}45}$ *TCR* + *CD8* mRNA (T37+CD8) condition in red. As a negative control, T cells were cultured in the absence of target cells (T cells only). Graphs represent mean ± SD values for 3-11 independent donors and were analyzed using two-way ANOVA followed by Tukey's multiple comparison test (ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P \leq 0.0001$).

## Co-electroporation of *CD8* and *CD3* mRNA in TCR-redirected CD8 T cells improves antigen-specific recognition of cancer cells

Next, we assessed whether transfection of TCR-engineered conventional CD8 T cells with mRNAs encoding CD8 and CD3 co-receptors correlated with improved antigen-specific recognition of target cells. As with CD4 T cells, conventional CD8 T cells were subjected to DSE protocol [11,22]. In resting CD8 T cells, high levels of T37 TCR surface expression were detected via $WT1_{37\text{-}45}$/HLA-A*02:01 tetramers when resting CD8 T cells were transfected with WT1-*TCR* mRNA together with *CD8* mRNA (68.5 ± 22.0 %**)** or in combination with *CD8* and *CD3* mRNA (77.9 ± 15.0 %; **Figure S7A**). Similar values of TCR expression were observed with a $WT1_{126\text{-}134}$-specific (T126) TCR in both ST126+CD8 (60.0 ± 19.4 %) and ST126+CD8+CD3 conditions (66.1 ± 14.4 %). However, no significant differences were detected between TCR+CD8 and TCR+CD8+CD3 conditions for both T37 and T126. Moreover, these results are similar to those previously reported by our group in ST37-engineered and ST126-engineered resting CD8 T cells [11,22]. In terms of antigen-specific activation, no significant upregulation of CD69 and CD137 T-cell activation markers was detected after co-culture of RNA-transfected resting CD8 T cells and target tumor cells that were pulsed with the relevant WT1 peptides (**Figure S7B**). Despite the absence of significant antigen-specific T-cell activation, higher expression of activation markers in response to peptide-pulsed target cells was observed in ST37+CD8+CD3 resting CD8 T cells compared to their ST37+CD8-engineered counterpart. These observations were not replicated in resting CD8 T cells engineered with T126 TCR.

To analyze whether the lack of WT1-specific responses was caused by the resting status of the CD8 T cells, we activated and expanded CD8 T cells from isolated and cryopreserved bulk CD8 T-cell samples. We used a short-term expansion protocol based on anti-human plate-bound CD3 and anti-human soluble CD28 monoclonal antibodies that included addition of interleukin (IL)-2 and IL-15 (**Figure S8**). At the beginning of the expansion, CD8 populations were mostly comprised of naïve CD8 T cells (57.7 ± 15.0 %; (**Figure S8A**). After one week of expansion, at passage number 4, CD8 T-cell populations started to differentiate into effector memory (EM; 13.1 ± 6.5 %) and terminally differentiated effector memory (EMRA; 25.2 ± 6.5 %) T cells, whereas the percentage of naïve and central memory (CM) T cells decreased (50.6 ± 10.1 % and 10.9 ± 10.5 %, respectively). Two weeks after the initiation of the expansion, at passage number 8, CD8 T-cell cultures were mostly comprised of EMRA T cells (57.5 ± 12.6 %). Activation of CD8 T cells with the aforementioned protocol was confirmed by

upregulation of CD69 and CD137 activation markers after 2-3 days (89.6 ± 7.8 %; 88.5 ± 7.7 %, respectively; **Figure S8B**). CD137 expression returned to levels similar to those observed at the beginning of the expansion protocol after more than one week (passage number 5; 5.7 ± 2.3 %), whereas CD69 expression dropped but did not reach background levels (passage number 5; 13.6 ± 5.6 %). Expanded CD8 T cells were electroporated with WT1-*TCR* mRNA in combination with *CD3* and *CD8* mRNA using our DSE protocol one week after the initiation of the expansion protocol. We first hypothesized that the addition of increasing amounts of *CD3* mRNA co-electroporated with *TCR* and *CD8* mRNA could improve the expression and stabilization of the TCR complex on expanded CD8 T cells. However, an increase in the amount of co-electroporated *CD3* mRNA did not lead to a significant improvement of WT1-TCR expression (**Figure S9A and D**), CD8 expression (**Figure S9B and E**) or CD3 expression (**Figure S9C and F**), nor did it lead to improved antigen-specific T-cell recognition of target cells (**Figure S10A and B**).

Therefore, following experiments were performed with equal amounts of *TCR*, *CD8* and *CD3* mRNA (**Figure 6 and 7**). T37 TCR expression reached maximum levels in ST37 (92.3 ± 5.6 %), ST37+CD8 (88.9 ± 8.8 %) and ST37+CD8+CD3-engineered (91.8 ± 4.7 %) expanded CD8 T cells compared to mock control (2.3 ± 2.1 %; $P \leq 0.001$; **Figure 6A**). No significant differences were detected in conditions in which WT1-*TCR* mRNA was co-electroporated with *CD8* mRNA alone or in combination with *CD3* mRNA. Regarding CD8 and CD3 expression (**Figure 6B and C**), mock, ST37 and ST37+CD8 conditions had similar CD8 and CD3 expression levels. Surprisingly, CD8 expression was significantly reduced in ST37+CD8+CD3 condition ($P \leq 0.001$; **Figure 6B**), whereas CD3 expression remained similar to the expression levels observed in the other electroporation conditions (**Figure 6C**). In terms of antigen-specific functionality (**Figure 7**), both ST37+CD8 and ST37+CD8+CD3 expanded CD8 T cells significantly upregulated CD137 expression upon encounter of $WT1_{37\text{-}45}$ peptide-pulsed and WT1-expressing tumor cells compared to mock and ST37 expanded CD8 T cells ($P \leq 0.0001$; **Figure 7A**). Compared to their ST37+CD8 counterpart (23.5 ± 10.6 %), ST37+CD8+CD3 expanded CD8 T cells showed significant and greater upregulation of CD137 against WT1-expressing Raji-A2-WT1 tumor cells (36.6 ± 8.2 %; $P < 0.05$; **Figure 7A**). Similarly, we observed improved granzyme B secretion in ST37+CD8 and ST37+CD8+CD3 expanded CD8 T cells versus mock and ST37 cells in co-cultures with $WT1_{37\text{-}45}$ peptide-pulsed Raji-A2 leukemic cells ($P < 0.05$; **Figure 7B**). More importantly, ST37+CD8+CD3 expanded CD8 T cells (2.2 ± 0.9 ng/mL), but not those engineered with ST37+CD8 (1.4 ± 0.6 ng/mL), secreted significant amounts of granzyme B against WT1-expressing Raji-A2-WT1 tumor cells in comparison with ST37 expanded CD8 T cells (0.8 ± 0.4 ng/mL; $P \leq 0.01$; **Figure 7B**).

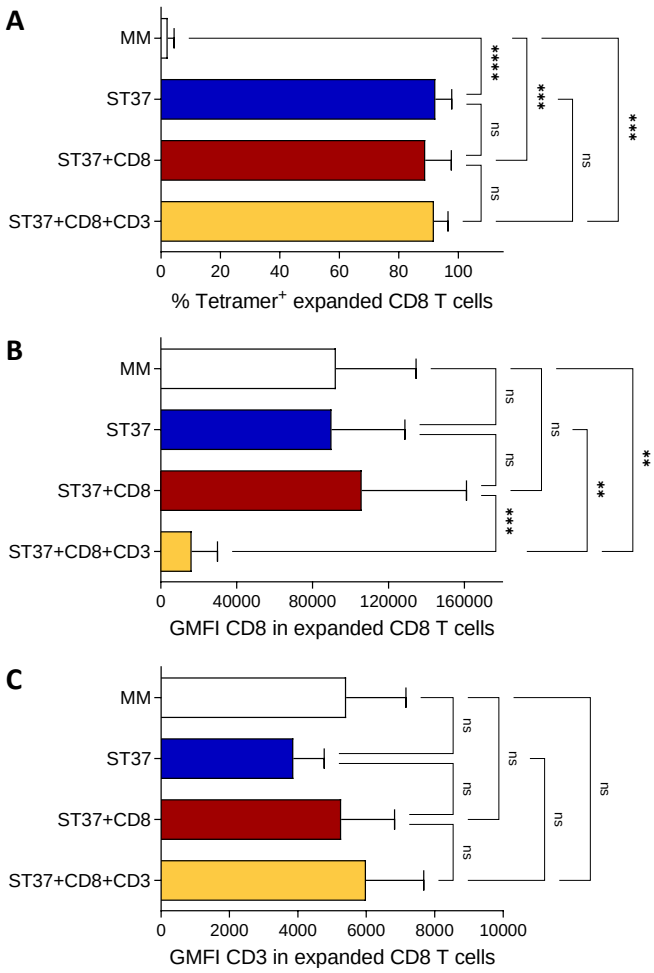



**Figure 6. Transgenic TCR, CD8 and CD3 expression in mRNA-electroporated primary expanded CD8 T cells**. T37 TCR (**A**), CD8 (**B**), and CD3 (**C**) expression was measured 24 h after electroporation with WT1$_{37\text{-}45}$-specific *TCR* mRNA with or without *CD8* and *CD3* mRNA. Graphs represent mean ± SD values for 7-18 independent donors that were analyzed using Kruskal-Wallis test followed by corrected Dunn's multiple comparison test (ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$). GMFI, geometric mean fluorescence intensity; MM, double sequential mock electroporation; S, DsiRNA against *TRAC* and *TRBC*; T37, WT1$_{37\text{-}45}$ *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with *TCR* mRNA; +CD3, *CD3* mRNA co-electroporated with *TCR* and *CD8* mRNA.

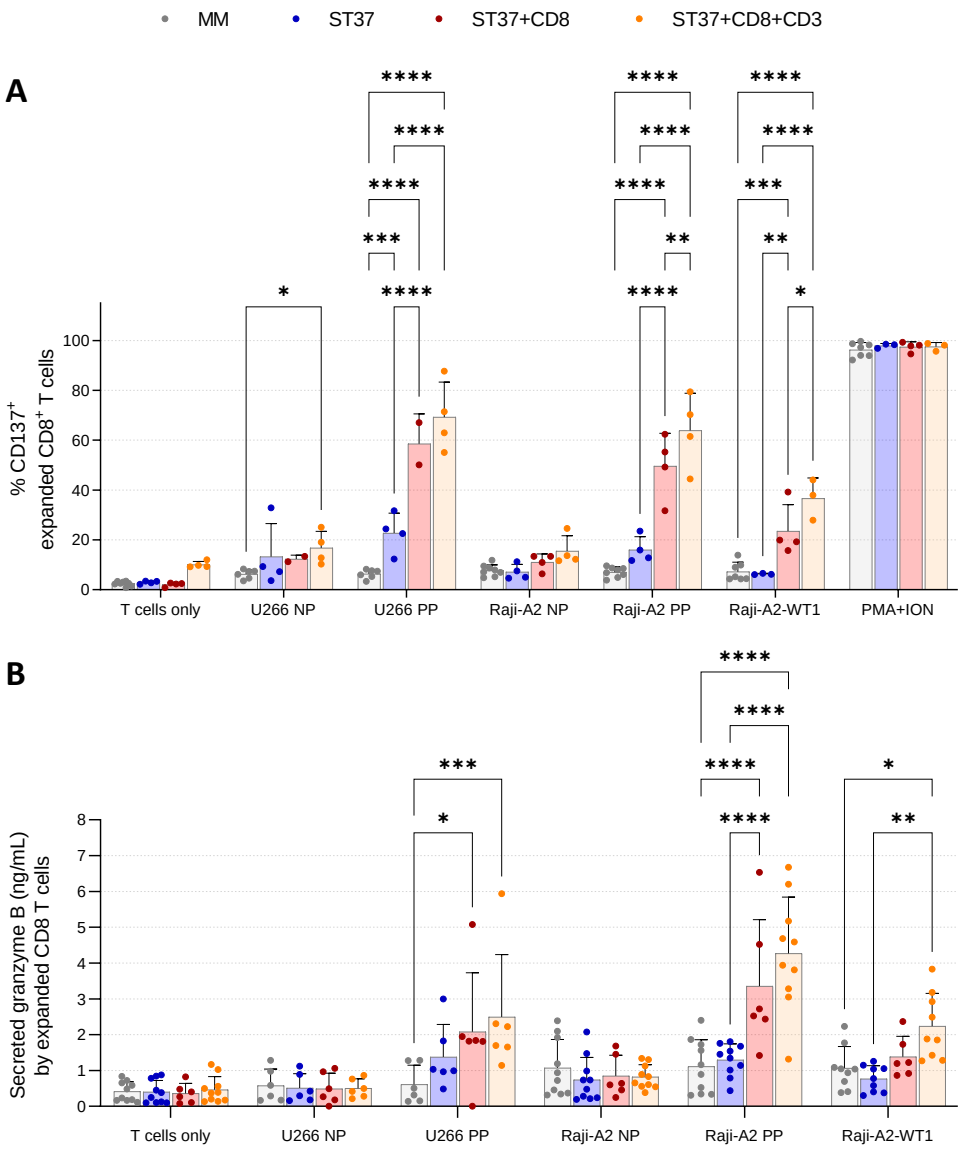



**Figure 7. Antigen-specific functional activity in expanded primary T37 TCR-redirected CD8 T cells in response to WT1-specific recognition.** (**A**) Upregulation of CD137 activation marker was analyzed 24 h after start of co-cultures of T37 TCR-redirected T cells and tumor cell lines that were pulsed with $WT1_{37\text{-}45}$ peptide (PP) or left unpulsed (NP). (**B**) Secretion of granzyme B was analyzed in supernatants from the same co-cultures using ELISA. As a negative control, T cells were cultured in the absence of target cells (T cells only). As a positive control, T cells were cultured in the presence of phorbol 12-myristate 13-acetate and ionomycin (PMA+ION). Graphs represent mean ± SD values for 3-10 independent donors and were analyzed using two-way ANOVA followed by Tukey's multiple comparison test (only statistically significant differences between groups are shown; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$). MM, double sequential mock electroporation; S, DsiRNA against *TRAC* and *TRBC*; T37, $WT1_{37\text{-}45}$ *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with *TCR* mRNA; +CD3, *CD3* mRNA co-electroporated with *TCR* and *CD8* mRNA.

# Discussion

The relevance of TCR-T cells as a therapeutic ally in the fight of cancer is gaining momentum as a robust complementary strategy to specifically target internally processed tumor antigens. Compared to CAR-T-cell therapies, which make use of immune receptors that are artificially designed, TCR-T-cell therapies take advantage of the natural TCR repertoires that are able to recognize TAAs. However, natural TCR repertoires are heavily edited in the thymus, and self-reactive T-cell clones are eliminated, thus limiting the amount of T cells able to recognize overexpressed TAAs, such as WT1, that can also be found in normal tissues, albeit at a lower level. Those that remain after negative selection of self-reactive T cells are usually characterized by low affinity towards the cognate peptide [5]. The limited repertoire of high-affinity TCRs against TAAs that also are self-antigens led investigators to artificially increase the affinity of TAA-reactive TCRs [5]. However, affinity-enhanced TCRs targeting tumor antigens have been linked to supraphysiological TCR affinities and unpredictable on-target off-tumor reactivities that have led to treatment-related fatalities [28]. Therefore, we sought to find a strategy that could improve TCR binding and anti-leukemic T-cell functionality using non-viral RNA-based methods. One of the key parameters that affect TCR binding is TCR avidity [5]. Contrary to TCR affinity, which is a direct measure of the capability of a TCR to bind its cognate peptide presented on MHC molecules, different factors may affect TCR avidity. These factors include the expression levels of the TCR itself and TCR co-receptors CD3 and CD8. Moreover, TCR mispairing and competition for TCR co-receptors between native and transgenic TCRs in TCR-engineered T cells reduce transgenic TCR expression and functionality. Our group recently developed a protocol for efficient, non-viral, and non-genotoxic genetic engineering of CD8 T cells [22]. This DSE protocol is based on the transfection of DsiRNAs for targeting native wild-type TCR sequences, followed by the transfection of a codon-optimized TAA-specific *TCR* mRNA. Therefore, the DSE protocol allows the production of TCR$\alpha\beta$-transgenic CD8 T cells while minimizing TCR$\alpha\beta$ mispairing by reducing the expression of endogenous TCRs. In this article, we show that the DSE protocol can also be successfully used for RNA-based TCR-engineering of expanded CD4 T cells, and less efficiently of resting CD4 T cells, with reduced mispairing. However, TCRs of low affinity are usually CD8 dependent, meaning that introduction of these TCRs into CD8-negative cells such as CD4 and $\gamma\delta$ T cells translates into poor T-cell functionality. Thus, different engineering strategies have addressed this issue by co-introducing CD8 when this TCR co-receptor is not present in the engineered immune cells, mostly using viral vectors [15,18,29]. Importantly, CD8 co-receptor can be formed by CD8$\alpha\alpha$ homodimers or by CD8$\alpha\beta$ heterodimers, but only the latter can positively

influence antigen recognition of CD8-dependent TCRs in low avidity CD8 T-cell clones [30]. As observed by Zhao *et al*., both CD8$\alpha$ and CD8$\beta$ chains are required for CD4 T cells engineered with tumor-specific TCR of intermediate affinity to achieve tumor-cell recognition at similar levels of those of CD8 T cells [15]. Otherwise, this can only be achieved by artificially enhancing TCR affinity [31]. These observations are in line with our results, where only TCR-engineered CD4 T cells co-electroporated with mRNA encoding for CD8$\alpha$ and $\beta$ chains were capable of recognizing WT1 epitope-loaded tumor cells, compared to those not transfected with *CD8* mRNA. Unfortunately, recognition of intracellularly processed WT1 peptide was still elusive, pointing at the need of greater TCR affinities and/or avidities to achieve tumor recognition in this type of setting. Nevertheless, MHC class I-restricted TCR-engineered and CD8-engineered expanded CD4 T cells exhibited an antigen-specific cytotoxic profile, as evidenced by secretion of granzyme B, a surrogate marker for cell-mediated cytotoxicity. Others have shown that MHC class I-restricted TCR-engineered CD4 T cells can display an effector and helper profile [32]. Indeed, Ray *et al*. reported that redirection of CD4 T cells with MHC class I-restricted TCRs elicited both cytotoxic and helper in vitro activity against melanoma [32], which reflects the versatility and relevance of these cells for TCR-T-cell therapies. Our aim was to analyze the relevance of de novo expression of CD8$\alpha\beta$ together with transgenic TCR in improving transgenic TCR avidity to the point of recognizing endogenously processed WT1 peptides; thus, additional research of our $WT1_{37\text{-}45}$ TCR-engineered CD4 T cells will be required to assess whether these cells also exert WT1-specific helper activity. In addition, it remains to be investigated whether Forkhead box P3 (FoxP3)-positive regulatory T cells (Tregs) are present in bulk anti-CD3 and anti-CD28 antibody-expanded CD4 T-cell populations and the potential immunosuppressive effect that these may have. Tregs are commonly identified as CD127-negative and CD25-positive CD4 T cells, and defined by high intracellular expression of FoxP3 transcription factor. CD25, or IL-2 receptor $\alpha$, is also considered a late activation marker in non-regulatory CD4 T cells, upregulated approximately 24 h after T-cell stimulation [33]. CD127, or IL-7 receptor $\alpha$, is a receptor at the heart of maintaining T-cell homeostasis, especially in relation to TCR-signaling [27]. Due to the limited availability of IL-7 in vivo, expression of CD127 is tightly controlled, also after TCR stimulation, inducing CD127 downregulation to maximize IL-7-dependent T-cell survival [27]. Interestingly, expression of CD127 inversely correlates with FoxP3 expression, both in the presence or absence of CD25 [34]. Transient upregulation of FoxP3 can occur in activated CD4 and CD8 T cells, accompanied by a transient immunosuppressive profile [35]. Moreover, while CD127 is downregulated in all CD4 T-cell subsets following activation, only actual Tregs remain CD127-negative [34]. Our data show that CD25 upregulation and CD127 downregulation only occur upon TCR-

mediated CD4 T-cell activation; however, TCR-engineered CD4 T cells still remain CD127-positive. This indicates that CD127 downregulation is most likely due to antigen-specific stimulation and not conversion to an immunosuppressive Treg phenotype. Nevertheless, additional evaluation of the downregulation of CD127, as well as expression of FoxP3, in TCR-engineered CD4 T cells will be required to elucidate whether these cells show an immunosuppressive profile that would be undesirable in TCR-T-cell cancer therapies.

Compared to CD4 T cells, the usage of γδ T cells in TCR-redirected cellular cancer immunotherapies is even more recent, with a handful of studies evaluating this approach [19,36-39], of which only one has made use of RNA-based methods for γδ T-cell engineering [39]. Consistent with their MHC-independent antigen recognition, the majority of γδ T cells either do not express CD4 or CD8 co-receptors [40]. Some CD8-positive γδ T-cell subpopulations have been observed in higher frequencies in intestinal epithelial tissue [41] and bone marrow grafts from cytomegalovirus (CMV) seropositive individuals [42]. In particular, γδ T cells expressing CD8αα co-receptor have been linked to anti-leukemia reactivities after CMV reactivation post allogeneic hematopoietic cell transplantation [43]. The CD8αα co-receptor appeared to have a co-stimulatory effect in leukemia-reactive Vδ1 TCRγδ T cells [43]. However, the existence of a subpopulation of CD8αβ-positive γδ T cells has been only reported in patients with inflammatory bowel disease, inversely correlating with degree of disease activity [44]. Since the majority of γδ T cells in peripheral blood pertain to the CD8-negative Vδ2 subset, introduction of CD8 in combination with low or intermediate affinity TCRs is, thus, still required to achieve antigen-specific tumor reactivities. Due to their MHC-independent intrinsic antitumor properties, γδ T cells are a very attractive T-cell subset to be exploited for TCR-T-cell therapies. In general, γδ T cells showed potent cytotoxic activity against WT1 peptide-loaded target cells at a single-cell level via flow-cytometric analysis. However, no significant differences were observed in terms of granzyme B secretion. This suggests that alternative granzyme B-independent cytotoxic mechanisms may be taking place. Potential mechanisms include the activation of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) pathways [45]. Similar to CD4 T cells, despite the recognition and killing of WT1 peptide-loaded target cell lines, TCR and CD8-enginered γδ T cells were not capable of killing WT1-expressing cells, neither those engineered to express WT1 (*WT1* mRNA-electroporated U266 cells and Raji-A2-WT1) nor those known to express the tumor antigen naturally (K562 and THP1). Nonetheless, the fact that CD8-engineered γδ T cells were not capable of inducing tumor cell killing of WT1-positive cell lines in the presence of WT1-specific TCR suggests that, as seen with CD4 T cells, further

improvement of TCR avidity may be required when using low or intermediate tumor antigen-specific TCRs in this context.

Considering the results obtained from CD4 and γδ T cells, we hypothesized whether upregulation of both CD3 and CD8 co-receptors in WT1-specific TCR-engineered TCR$\alpha\beta$-positive CD8 T cells would mediate better TCR avidities compared to T cells only modified with the WT1-specific TCR or together with *CD8* mRNA. In terms of transfection efficiency of co-electroporated *TCR*, *CD3*, and *CD8* mRNAs, measured by percentage of tetramer-positive CD8 T cells for WT1-specific *TCR* mRNA and by GMFI levels for *CD3* and *CD8* mRNAs, no detrimental effects were observed in WT1-specific TCR and CD3 expression. However, CD3 levels were significantly downregulated when the three mRNA were introduced. The *CD3* mRNA had a final length of approximately 3700 base pairs, greatly exceeding that of WT1-specific *TCR* and *CD8* mRNAs used in our study (~2800 base pairs both). This fact could have had a negative effect in transfection efficiency of *CD3* mRNA, as larger transcripts may have more difficulties in diffusing through the pores formed during electroporation, but we did not detect any deleterious effect in CD3 levels. Moreover, others have reported the use of a combination of various mRNA for the electroporation of dendritic cells without notable detrimental effects on protein expression [46,47]. We did see a decrease in CD8 surface levels in ST37+CD8+CD3-engineered CD8 T cells; however, contradictorily, increasing concentrations of *CD3* mRNA led to an increase in CD8 expression. Another factor to consider is steric hindrance between the different antibodies and tetramers used during staining, a well-known issue in flow cytometry that is not usually taken into account [48]. Regarding antigen-specific tumor recognition, we observed a remarkable increase in antigen-specific T-cell activation, via upregulation of the activation marker CD137, and CD8 T-cell cytotoxic capabilities, via granzyme B secretion, in T37+CD8 and T37+CD8+CD3-engineered CD8 T cells compared to CD8 T cells only transfected with *TCR* mRNA. This suggests that, indeed, the upregulation of TCR co-receptors positively affected TCR functional avidity. However, only the combination of *TCR*, *CD8*, and *CD3* mRNAs were capable of inducing the recognition of WT1-positive (unpulsed) tumor cells, which indicates the central role that CD3 plays in stabilizing transgenic TCRs on the cell membrane. Interestingly, increasing concentrations of CD3 did not significantly impact transgenic TCR levels—or antigen-specific T-cell activation—probably due to already TCR maximal expression in expanded CD8 T cells.

# Conclusion

The transient nature of RNA-based methods for TCR engineering has traditionally favored the employment of stable methods of genetic engineering, usually based on viral vectors. Transposons and CRISPR-Cas9-based strategies have opened the way for stable, non-viral and more targeted genome engineering. However, RNA-based protocols will still hold a place in the TCR-engineering domain as a system for rapid testing of newly developed TCRs. Moreover, the transient nature of this system allows reverting to the original state of the engineered T cells in case of TCR-T-mediated toxicities caused by allogeneic TCRs, as can happen with the previously mentioned affinity-enhanced TCRs. We successfully generated MHC class I-restricted TCR-engineered CD8 T cells, CD4 T cells and γδ T cells by co-transfection of *TCR* with *CD8* mRNA alone or together with *CD3* mRNA without loss of transgenic TCR expression, and reduced mispairing in the case of αβ T cells. We provide a non-viral RNA-based engineering protocol to increase the functional avidity of TCR-engineered T cells by upregulation of TCR co-receptors. Engineered CD4 and γδ T cells showed tumor recognition when co-transfecting *TCR*-encoding mRNA with *CD8* mRNA, which was also replicated in CD8 T cells. In the case of CD8 T cells, co-introduction of *CD3* mRNA further improved functional avidity and antigen-specific antitumor activity, being able to detect and respond to the intracellularly processed WT1 epitope. Therefore, our study highlights the importance of TCR co-receptors in the context of TCR-T-cell therapies based on TCRs derived from natural repertoires against tumor self-antigens. The proposed electroporation protocol could be valuable as a stand-alone improved TCR-T-cell therapy against WT1 and for screening potential transgenic TCR cross-reactivities in early-phase clinical trials.

# Materials and methods

### T-cell isolation and expansion

Blood samples from healthy anonymous donors were purchased from the Blood Service of the Flemish Red Cross (Mechelen, Belgium) following the approval by the Ethics Committee of the Antwerp University Hospital and the University of Antwerp (reference number 16/35/357). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). CD4 and CD8 T cells were positively selected using CD4 or CD8 magnetic microbeads for magnetic-activated cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotec). Subsequently, CD4 and CD8 T cells were used either resting (no treatment) or were cryopreserved. For cryopreservation, T cells were centrifuged and resuspended in a cryopreservation medium consisting of fetal bovine serum (FBS; Life Technologies) supplemented with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich). Aliquots of 20–35 × $10^6$ cells/mL were transferred to freezing containers and kept in a −80°C freezer before transferring to liquid nitrogen containers. Aliquots were thawed in pre-warmed

Iscove's Modified Dulbecco's Medium (IMDM; Gibco Invitrogen) supplemented with 5% human AB serum (hAB; Life Technologies) and rested for at least one hour in a humidified 5% $CO_2$ incubator at 37°C. CD4 and CD8 T cells were expanded with purified no azide low endotoxin (NALE) plate-bound anti-human CD3 and soluble anti-human CD28 monoclonal antibodies (mAbs; BD Biosciences). Briefly, cultured flasks were incubated for 2 hours (h) at 37°C with NALE CD3 mAb diluted in phosphate-buffered saline (PBS; Gibco Invitrogen) at a concentration of 5 μg/mL. After incubation, flasks were washed with PBS. Subsequently, CD4 or CD8 T cells were resuspended in IMDM supplemented with 5% hAB, 50 IU/mL (5 μL/mL) recombinant human (rh) interleukin (IL)-2 (10 IU/μL; ImmunoTools) and 10 ng/mL (10 μL/mL) rhIL-15 (1 ng/μL; ImmunoTools). On day 0, 1 μg/mL NALE CD28 mAb was added to culture media. Cells were passaged every 2-3 days with cytokine-supplemented medium and were incubated for 1 or 2 weeks before use. The purity of CD4 and CD8 was analyzed by staining with anti-human CD3-peridinin-chlorophyll-Protein (PerCP), CD4-R-phycoerythrin (PE) and CD8-fluorescein (FITC) mAbs (BD Biosciences). CD8 T cells were additionally phenotyped at every passage to analyze differentiation subsets, using CD8-PerCP, CD45RA-FITC and C-C chemokine receptor type 7 (CCR7)-PE (BD Biosciences), and upregulation of CD69 and CD137 activation markers, using anti-human CD8-PerCP, CD69-FITC and CD137-PE mAbs (BD Biosciences). γδ T cells were expanded from PBMC with 1 μL/mL (5 mM) zoledronic acid (StemCell Technologies), 10 μL/mL rhIL-2 (10 IU/μL), and 10 μL/mL rhIL-15 (1 ng/μL) in IMDM supplemented with 5% hAB. Cells were passaged every 2-3 days with cytokine-supplemented medium and were incubated for 2 or 3 weeks before use. The purity of γδ T cells was analyzed by staining with anti-human TCRγδ-FITC, CD3-PerCP and CD8-PE mAbs or with TCR Vδ2 mAb (BD Biosciences). Purity values of 80% or higher were considered acceptable for expanded γδ T cells. Samples were measured on a CytoFLEX flow cytometer (Beckman Coulter).

### Cell lines

The HLA-A*02:01-positive WT1-negative human lymphoblastoid T2 cell line, with a deficiency in transporter associated with antigen presentation (TAP), was kindly provided by Dr. Pierre Van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium). The HLA-A*02:01-positive, WT1-negative multiple myeloma cell line U266 was kindly provided by Dr. Wilfred T.V. Germeraad (GROW School for Oncology & Developmental Biology, Maastricht University, Maastricht, The Netherlands). The HLA-A*02:01-transduced Burkitt's lymphoma Raji-derived Raji-A2 cell line and the HLA-A*02:01- and WT1-transduced Raji-A2-WT1 cell line were kindly provided by Dr. Mirjam Heemskerk (Leiden University Medical Center, Leiden, The Netherlands). The HLA-A*02:01-transduced WT1-positive human chronic myelogenous leukemia K562-derived K562-A2 cell line was a kind gift from Dr. Cedrik Britten (R&D Oncology, GlaxoSmithKline, Stevenage, UK). The HLA-A*02:01-positive WT1-positive human monocytic THP1 cell line was purchased from ATCC. The HLA-A*02:01-positive WT1-positive human B-cell precursor leukemic BV173 cell line was a kind gift of Dr. Hans Stauss (Institute of Immunity and Transplantation, University College London, London, UK). The HLA-A*02:01-positive WT1-positive Epstein–Barr virus (EBV)-immortalised B-cell lymphoblastoid JY (JY-EBV) cell line was a kind gift of Dr. Stefan Stevanovic (Institute for Immunology, University of Tübingen, Tübingen, Germany). T2 and K562-A2 cell lines were maintained in IMDM supplemented with 10% FBS. All Raji-derived, U266, THP1, BV173 and JY-EBV cell lines were maintained in Roswell Park Memorial Institute 1640 (RPMI) culture medium (Gibco Invitrogen) with 10% FBS. All cell lines were maintained in logarithmic growth phase at 37°C in a humidified atmosphere supplemented with 5% $CO_2$.

### mRNA synthesis

pST1 DNA plasmids containing HLA-A*02:01-restricted $WT1_{37-45}$-specific TCRβ-P2A-TCRα and HLA-A*02:01-restricted $WT1_{126-134}$-specific TCRβ-P2A-TCRα constructs were obtained as previously described [22]. pST1 DNA plasmids containing full-length WT1 [49], CD8α-P2A-CD8β and CD3δ-F2A-CD3γ-T2A-CD3ε-E2A-CD3ζ constructs were obtained from GeneArt (Life Technologies). SoloPack Golden super-competent *E. coli* cells were transformed with pST1 DNA plasmids according to the manufacturer's instructions. Transformed *E. coli* cells were cultured in Luria-Bertani (LB)-kanamycin agar plates and incubated overnight at 37°C and amplified in LB-kanamycin cultures at 37°C under constant motion. Plasmid DNA isolation and purification from bacterial cells were performed using the Nucleobond Xtra Midi EF and Nucleobond finalizer kits (Macherey-Nagel). Next, plasmid DNAs were digested with Sap-I restriction enzyme (Thermo Fisher Scientific) for 16 h at 37°C. Capped in vitro transcribed (IVT) mRNAs were synthesized from linearized plasmids and purified by DNase digestion and LiCl precipitation using a mMessage mMachine T7 or a mMessage mMachine T7 Ultra in vitro transcription kit (Life Technologies) following the manufacturer's recommendations.

### RNA electroporation

Double sequential electroporation of human primary CD4 and CD8 T cells with WT1-specific *TCR* mRNA was performed as previously described [22]. Briefly, 10-20 × $10^6$ fresh or thawed viable human primary CD4 or CD8 T cells were resuspended in 200-400 µL of serum-free Opti-MEM I reduced serum medium (Gibco Invitrogen) and transferred to a 4.0-mm electroporation cuvette (Cell Projects). Next, cells were electroporated with 16-32 µL of a pool containing 100 µM of T-cell receptor alpha constant (*TRAC*)- and T-cell receptor beta constant (*TRBC*)-specific DsiRNAs (Integrated DNA Technologies) in a ratio of 1:1. After electroporation, cells were transferred to pre-warmed IMDM medium supplemented with 10% hAB, rested at 37°C in a humidified atmosphere supplemented with 5% $CO_2$ for at least 20 min, centrifuged (300×*g*, 3 min), transferred to 6-well plates, and then incubated for 24 h. Second electroporation with in vitro transcribed *TCR*, *CD8* and/or *CD3*-encoding mRNA was performed following the same protocol, using 1 µg of IVT mRNA per mRNA and per $10^6$ cells. For single electroporation with mRNA (γδ T cells with *TCR* and/or *CD8*-encoding mRNA and U266 cells with full-length *WT1*-encoding mRNA), 5-10 × $10^6$ viable cells were washed once in cold serum-free Opti-MEM I reduced serum medium, resuspended in 200 µL of the same medium and transferred to a 4.0-mm electroporation cuvette (Cell Projects). Next, 1µg of IVT mRNA per mRNA and per $10^6$ cells was added to the cuvette. Electroporations were performed in a Gene Pulser Xcell™ device (Bio-Rad Laboratories). For T cells, a square wave protocol (500 V, 5 ms, 0 gap, 1 pulse) was used. For U266 cells, a time constant protocol (300 V, 8 ms, 1 pulse) was used. As a negative control, cells were electroporated under the same conditions without the addition of any RNA ("mock"). Immediately after electroporation, cells were transferred to 10 mL of IMDM medium supplemented with 10% hAB (γδ T cells) or IMDM medium supplemented with 10% FBS (U266 cells) and rested for at least 20 min in a humidified 5% $CO_2$ incubator at 37°C.

### Analysis of transgenic TCR, CD8, and CD3 surface expression

γδ T cells were harvested after electroporation and stained with the following mAbs: anti-human anti-pan TCRαβ-PE (Miltenyi Biotec), CD3-PerCP, CD8-FITC for 15 min at room temperature. After washing, samples were resuspended in 200 µL of FACS buffer (FACSFlow sheath fluid, BD Biosciences; 0.1% bovine serum albumin (BSA), Sigma-Aldrich; 0.05% sodium azide, Merck) and measured on a CytoFlex flow cytometer. CD4 and CD8 cells were incubated with $WT1_{37–45}$/HLA-A*02:01 tetramer-allophycocyanin (APC) or $WT1_{126–134}$/HLA-A*02:01 tetramer-PE (monomers kindly provided by Dr. David A. Price, Division of Infection and Immunity, Cardiff University

School of Medicine, Cardiff, UK) for 30 min at 37°C, washed and stained with anti-human CD4- or CD8-FITC and, CD3-PerCP mAbs for 15 min at room temperature. Alternatively, CD4 T cells were stained with TCR Vβ21.3 (Miltenyi Biotec) instead of tetramers. After washing, cells were resuspended in 200 μL of FACS buffer for flow cytometric analysis using a CytoFLEX flow cytometer.

### RT-qPCR analysis

Twenty-four hours after one or two electroporations, total RNA was extracted from primary resting CD4 T cells using RNeasy Micro kit (QIAGEN), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthetized by reverse transcription from total RNA samples using iScript cDNA synthesis kits (Bio-Rad) and diluted in water to a final concentration of 5 ng/μL. Real-time PCR reactions were performed in duplicate or quadruplicate on a CFX96TM real-time PCR detection system(Bio-Rad) using SsoAdvanced TM Universal SYBRR Green Supermix (Bio-Rad) and PrimePCR primers (Bio-Rad) to detect and quantify the relative abundance of *TCR* constant region mRNA (*TRAC*; forward primer: 5′-CTGTCTGCCTATTCACCGATT-3′, reverse primer: 5′-GTCAGATTTGTTGCTCCAGG-3′) and T-cell receptor beta constant region mRNA (*TRBC*; forward primer: 5′-GGTGAATGGGAAGGAGGTG-3′, reverse primer: 5′-GTATCTGGAGTCATTGAGGGC-3′) transcripts. Importin-8 (*IPO8*, Hs.505136) and ribosomal protein L13A (*RPL13A*, Hs.523185) were chosen as reference genes [50]. Results were analyzed using CFX Manager (v3.1, Bio-Rad).

### Peptide pulsing of target tumor cells

Viable target cell lines were harvested, washed once in serum-free IMDM medium, and resuspended using the same medium at a final concentration of $10^6$ cells/mL. Cells were split in tubes and pulsed with $WT1_{37–45}$ (VLDFAPPGA), or $WT1_{126–134}$ peptide (RMFPNAPYL) where applicable, (JPT Peptide Technologies) at a concentration of 10 μg/mL for 60 min at room temperature under constant motion. After incubation, cells were washed and resuspended in IMDM supplemented with 5% hAB.

### Cytotoxicity assay

The killing capacity of RNA-engineered human primary T cells against peptide-pulsed or unpulsed target tumor cells was determined using a flow cytometry-based protocol as described previously, with minor modifications [51]. Briefly, before co-culture, tumor cells were stained with PKH67 green fluorescent cell linker dye (Sigma- Aldrich) according to the manufacturer's protocol. PKH67-positive tumor cells were peptide pulsed as described above. Next, target tumor cells were cultured alone or with electroporated human primary T cells for 4 h at an effector-target ratio of 5:1. After co-culture, samples were stained with propidium iodide (PI) and APC-labeled annexin V (BD Biosciences). Samples were analyzed using a FACSAria II flow cytometer (BD Biosciences). Cytotoxicity was calculated based on the survival of PKH67-positive target tumor cells using the following equation:

$$\%\ Cytotoxicity = 100 - \left[\left(\frac{\%\ Annexin\ V^{-}PI^{-}target\ cells\ co\text{-}cultured\ with\ T\ cells}{\%\ Annexin\ V^{-}PI^{-}target\ cells\ co\text{-}cultured\ without\ T\ cells}\right) x\ 100\right]$$

### Flow cytometric analysis of activation markers

For the analysis of upregulation of antigen-specific activation markers, CD4 and CD8 T cells were co-cultured with peptide-pulsed or unpulsed target cells at an effector-target ratio of 4:1 and incubated for 18-20 h at 37°C and 5% $CO_2$. After incubation, supernatants were collected for analysis of cytokine secretion. CD4 T cells were stained with anti-human CD127-FITC (Miltenyi),

CD154-PE (BioLegend), CD69-APC-Cy7 (BD Biosciences), CD8-Pacific blue (Life Technologies), CD137-PerCP-Cy5.5 (BioLegend), CD4-PE-Cy7 (BioLegend), and CD25-APC (BioLegend) mAbs for 30 min at 4°C. Cells were washed and analyzed using a FACSAria II flow cytometer. CD8 T cells were stained with anti-human CD3-PerCP (BD Biosciences), CD8-PE-Cy7 (BioLegend), CD69-FITC (BD Biosciences) and CD137-PE (BD Biosciences) mAbs for 30 min at 4°C. Cells were washed and analyzed using a CytoFLEX flow cytometer.

### Cytokine secretion assays

Secretion of granzyme B by electroporated human primary T cells was determined by enzyme-linked immunosorbent assay kits (ELISA; R&D Systems) following manufacturer's instructions in supernatants of co-cultures used for the analysis of activation markers (CD4 and CD8 T cells) or of cytotoxicity (γδ T cells). All ELISA plates were measured using a Victor 3 multilabel plate reader (Perkin Elmer).

### Statistical analysis

Flow cytometry data were analyzed using FlowJo software (v10.2, TreeStar Inc). Prism software (v9, GraphPad) was used for graphing and statistical calculations. Information on the statistical tests used can be found in captions of figures. Results were considered statistically significant when *P*-value was less than 0.05. * indicates $P < 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$ and **** indicates $P \leq 0.0001$.

# Supplementary material

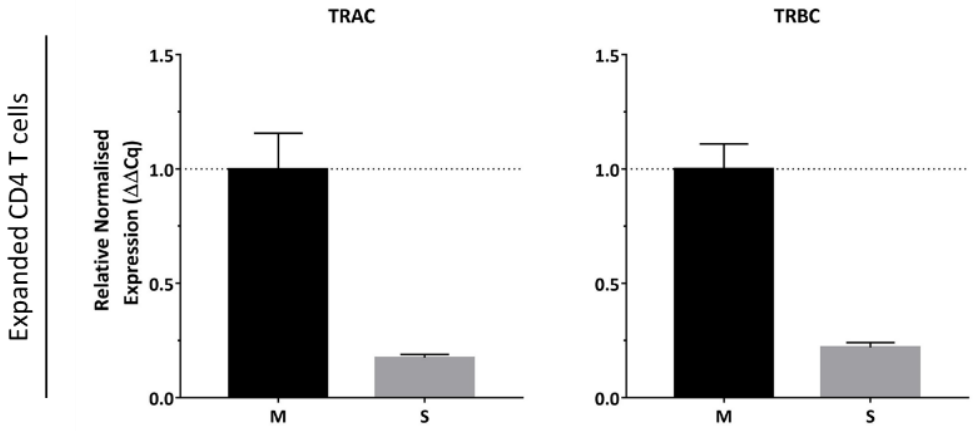



**Figure S1. RT-qPCR analysis of DsiRNA-mediated native TCR silencing capacity in expanded CD4 T cells.** DsiRNA targeting the alpha and beta constant regions of the wild type *TCR* mRNA (*TRAC* and *TRBC*, respectively) significantly downregulate *TRAC* and *TRBC* expression in expanded CD4 T cells 24 h after DsiRNA electroporation. Graphs show the average relative normalized expression (ΔΔCq) ± SEM for 2 donors. M, mock; S, DsiRNAs against *TRAC* and *TRBC* sequences.

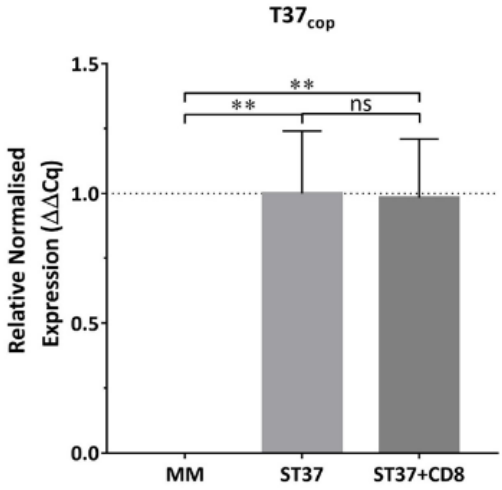



**Figure S2. RT-qPCR analysis of T37 *TCR* mRNA transfection efficiency in double-sequential electroporated resting CD4 T cells.** Codon-optimized T37 *TCR* mRNA was efficiently transfected after DSE with (ST37+CD8) and without (ST37) concomitant electroporation with *CD8* mRNA as shown by the significantly increased in T37 *TCR* mRNA levels in both treatment conditions. Graphs show the average relative normalized expression (ΔΔCq) ± SEM for 6 donors (ns, not significant; **, $P \leq 0.01$). MM, double sequential mock electroporation; S, DsiRNA against *TRAC* and *TRBC* sequences; T37, $WT1_{37\text{-}45}$-specific *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with *TCR* mRNA.

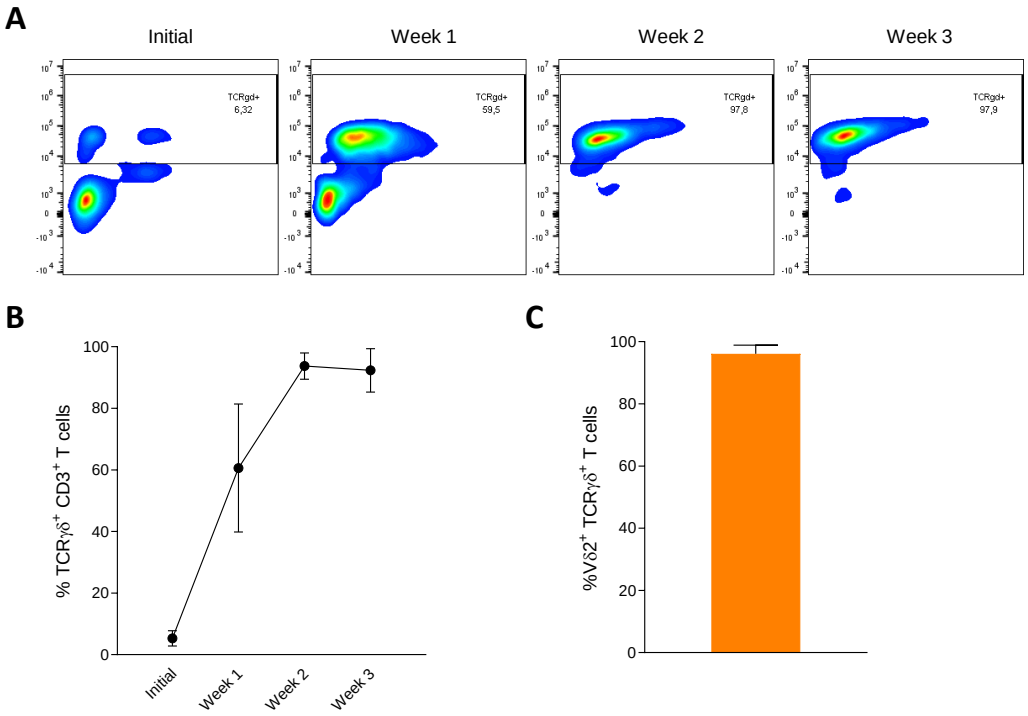



**Figure S3. Purity of γδ T cells expanded with zoledronic acid, interleukin (IL)-2 and IL-15.** (**A, B**) Purity of γδ T cells, represented as % of TCRγδ+CD3+ T cells, was measured during the three weeks of expansion. (**C**) Expanded γδ T cells were phenotyped according to their expression of Vδ2+ TCR chain after expansion. Graphs represent mean ± SD values for 7-12 (**B**) and 3 (**C**) independent donors.

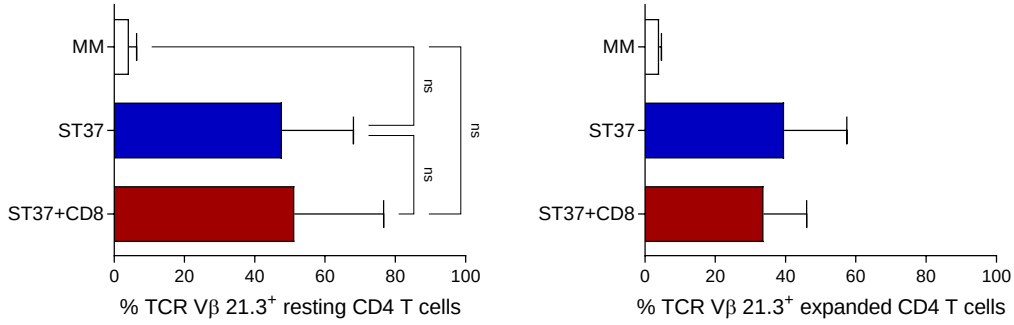



**Figure S4. Transgenic T37 TCR expression in mRNA-electroporated primary CD4 T cells**. T37 TCR expression was measured 24 h after electroporation with WT1$_{37\text{-}45}$-specific *TCR* mRNA with or without *CD8* mRNA by using a monoclonal antibody targeting the TCR Vβ21.3 variant of the TCR β chain. Graphs represent mean ± SD values for 2 (expanded CD4 T cells) or 3 (resting CD4 T cells) independent donors that were analyzed using Kruskal-Wallis test followed by corrected Dunn's multiple comparison test (ns, not significant). MM, double sequential mock electroporation; S, DsiRNA against *TRAC* and *TRBC*; T37, WT1$_{37\text{-}45}$ *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with *TCR* mRNA.

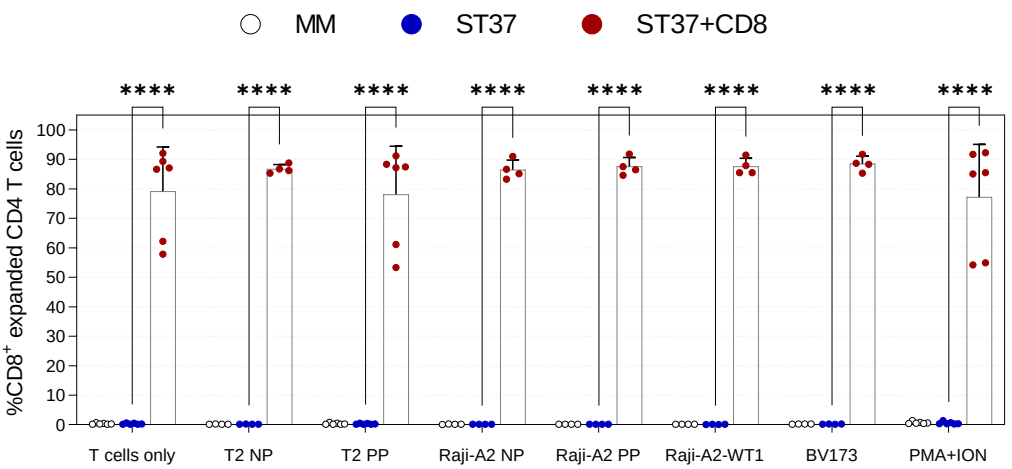
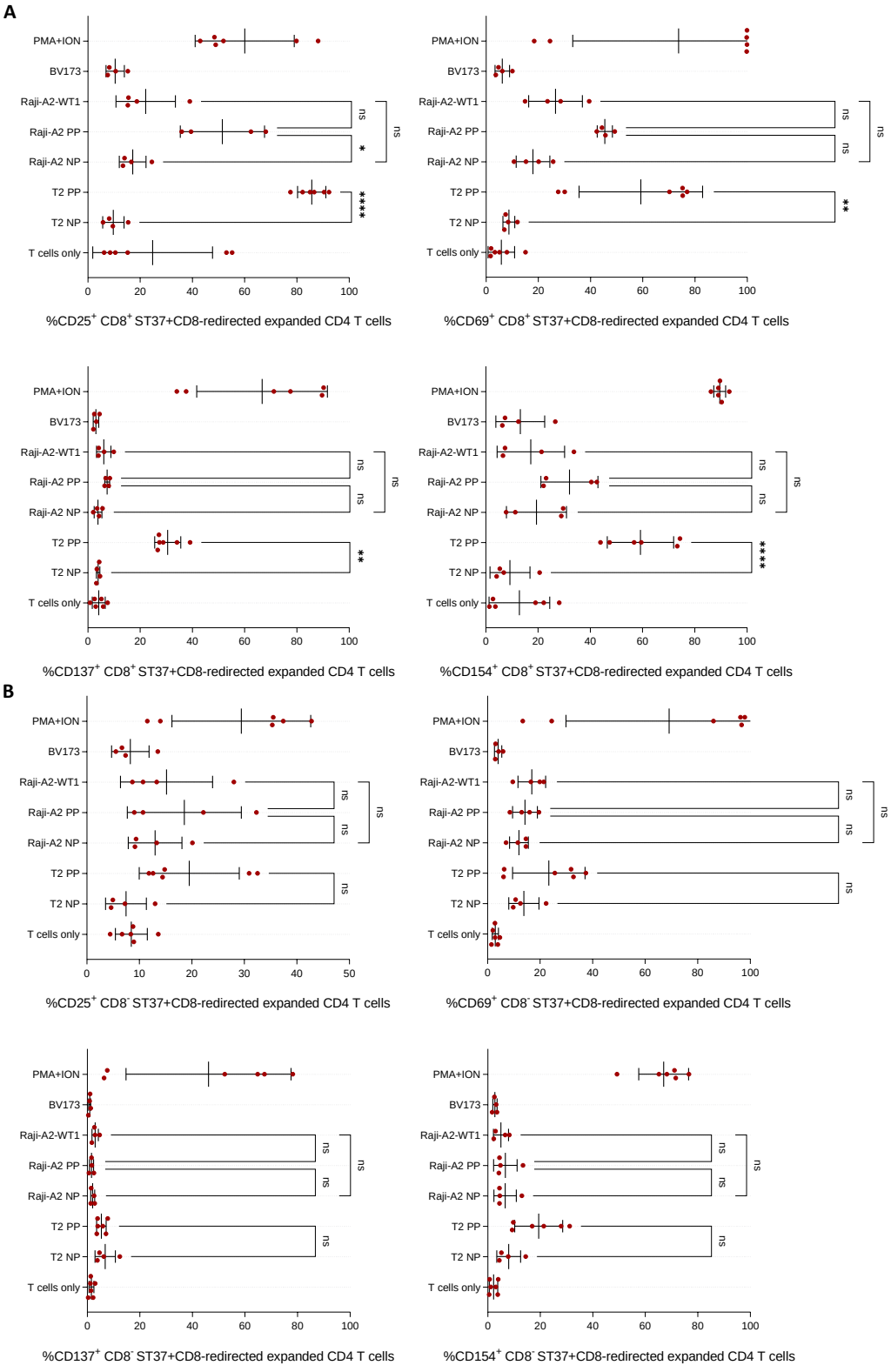



**Figure S5. CD8 expression in expanded primary ST37+CD8-electroporated CD4 T cells.** De novo expression of CD8 in ST37+CD8-electroporated CD4 T cells was measured after co-culture with tumor cell lines that were pulsed with $WT1_{37\text{-}45}$ peptide (PP), left unpulsed (NP). As a negative control, T cells were cultured in the absence of target cells (T cells only). As a positive control, T cells were cultured in the presence of phorbol 12-myristate 13-acetate and ionomycin (PMA+ION). Graphs represent the mean ± SD values for 4-6 independent donors and were analyzed using two-way ANOVA followed by Tukey's multiple comparison test (****, $P \leq 0.0001$). MM, double sequential mock electroporation; S, DsiRNA against *TRAC* and *TRBC*; T37, $WT1_{37\text{-}45}$ *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with the *TCR* mRNA.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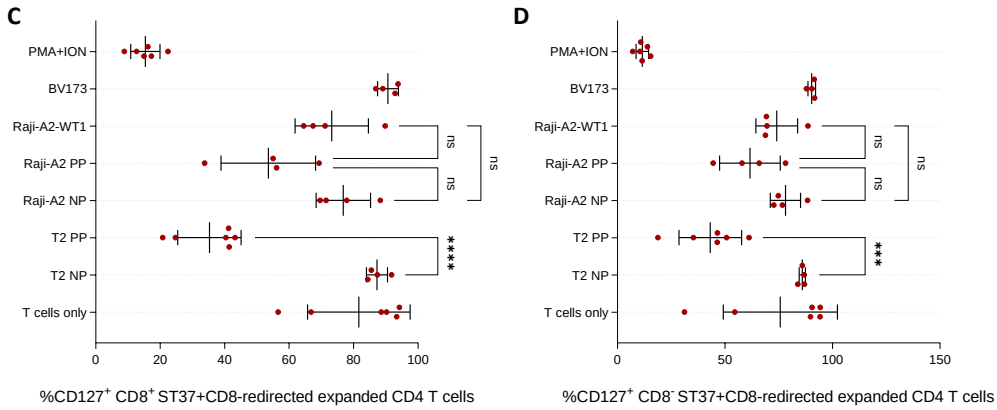


**Figure S6. Expression of activation markers in response to WT1-specific recognition in expanded primary ST37+CD8-electroporated CD4 T cells.** Upregulation of CD25, CD69, CD137, and CD154 expression in CD8-positive (**A**) and CD8-negative (**B**) subpopulations and downregulation of CD127 expression in CD8-positive (**C**) and CD8-negative (**D**) subpopulations of ST37+CD8-electroporated CD4 T cells was measured after co-culture with tumor cell lines that were pulsed with $WT1_{37\text{-}45}$ peptide (PP), left unpulsed (NP). As a negative control, T cells were cultured in the absence of target cells (T cells only). As a positive control, T cells were cultured in the presence of phorbol 12-myristate 13-acetate and ionomycin (PMA+ION). Graphs represent the mean ± SD values for 4-6 independent donors and were analyzed using two-way ANOVA followed by Tukey's multiple comparison test (ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$). S, DsiRNA against *TRAC* and *TRBC*; T37, $WT1_{37\text{-}45}$ *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with the *TCR* mRNA.

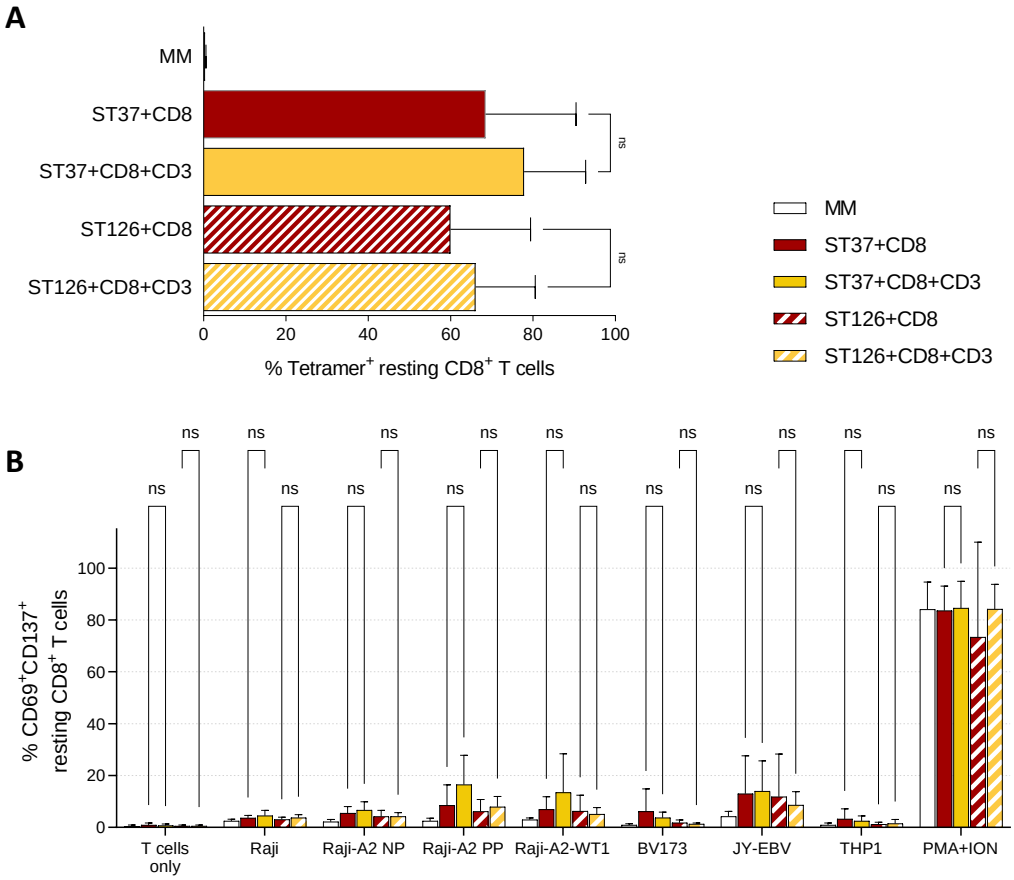


**Figure S7. Transgenic WT1-TCR expression and WT1-specific functionality of RNA-electroporated primary resting CD8 T cells.** (**A**) Transgenic TCR expression was measured 24 h after electroporation with $WT1_{37\text{-}45}$-specific *TCR* mRNA or $WT1_{126\text{-}134}$-specific *TCR* mRNA with *CD3* and *CD8* mRNA. (**B**) After transfection, upregulation of CD69 and CD137 activation markers was measured in T37 TCR-redirected or T126 TCR-redirected resting CD8 T cells 24 h after co-culture with tumor cell lines that were pulsed (PP) with $WT1_{37\text{-}45}$ and $WT1_{126\text{-}134}$ peptides or left unpulsed (NP). As a negative control, T cells were cultured in the absence of target cells (T cells only). As a positive control, T cells were cultured in the presence of phorbol 12-myristate 13-acetate and ionomycin (PMA+ION). Graphs represent the mean values for 6-8 independent donors. Data were analyzed using (**A**) Kruskal-Wallis test followed by corrected Dunn's multiple comparison test or (**B**) two-way ANOVA followed by Tukey's multiple comparison test (ns, not significant). MM, double sequential mock electroporation; S, DsiRNA against *TRAC* and *TRBC*; T37, $WT1_{37\text{-}45}$ *TCR* mRNA; +CD8, *CD8* mRNA co-electroporated with *TCR* mRNA; +CD3, *CD3* mRNA co-electroporated with *TCR* and *CD8* mRNA.

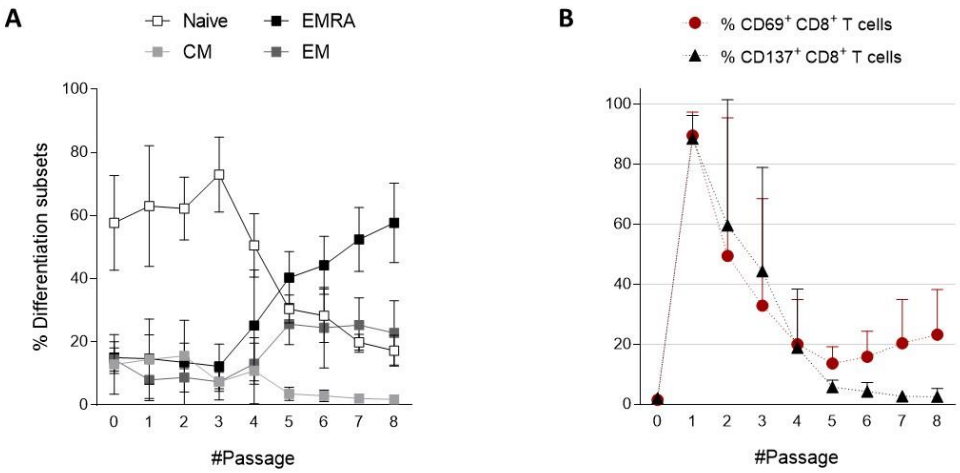


**Figure S8. Kinetics of phenotype of primary human CD8 T cells expanded with plate-bound anti-CD3 monoclonal antibody (mAb), soluble anti-CD28 mAb, interleukin (IL)-2 and IL-15 from isolated and cryopreserved CD8 T-cell samples.** (**A**) Percentage of differentiation subsets based on the expression of CD45RA and CCR7 in expanded CD8 T cells. Expanded CD8 T cells are divided into four differentiation status: naïve ($CD45RA^+$ $CCR7^+$), central memory (CM; $CD45RA^-$ $CCR7^+$), effector memory (EM; $CD45RA^-$ $CCR7^-$) and effector memory terminally differentiated (EMRA; $CD45RA^+$ $CCR7^-$) CD8 T cells. (**B**) Expression of activation markers CD69 and CD137 following activation via anti-CD3 mAb and soluble anti-CD28 mAb. Cells were passaged every 2-3 days. Graphs represent mean ± SD values for 4 independent donors.

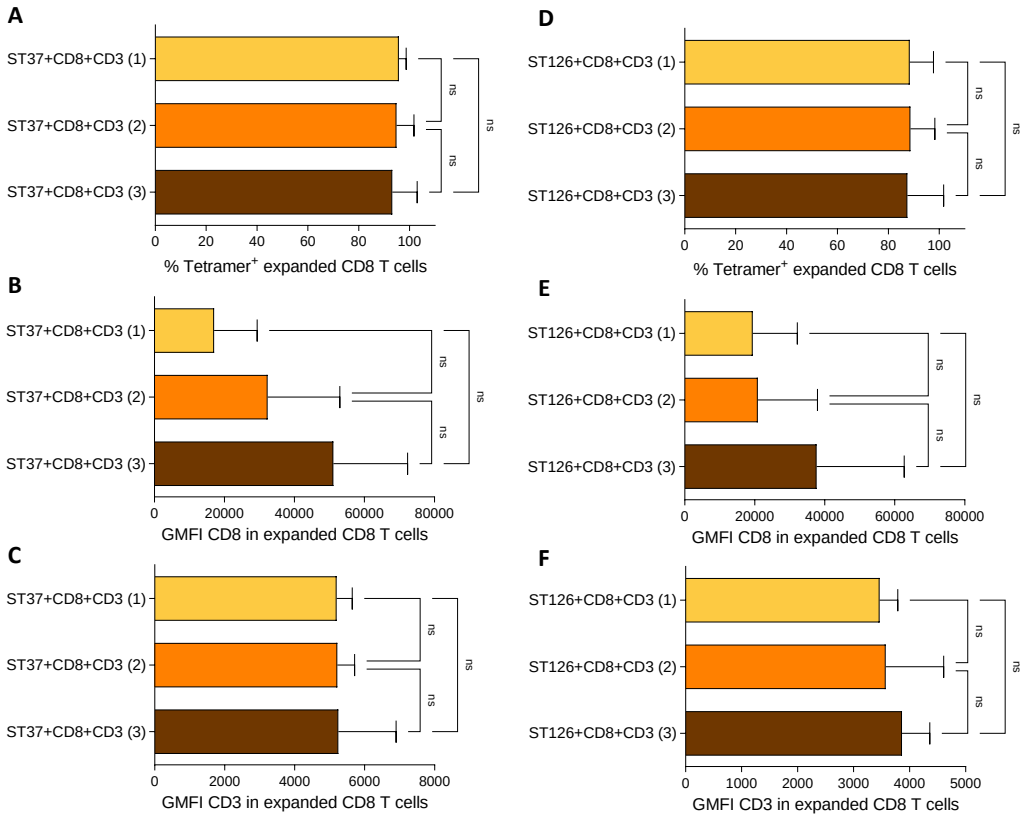


**Figure S9. Transgenic TCR and CD8 expression in mRNA-electroporated primary expanded CD8 T cells**. T37 TCR (**A**), CD8 (**B**), and CD3 (**C**) expression was measured 24 h after electroporation with WT1$_{37\text{-}45}$-specific *TCR* mRNA with *CD8* mRNA and increasing concentrations of *CD3* mRNA. (1) Refers to 1 µg *CD3* mRNA per $10^6$ cells (50 µg mRNA/mL), (2) refers to 2 µg *CD3* mRNA per $10^6$ cells (100 µg mRNA/mL), and (3) refers to 3 µg *CD3* mRNA per $10^6$ cells (150 µg mRNA/mL). T126 TCR (**D**), CD8 (**E**), and CD3 (**F**) expression was measured 24 h after electroporation with WT1$_{126\text{-}134}$-specific *TCR* mRNA with *CD8* mRNA and *CD3* mRNA. (**A**) T37 TCR expression was measured via WT1$_{37\text{-}45}$/HLA-A*02:01 staining and (**D**) T126 TCR expression via WT1$_{126\text{-}134}$/HLA-A*02:01 staining. Graphs represent mean ± SD values for 3-4 independent donors that were analyzed using Kruskal-Wallis test followed by corrected Dunn's multiple comparison test (ns, not significant). GMFI, geometric mean fluorescence intensity; M, mock; S, DsiRNA against *TRAC* and *TRBC*; T37, WT1$_{37\text{-}45}$ *TCR* mRNA; T126, WT1$_{126\text{-}134}$ *TCR* mRNA;+CD8, *CD8* mRNA co-electroporated with *TCR* mRNA; +CD3, *CD3* mRNA co-electroporated with *TCR* and *CD8* mRNA.

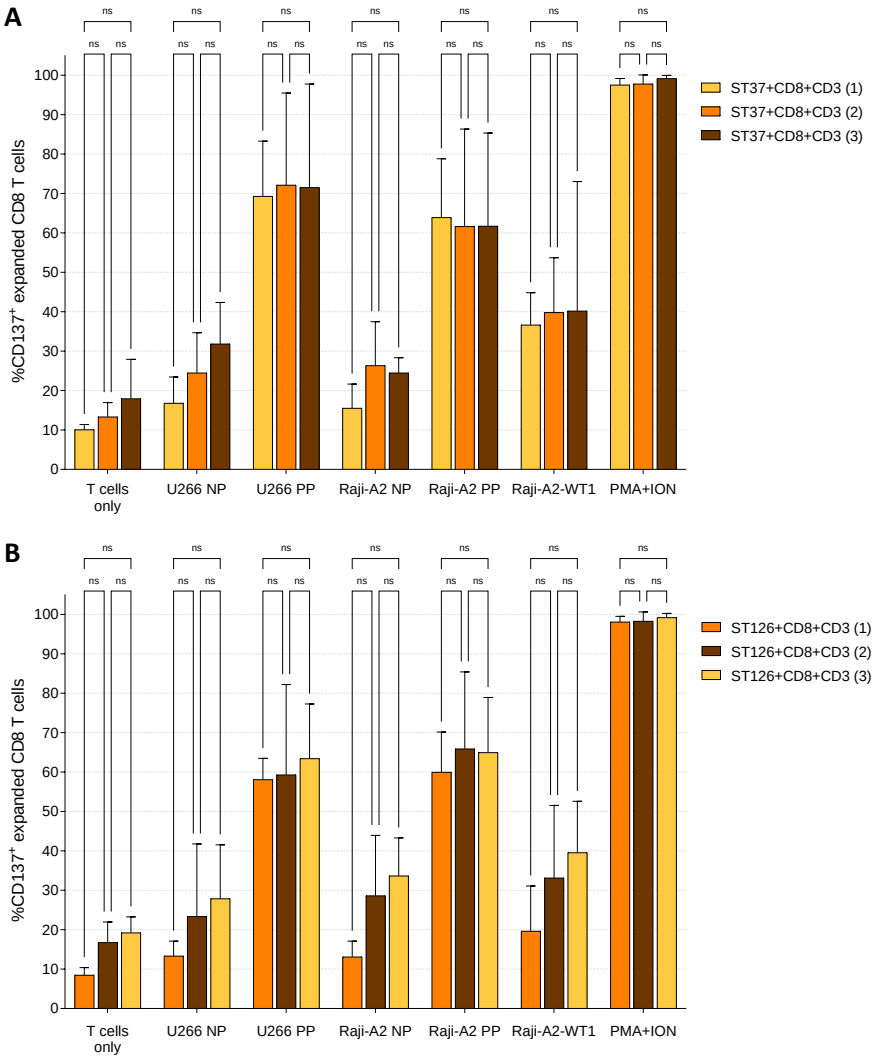


**Figure S10. Expression of activation marker CD137 in response to WT1-specific recognition in expanded primary CD8 T cells electroporated with WT1-*TCR* mRNA, *CD8* mRNA, and increasing concentrations of *CD3* mRNA.** Upregulation of CD137 expression in T37 TCR-redirected expanded CD8 T cells (**A**) or T126 TCR-redirected expanded CD8 T cells (**B**) was measured 24 h after co-culture with tumor cell lines that were pulsed (PP) with $WT1_{37\text{-}45}$ peptide (in **A**) or with $WT1_{126\text{-}134}$ peptide (in **B**) or left unpulsed (NP). As a negative control, T cells were cultured in the absence of target cells (T cells only). As a positive control, T cells were cultured in the presence of phorbol 12-myristate 13-acetate and ionomycin (PMA+ION). (1) Refers to 1 µg *CD3* mRNA per $10^6$ cells (50 µg mRNA/mL), (2) refers to 2 µg *CD3* mRNA per $10^6$ cells (100 µg mRNA/mL), and (3) refers to 3 µg *CD3* mRNA per $10^6$ cells (150 µg mRNA/mL). Graphs represent mean ± SD values for 2 (only ST37-8-3 (3) co-cultured with Raji-A2-WT1 or PMA+ION conditions) to 4 independent donors that were analyzed using two-way ANOVA followed by Tukey's multiple comparison test (ns, not significant). M, mock; S, DsiRNA against *TRAC* and *TRBC*; T37, $WT1_{37\text{-}45}$ *TCR* mRNA; T126, $WT1_{126\text{-}134}$ *TCR* mRNA +CD8, *CD8* mRNA co-electroporated with *TCR* mRNA; +CD3, *CD3* mRNA co-electroporated with *TCR* and *CD8* mRNA.

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> **You keep using that word. I do not think it means what you think it means.**
>
> — Iñigo Montoya (*The Princess Bride,* William Goldman)

# 7

# The quest for the best: How TCR affinity, avidity and functional avidity affect TCR-engineered T-cell antitumor responses

This chapter has been published in:

Campillo-Davo D, Flumens D, Lion E.
*Cells* (2020);9(7):1720.

# Abstract

Over the past decades, adoptive transfer of T cells has revolutionized cancer immunotherapy. In particular, T-cell receptor (TCR) engineering of T cells has marked important milestones in developing more precise and personalized cancer immunotherapies. However, to get the most benefit out of this approach, understanding the role that TCR affinity, avidity, and functional avidity play on how TCRs and T cells function in the context of tumor-associated antigen (TAA) recognition is vital to keep generating improved adoptive T-cell therapies. Aside from TCR-related parameters, other critical factors that govern T-cell activation are the effect of TCR co-receptors on TCR–peptide-major histocompatibility complex (pMHC) stabilization and TCR signaling, tumor epitope density, and TCR expression levels in TCR-engineered T cells. In this review, we describe the key aspects governing TCR specificity, T-cell activation, and how these concepts can be applied to cancer-specific TCR redirection of T cells.

# Introduction to TCR affinity, avidity and functional avidity

From tumor infiltrating lymphocytes to T-cell receptor (TCR) and chimeric antigen receptor (CAR) T-cell engineering, T cells have marked important milestones in cancer immunotherapy [1]. T cells recognize short peptide epitopes in the context of the major histocompatibility complex (MHC) thanks to their TCR. This receptor is a heterodimer of the immunoglobulin gene superfamily composed of two different alpha and beta polypeptides in conventional αβ T cells. The extracellular domain, which is involved in antigen recognition, comprises a variable region, a constant region, and a hinge, where a disulfide bridge is located to stabilize the interaction between the TCR chains. It continues into the transmembrane region and the intracellular domain, which interacts noncovalently with CD3 γ, δ, ε, and ζ proteins to form the TCR–CD3 complex. When a TCR correctly identifies the cognate peptide-MHC complex (pMHC), including the correct matching between MHC type and CD4/CD8 co-receptor, the TCR undergoes a series of conformational changes that lead to a first activation signal [2]. Three different TCR parameters are the major players governing this pMHC recognition and posterior T-cell activation, namely, TCR affinity, avidity, and functional avidity (**Figure 1**). TCR affinity is a key factor in controlling the sensitivity of the T cells towards the antigen and it is defined as the strength of the interaction between a single TCR and the pMHC ligand [3]. It is usually determined by the association ($k_{on}$) and dissociation rates ($k_{off}$) and represented as the equilibrium dissociation constant ($K_D$) [3]. If TCR affinity relates to single receptors, TCR avidity measures the strength of multiple TCR–pMHC engagements and considers the effect of other molecules such as TCR co-receptors in the interaction, whereas functional avidity represents the T-cell fitness and activity at different concentrations of peptide epitope. The mean functional avidity is usually described as an EC50 concentration, representing the peptide dose at which a half-maximal activation of the T-cell population is reached [4]. Although physiological TCR affinities can range from 1 μM to 100 μM [5,6], several studies have marked the threshold of affinity for maximal T-cell activity, including antitumor T-cell responses, at 5–10 μM of peptide epitope [3,6-8]. In a comparative study of native TCRs with a TCR-like CAR, it was evidenced that the affinity of the antibody fragment in the TCR-like CAR was determinant to achieve better T-cell responses. In this study, and similarly to the ones performed by Zhong *et al.* [6], TCR affinity could not be improved above 5 μM, whereas TCR-like CARs would display an improved affinity threshold in the nM range [7]. Conversely, a comparison between conventional high-affinity single-chain TCRs and TCR-like CARs revealed that, although TCR-like CAR expression levels were

higher, they were less sensitive in recognizing the ligand, which can be potential attributed to their signaling kinetics [9]. In a panel of TCRs with enhanced affinities within the physiological range against the cancer/testis antigen (CTA) New York esophageal squamous cell carcinoma 1 (NY-ESO-1), TCR-transduced T cells could respond to affinities above 5 μM, showing that affinity can limit the maximal activation of T cells [8]. This fact is likely caused by a reduced contribution of the TCR affinity to TCR avidity above the threshold [6]. Furthermore, a computational analysis from 12 phenotypic models of TCR–pMHC interactions showed that TCR affinity would not be a reliable marker for T-cell responses [10].

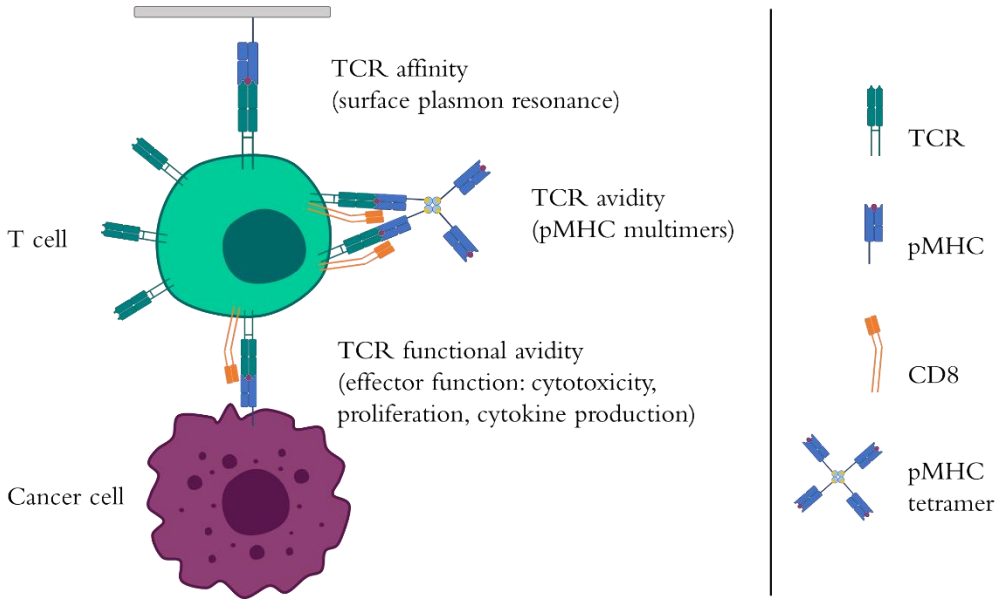


**Figure 1. The interaction between the T-cell receptor (TCR) and the peptide-major histocompatibility complex (pMHC).** T cells recognize tumor peptide epitopes via the pMHC. Different parameters affect the sensitivity that T cells, including T-cell receptor (TCR)-engineered T cells, will display against the pMHC. TCR affinity describes the strength of the interaction between a single TCR and pMHC. It is commonly measured using a technique named surface plasmon resonance. TCR avidity, on the other hand, reflects the contact of multiple TCRs and pMHCs. For this reason, multimers consisting of a number of pMHCs linked via streptavidin–biotin complexes to a fluorochrome are used to stain antigen-specific T cells and measure their TCR avidity. This parameter also takes into account the effect of T-cell co-receptors such as CD8 in the stabilization of TCR–pMHC binding. Closely related to TCR avidity, functional avidity shows the T-cell fitness to a target antigen in terms of its activation and effector functions, namely, T-cell proliferation, antitumor cytotoxicity, cytokine production, upregulation of activation markers, among others.

The TCR acquires its specificity in a maturation process that is based on somatic rearrangements of the variable (V), joining (J), and, only in the β chain, the diversity (D) TCR segments [11]. These rearrangements give rise to an almost infinite repertoire of TCRs with different specificities, including TCRs that recognize self-antigens, i.e., antigens naturally expressed in the human body. Many tumor-associated antigens (TAAs) targeted in adoptive T-cell therapies are self-antigens that can also be present in healthy tissues. Due to mechanisms of negative selection of auto-reactive lymphocytes, T-cell clones of high affinity against self-antigens are usually eliminated. Therefore, the frequency of high-affinity TCRs towards TAAs in circulating T-cells is low. In fact, natural cancer-specific TCRs usually promote an inferior T-cell response to physiological epitope densities, which would explain why tumors are able to avoid recognition by T cells [12]. On the contrary, TCRs with higher affinities and longer half-lives of TCR–pMHC binding kinetics commonly generate better T-cell responses because they can sense lower peptide epitope densities [12]. As the T-cell repertoire is edited and the affinity of circulating T cells against self-TAAs is usually low, in vitro affinity maturation is a potent tool to increase the ability of T cells to recognize low doses of peptide epitopes, which can even result in a 700-fold affinity increase [13]. However, it is important to highlight that affinity maturation may not always solve the problem of no recognition of low epitope densities, as it has been shown that affinity-matured TCRs with very high affinities improve the speed at which a T cell responds, but fail to respond to low density of pMHC [13]. This lack of recognition would be restored with lower TCR affinities that would lead to half-lives of more than 10 s, but half times ranging from $10^2$ to $10^3$ s would result in loss of sensitivity [13]. In a study analyzing the $k_{off}$ rates of a library of low- and high-avidity cancer-specific T-cell clones after vaccination with different peptides, the dissociation rate was correlated with target recognition and $Ca^{2+}$ mobilization [14]. More importantly, the affinity of the peptide used for the vaccine had a big impact on the avidity of the T-cell clones that were generated in patients after vaccination, with native and low-affinity peptides promoting the differentiation of cancer-specific T cells with higher avidity [14].

# The role of epitope density

T-cell activation is dependent on the binding kinetics of the TCR–pMHC, which in turn is influenced by the epitope density on the membrane of the tumor cell or antigen-presenting cell (APC) [15]. TAAs are processed intracellularly, bound to MHC molecules to form the pMHC, and presented on the cell membrane. The binding affinity between the tumor peptide and the MHC molecules has been linked with how T cells will respond. It appears that peptide-MHC affinities of 10 nM or higher are needed for

tumor regression [16]. However, tumor peptide antigens are usually expressed in small amounts on the surface of tumor cells due to defects in their antigen processing and presentation machinery, such as downregulation of the levels of human leukocyte antigen (HLA) molecules [17]. In many cases, TAA levels are analyzed using mRNA-based techniques, which may misrepresent the actual pMHC numbers available for T cells [18]. In a peptidome analysis of predicted alternative splice forms, it was observed that peptides that are overabundant in cancer splice variants represent a minority of HLA class I epitopes in comparison to normal transcripts [19]. Moreover, hydrophilic amino acids were found to be more abundant in transcripts from cancer tissues, which may explain why cancer-specific peptides are less prone to be predicted as MHC epitopes [19]. Some studies have tried to understand the immunogenic profile of tumor cells in relation with the epitope density by using high-affinity soluble TCRs against immunodominant epitopes of CTAs NY-ESO-1 and L antigen family member 1 (LAGE-1), overexpressed TAAs, or differentiation-associated TAAs [20-22]. This technique has shown that naturally-processed TAA peptide epitopes are usually presented at ratios of 10 to 150 copies per cell [20]. These numbers would be sufficient for antigen-specific T cells as it has been demonstrated that one single TCR–pMHC interaction can induce T-cell activation in helper T cells [23]. This pMHC can engage with different TCR molecules and trigger T-cell activation after engaging with approximately 200 TCRs [24]. Moreover, three pMHC complexes are enough to promote cytotoxic T-cell killing [25]. However, more recent observations increased the number of pMHC ligands needed for correct T-cell activation to a minimum of 90 [26].

Although TCR affinity is directly correlated with the ability of the T cells of sensing lower densities of the antigen, TCR (functional) avidity predicts the capacity of a TCR-engineered T cell to induce a tumor-specific reaction when the number of pMHC is poor. Some evidence suggests that epitope density and not TCR affinity or avidity would play a major role in eliciting cancer-specific T-cell responses. In a non-Hodgkin B cell lymphoma mouse model, Segal and colleagues observed that avidity had not a major role in eliminating tumor burden [27]. Both high- and low-affinity TCRs successfully eradicated small tumors and were unable to respond against bigger tumors. Importantly, numbers of high-affinity T cells were reduced compared to low-affinity T cells, most probably due to the induction of apoptosis in the first group. T-cell fitness could be restored by changes in epitope density aiming to lower avidity from the side of the tumor. Similar observations have been described by Dougan and collaborators against the endogenous melanoma antigen tyrosinase-related protein 1 (TRP1) [28]. Another report argues that avidity is the major factor in eliminating leukemic cells in vivo, and not epitope density, the peptide-MHC affinity, nor the

stability of the pMHC [29]. These findings support that there is a threshold of affinity and avidity above which further affinity enhancement or selection of supraphysiological avidities in T-cell clones would not translate in better in vivo responses. Hence, this challenges the way T-cell clones and TCRs are selected for preclinical and clinical testing. However, a study by Jaigirdar and colleagues indicated that high-avidity TCRs against the leukemia antigen Wilms' tumor 1 (WT1) could not recognize naturally processed WT1 peptides [30]. These divergent studies highlight the complexity of TCR–pMHC interactions in the context of cancer recognition and the risk of oversimplifying the selection of T-cell clones or TCRs for TCR-engineering to the best TCR affinity or avidity.

# The role of TCR co-receptors

Once a TCR has engaged the corresponding pMHC, TCR co-receptors CD4 and CD8 bind to the invariant region of MHC class II and class I molecules, respectively. It is generally known that these co-receptors augment T-cell sensitivity and responses as the result of two main effects: (1) stabilization of weak interactions between the TCR and a cognate pMHC [31-33]; and (2) intracellular recruitment of the co-receptor-associated tyrosine kinase Lck to the vicinity of the TCR signaling complex, thereby enhancing the initiation of the TCR signaling cascade [34,35]. However, whereas numerous studies supported the role of CD8 in the latter effects, with TCR affinity threshold for CD8 dependence ranging from 60 to 120 μM [36], CD4 only acts to accelerate TCR-triggered signaling and not to stabilize TCR–pMHC interactions [37,38]. This ability is disputed by the extremely low affinity of CD4 for MHC molecules [39]. Nevertheless, the importance of co-receptor engagement in TCR binding to pMHC is illustrated by the fact that anti-CD4 and anti-CD8 antibodies can decrease or block and in the case of some antibody clones even enhance the extent to which the TCR interacts with pMHC [40,41]. This antibody blockade or enhancement is even more pronounced when a TCR binds with a low-affinity to pMHC [41]. Moreover, stabilization afforded by the extracellular domain of the CD8 co-receptor appears to be indispensable for enhanced activation of T cells with low-affinity TCRs, but not for T cells with high-affinity TCRs [42]. The CD8 co-receptor has been found to augment the binding efficiency at suboptimal TCR–pMHC affinities by altering both the association and dissociation rate of the TCR–pMHC interaction [43,44]. In addition, CD8 regulates the TCR sensitivity or triggering threshold by mobilizing TCR–pMHC class I complexes to membrane microdomains at a rate depending on the affinity of CD8 for MHC [44]. In contrast to the extracellular domain, the intracellular signaling domain of CD8 is critical for enhanced T-cell activation independent of the strength of the TCR [42]. Reduction of this CD8/Lck-

dependent tyrosine kinase activity lowers the sensitivity of the TCR, and, therefore, impedes T-cell effector functions [45-47]. Based on these findings, the degree of dependency on CD8 to enhance T-cell functions differs depending on the affinity of its TCR for cognate pMHC. Furthermore, studies using pMHC multimers indicate the critical role of CD8 in antigen-specific TCR binding. Tetramers bearing a mutation in the CD8 binding site selectively bind to higher avidity T cells, but bind not to low avidity T cells [48]. Moreover, CD8 co-receptor engagement strengthens the avidity and stability of the interaction between T cells and their cognate multimers [48,49]. The aforementioned observations highlight how the presence or absence of TCR co-receptors impacts the interaction between T cells and cognate pMHC molecules. In addition, alterations in co-receptors expression levels or MHC binding capacity affect T-cell functionality as well. This is demonstrated by artificial mutations in the $\alpha$3 domain of HLA-A2 that abrogate CD8 co-receptor binding, which resulted in inhibition of T cell-mediated specific lysis of target cells, without disturbing the TCR–pMHC interaction [50]. On the other hand, artificial altered HLA-A*68 molecules with enhanced CD8-binding ability induced an increase in T-cell proliferation and cytokine secretion [51]. The functional effects of a CD8–pMHC interaction are also underlined by the fact that IFN-$\gamma$ secretion and CD107a surface expression of lower affinity pMHC-stimulated T cells could be achieved only in the presence of co-receptor engagement [43]. Lastly, CD8 synergy with low-affinity TCRs presents the issue of undesirable autoreactivity against self-peptides. However, T cells have the ability to reduce their functional avidity and thereby their autoreactive potential by downregulating CD8 membrane expression [52,53].

# Selection of cancer-specific TCRs

A good starting point for searching cancer-specific TCR candidates is to isolate them from patients who have responded after treatment with peptide-based vaccines or dendritic cells (DCs) that have been engineered to express the full tumor antigen or pulsed with the target peptide (reviewed by [54]). The application of peptide-based or antigen mRNA-based cancer vaccines using DCs focuses on the increment of epitope density on the surface of antigen-presenting cells to boost the immune system against one or multiple TAAs (reviewed by [55,56]). When patient cells are not available, using donor material is another alternative. High-avidity T-cell clones from a naïve repertoire can be isolated using autologous peptide-loaded monocyte-derived DCs, followed by subsequent restimulation with peptide-loaded peripheral blood mononuclear cells (PBMC) [57]. Although this can be difficult to achieve due to the scarcity of highly reactive clones against self-antigens. Another source of tumor-reactive T-cell clones is

allogeneic material. In this case, cells from mismatched donors are used aiming to achieve alloreactive T cells specific towards the full pMHC rather than the peptide alone [58-60]. Alternatively, transgenic mice that have been vaccinated with the target peptide represent a source of murine TCRs usually defined by a high affinity towards the ligand [61-63]. However, one drawback of this strategy is that allogeneic TCRs can show epitope promiscuity and could potentially cause off-target reactivities [58]. To improve the specificity and the affinity of the TCR candidates, viral antigens can be used from virus-associated malignancies (reviewed by [64]), but the usage of reactive T cells against these epitopes will be limited to a certain number of patients. T-cell clones reactive to tumor neoantigens are gaining momentum since the latter are truly cancer epitopes that are not found in healthy tissues [65]. These neoantigen-specific T cells provide a source of highly specific tumor-reactive TCRs for genetic transfer [66,67]. Nevertheless, this approach presents some challenges related to the correct identification of candidate neoepitopes, and thus to that of neoantigen-specific T-cell clonotypes, as well as other challenges related to the heterogeneity of tumor mutations and the epitope density of these antigens [68].

Regardless of their origin, the selected TCR candidates should undergo further testing to ensure their specificity and efficacy, by both binding assays with pMHC multimers and functional assays [69] (**Figure 1**). This is especially important due to the weaker binding strength of TCR against self-antigens versus, for example, viral antigens [69]. This correlation between TCR affinity and T-cell immune responses is clearly evidenced by the difference in how T cells engineered with virus (higher affinity) or cancer-specific (lower affinity) TCRs respond [69]. In addition, high-affinity TCRs tend to rely less on the effect of CD8 co-receptor binding than low affinity TCRs [69]. The use of pMHC multimers has been extensively used as the first method of choice to analyze TCR avidity, especially for CD8-positive T cells, as the detection of antigen-specific CD4 T cells using pMHC class II multimers is still challenging [70,71]. However, as described before, pMHC multimers do not provide information on functional avidity or may not even identify important antigen-specific TCR repertoires [72]. For this purpose, Morimoto and colleagues developed a TCR-deficient CD8-positive Jurkat-derived cell line to rapidly and uniformly evaluate the functional avidity of cloned TCRs [73]. This cell line, called 2D3, provides a way to homogenize/standardize the measurement of T-cell functional avidity. It is provided with a nuclear factor of activated T-cells (NFAT)-driven enhanced green fluorescent protein (EGFP) reporter gene so that TCR activation can be linked to EGFP expression [73]. One of the advantages of this cell line is that it can be easily genetically modified with DNA or mRNA encoding the TCR using any type of engineering method [73,74]. Rosskopf and colleagues went further by adding

three fluorescent proteins: EGFP, cyan fluorescent Protein (CFP), and mCherry to another Jurkat-derived cell line. With this triple parameter reporter platform, up to three transcription factors—NFAT, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and activator protein 1 (AP-1), which play key roles in T-cell activation—can be analyzed at the same time to evaluate TCR function [75]. CD137 is an activation marker upregulated 24 h after stimulation of CD8 T cells and can be used as an enrichment marker for high-avidity T-cell clones of different expanded T-cell subsets from a naïve repertoire [76]. One of the greatest challenges of selecting affinity-optimized TCRs is to diminish the risk of on-target or off-target cross-reactivities. Border *et al.* described a scanning method with which effective TCRs could be identified while pinpointing those that could be potentially dangerous TCRs [77]. This scanning method is based on a first selection of natural TCRs based on affinity and functional avidity followed by the affinity enhancement of those TCRs and further affinity and functional characterization. The final candidates are then compared by using an X-scan, a system in which all the residues of the peptide of interest are mutated into every amino acid possible. This extensive screening ensures that the candidates will not recognize other potential peptides but the target one.

Our group has also highlighted the importance of selecting the correct APC to correctly analyze TCR avidity [4]. To analyze TCR avidity and to predict the sensitivity of cancer-specific TCR-engineered T cells, APCs are pulsed with different concentrations of peptide antigens, usually in the micromolar range. In particular, the T2 cell line, a T cell-B cell hybridoma, has become the gold standard in this type of assay. This cell line presents a deficiency in transporter associated with antigen processing (TAP) proteins, which leads to the presence of "empty" HLA molecules on the cell surface. Although this feature is desirable in peptide-pulsing assays, the overabundance of the pulsed peptide above physiological levels compared to those of naturally-processed TAA peptides may lead to misrepresentation of the TCR avidity. In fact, when peptide-pulsing assays are commonly performed using micromolar amounts of peptide [74,78], T2 cells would need to be pulsed with low nanomolar concentrations to resemble physiological amounts of epitopes [21]. Certainly, other cell lines and assays to investigate tumor killing, cytokine production (important for adverse effects related to cytokine storms), and, in general, any other indicator of T-cell fitness and specificity for antitumor responses are possible.

# Improvement of TCR-engineered T-cell antitumor responses

Despite some divergences in the correlation between TCR affinity and T-cell activity, the selection of high-affinity TCRs or the affinity enhancement of low-affinity TCRs constitutes a mean to improve antitumor responses (**Figure 2**). Different techniques are employed for TCR affinity maturation, including the phage display system—which can achieve TCR affinities in the picomolar range [3,79,80], the yeast TCR display system [81], a mammalian retroviral display system coupled with an alanine-scanning approach to identify key amino acid residues [82], the substitution of key amino acids in the TCR complementarity-determining regions (CDRs) [83-85], or the use of somatic hypermutation [86]. On another note, enhancement of transgenic TCR dimerization and TCR availability on the surface of the T cells represents a way to improve TCR avidity and, hence, T-cell functionality [36]. One of the pitfalls in TCR engineering is the low expression of transgenic TCRs due to mispairing with native TCRs, which in turn can give rise to deleterious reactivities, and competition for the TCR complex machinery [87-90]. Multiple techniques have been developed over the years to solve this problem, focusing on different aspects of the TCR machinery (**Figure 2**). A way to improve the amount of transgenic TCRs available on the cell surface, while reducing the presence of the native TCRs is by silencing the native TCR sequences using short hairpin RNAs either included in the same vector where the transgenic TCR is located [91-94] or by transfection of silencing RNAs (siRNAs). In both cases, the siRNAs are directed against the constant regions of the TCR chains to target multiple native TCR sequences at a time. The complete removal of the native TCR can be achieved by techniques such as zinc-finger nucleases (ZFNs) [95], transcription activator-like effector nucleases (TALENs) [96-98], or, more recently, the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system [97-100]. Although native TCR inhibition is a simple way to reduce TCR mispairing, other strategies tackle the stability of the transgenic TCR and, by doing so, they reduce TCR mispairing (**Figure 2**). Thus, TCRs for genetic engineering of T cells have been modified with extra disulfide bonds [101-103], which has recently been employed in high-affinity soluble TCRs [104]. This is achieved by introducing cysteines in both the TCR alpha and beta chains. Alternatively, the constant domains of human TCR chains can be substituted for either murine TCR$\alpha\beta$ or human TCR$\gamma\delta$. With this strategy, the constant regions of the TCR$\alpha\beta$ chains are swapped to produce chimeric TCRs that retain their antitumor functionality [105-109]. Despite enhanced TCR antitumor functionality, the presence of xenogeneic material may result in immunogenicity that could hinder the effect of the cells. This issue can be

addressed by substituting key residues in the constant region of the TCR with those of murine origin [110]. Furthermore, while this strategy still produces mispaired TCRs, these are unable to bind to CD3 rendering them ineffective [105]. However, with these strategies, mispairing can still occur. To largely avoid incorrect pairing, single chain TCRs are based on the fusion of the variable regions of the TCR alpha and beta chains connected with a linker [111]. This structure is then joined to the TCR beta constant region to form the single chain TCR, whereas the constant TCR alpha is added separately to allow the recruitment of the CD3 complex. Similar to a full TCR, the addition of an extra disulfide bond in the variable region strengthens the stability of the molecule and even improves the functional activity of engineered cells [111]. These alterations of either the pool of native TCRs or the structure of the transgenic TCR can of course be combinable to further increase TCR avidity and promote better T-cell responses.

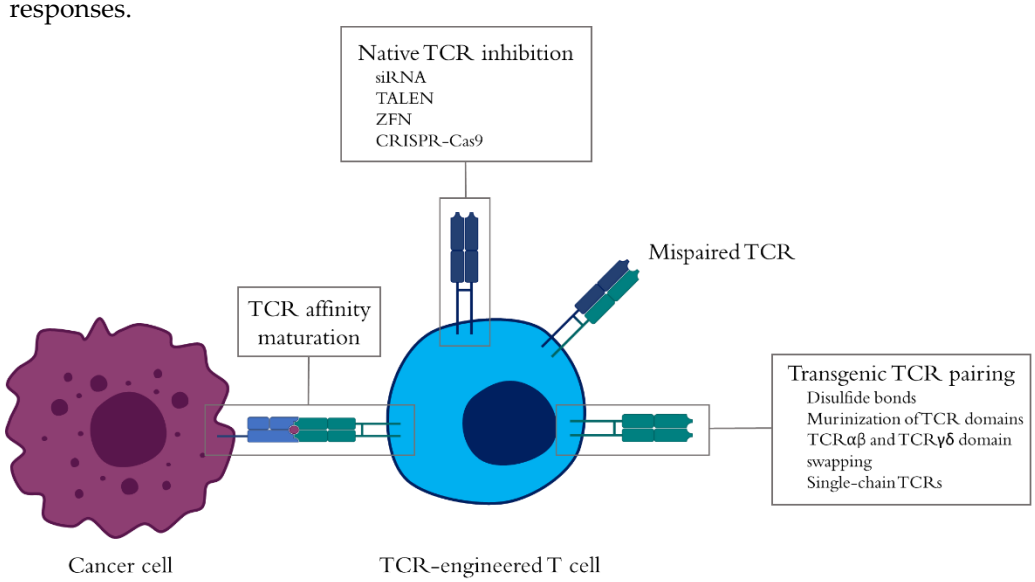


**Figure 2. Enhancement of tumor-specific T-cell receptor (TCR)-engineered T cells.** The antitumor functionality of TCR-engineered T cells can be leveraged by improving the affinity of the TCR–peptide-major histocompatibility complex (pMHC) interaction via TCR affinity maturation processes, such as phage display or the substitution of key amino acids in the complementarity-determining regions (CDRs) of the TCR. On another note, the presence of native and transgenic TCRs can lead to the mispairing of their TCR chains that reduce the levels of transgenic TCR on the surface of the T cells. To overcome this problem, the presence of native TCRs can be either downregulated by silencing RNAs targeting the TCR constant sequences in mRNA transcripts or completely abrogated with tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system. These techniques can be combined with the improvement of TCR pairing by addition of disulfide bonds, the murinization of TCR$\alpha\beta$ constant domains, or the use of TCR$\gamma\delta$ domains in the TCR$\alpha\beta$. Finally, systems in which the two TCR chains are transformed into one single TCR chain can also ensure that mispairing with the native TCRs does not occur without the need to abolish its expression.

# Clinical impact of TCR affinity and avidity in cancer-specific TCR-engineered T cells

Cancer-specific TCR-engineered T cells have been used in the clinic for more than a decade [112], concurrently with the idea that TCR affinity and avidity would have a major role in successfully eliminating cancer cells [113]. Affinity-matured TCR-engineered T cells have been successful in inducing clinical responses in tumors expressing the melanoma antigen recognized by T cells (MART-1) [114-116], glycoprotein 100 (gp100) [114], WT1 protein [117], carcinoembryonic antigen (CEA) [118], NY-ESO-1 [119-124], LAGE-1 [124], or the melanoma-associated antigen A (MAGE-A) family [125-128]. Antitumor affinity-enhanced TCRs, although they increase the recognition of tumor cells with low epitope density, they also increase the risk of cross-reactivity with antigens from normal tissues. Off-target recognition and cross-reactivity has been demonstrated in clinical trials using affinity-enhanced TCRs [118,125,127,128]. T cells engineered with an affinity-enhanced HLA-A*02-restricted TCR isolated from immunized mice with CEA peptide led to severe transient colitis [118]; whereas an affinity-enhanced HLA-A*02-restricted MAGE-A3/A9/A12-specific TCR derived from MAGE-A3-vaccinated transgenic mice caused neurotoxicity due to the recognition of MAGE-A12 expressed by brain cells [125]. Another high-affinity HLA-A*01-restricted MAGE-A3-specific TCR, developed against myeloma and melanoma, led to cardiogenic shock and ultimate death of the first two treated patients [127]. Preclinical studies showed no predicted off-target reactivities [128]; however, T cells engineered with this TCR caused severe cardiac tissue damage in patients due to the recognition of a striated muscle-specific titin-derived peptide [127,128]. Although lethal adverse events can also occur with TCRs that have not undergone affinity enhancement [129], this study showed the risks of using affinity-enhanced TCRs without extensive prior testing of cross-reactivities. To address this issue, Sanderson and colleagues developed an in vitro extensive preclinical testing protocol to evaluate the safety and efficacy of an affinity-enhanced MAGE-A4-specific TCR by using a wide range of testing material, including human tumor cell lines, primary tumor material, and panels of EBV-transformed B-lymphoblastic cell lines (B-LCLs) expressing multiple HLA alleles and molecular analysis [130]. After undergoing this testing procedure, Sanderson and colleagues obtained an affinity-enhanced TCR candidate with a safe clinical profile to test in clinical trials (NCT03132922, NCT04044768). Another issue involving affinity-enhanced TCRs revolves around the constant tonic signaling by recognition of the HLA molecules. Although this problem initially may not put the lives of patients at risk, it impairs the functional activity of the engineered T cells due to TCR–

CD3 downregulation and upregulation of inhibitory receptors [131]. On the bright side, this constant TCR activation may be prevented by fine-tuning the affinity of the TCR [131].

TCR mispairing between the endogenous and the transgenic TCR chains, although not limited to high-affinity TCRs, is a concern to be taken into consideration for the safety of adoptive TCR-engineered T cell therapies [88,89]. Even though adverse events caused by neoreactivities linked to TCR mispairing have not been reported so far, it is an underlying issue that can be solved by disruption of the endogenous TCR using multiple techniques (**Figure 2**), some of which have already been tested in the clinic with positive results [117,132,133]. In particular, the CRISPR-Cas9 system has revolutionized the way cells are genetically engineered for the treatment of cancer due to its simplicity, fidelity, and versatility [134]. Very recently, this method has been employed in refractory cancer patients to modify T cells with a cancer-specific TCR while suppressing the endogenous TCR chains and the negative immune checkpoint programmed cell death protein 1 (PD-1) genes in a multiplex system [132].

In patients where it is difficult to isolate cancer-specific TCRs, T cells from healthy donors can be a good alternative [135]. One of the advantages of this option is that an indefinite number of donors, whose T cell numbers are not compromised, can be screened until achieving the best high-affinity TCRs. However, the HLA repertoire of patients and donors should be matched to prevent alloreactivities from the endogenous donor TCR [135]. Another issue of this strategy is the potential off-target reactivities also caused by the donor TCR, which can be prevented using the same techniques employed to minimize TCR mispairing. Due to the potential severe toxicities of TCRs derived from cytotoxic CD8 T cells, high-affinity TCRs obtained from regulatory T cells (Tregs) [136] or helper CD4 T cells [137,138] represent an alternative source of cancer-specific TCRs. Although the use of Treg-derived TCRs raise concerns regarding the possibility of redirection of engineered helper CD4 T cells into Tregs in vivo, this was not observed in patients so far and instead induced tumor regression in metastatic cancer patients [139].

# Conclusion and future perspectives

The delicate interconnection between TCR affinity, avidity, the co-receptors, and the epitope density highlights the importance of finding a balance between increased TCR affinity or avidity to sense low epitope densities and supraphysiological T-cell activity to avoid potentially dangerous cross-reactivities. In this direction, new ways to produce

TCRs with fine-tuned affinities [140], de novo generation of tumor-specific TCRs [141] and the selection of neoantigens [142,143] or TAP-independent antigens [143,144] as epitopes for tumor targeting will be beneficial to produce more effective and safer TCR-modified T cells. The future of TCR therapies is increasingly becoming not limited to conventional T cells, as unconventional lymphocytes such as γδ T cells, and their TCRs, and natural killer cells are being explored in pre-clinical and clinical settings [145-148]. These cell types bypass concerns related to TCR mispairing and cross-reactivities, while having an intrinsic antitumor activity. They also offer the possibility of producing off-the-self allogeneic products due to their lack of graft-versus-host complications. Additionally, combinatorial approaches to improve T-cell activity with cytokines or immune checkpoints inhibitors may eliminate the need to produce TCRs with supraphysiological affinities that may cause severe adverse effects [132,149-151]. In summary, the complexity of the TCR–pMHC interactions, and thus that of T cell–tumor cell interactions, will require TCR genetic engineering to take a holistic approach to develop more precise and effective adoptive T-cell cancer therapies.

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> **Life can only be understood backwards; but it must be lived forwards.**
>
> — Søren Kierkegaard

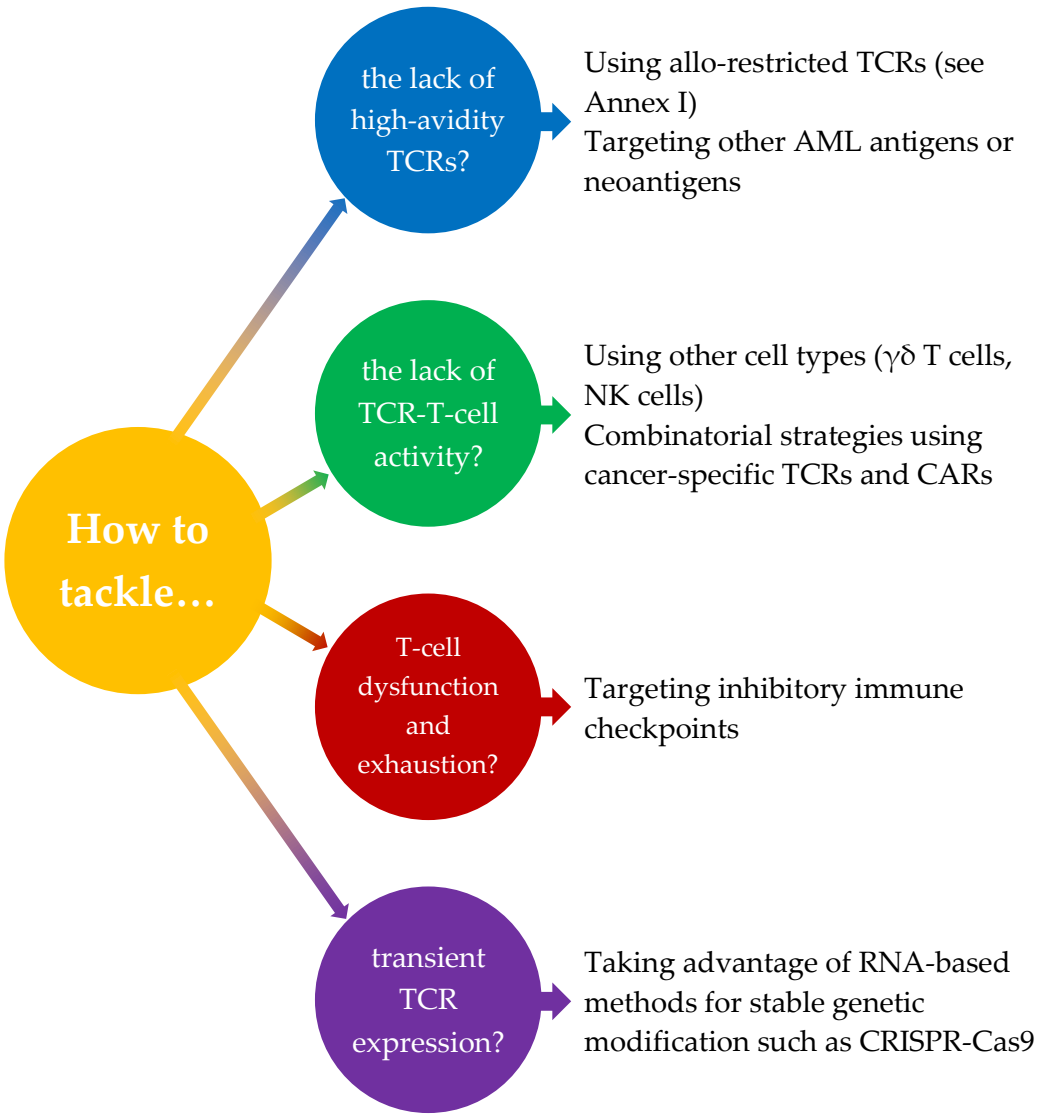
# Epilogue

## Future perspectives

## (or next steps on how to tackle different issues related to TCR-T-cell therapy)

# The fight against AML is far from over

Despite the work carried out in this doctoral thesis and recent translational and clinical advances, a cure for AML is still missing. There are different lines of research that can be undertaken in order to further improve TCR-T-cell therapies, based on different ways to tackle some of the challenges associated with this type of therapy:



## How do we tackle the lack of high-avidity TCRs?

Despite the efforts to improve TCR-T-cell therapies described in **Chapter 4** and **Chapter 6**, potent tumor-antigen recognition of cancer samples that endogenously express WT1 remains elusive (**Chapter 5**), potentially due to the affinity of the TCRs used. High-affinity TCRs against tumor self-antigens are difficult to obtain, mainly because TCRs that recognize self-antigens undergo clonal selection, as mentioned in **Chapter 7**. However, artificial enhancement of TCR affinity has led to cross reactivities with fatal consequences. An alternative strategy to obtain high-avidity TCRs is the isolation of **T-cell clones from HLA mismatched donors** that recognize the tumor peptide-MHC complex. TCRs from these clones usually display higher affinities since these cells do not recognize the tumor peptide-MHC complex as a self-antigen. Compared to affinity-matured TCRs, which have supraphysiological affinities for their ligand, allo-restricted TCR affinities are usually in the physiological range since they were naturally produced and underwent thymic selection in the donor. However, allo-restricted TCRs are difficult to obtain and can also show off-target promiscuity in mismatched patients [1]. As seen in **Annex I**, this line of research has been started in our group and data on the obtained WT1-specific allo-restricted TCR is being collected. An alternative to tackle the lack of recognition of self-antigens due to low affinity/avidity is targeting non-self AML-specific antigens such as **neoantigens**, such as the frequent fusions between CBFB and MYH11 genes which generate novel epitopes for which circulating TCRs have not been challenged and negative selected [2]. The importance of this specific fusion gene is that it is very common in AML blasts, making it a great target for TCR-T-cell therapy.

## How do we tackle the lack of TCR-T-cell activity?

As seen in **Chapter 6**, conventional T cells such as CD4 T cells and unconventional γδ T cells can be interesting candidates in TCR-T-cell therapy. Compared to CD8 and CD4 T cells (both αβ T cells), **γδ T cells** have the advantage of lacking TCR mispairing between the introduced TCRαβ and the native TCRγδ; the same is true for **NK cells**. This feature makes these cells good candidates for TCR-T therapy. The main difference between γδ T cells and NK cells in terms of TCR-therapy potential is the lack of CD3 expression in NK cells. The absence of CD3 expression can be corrected by co-transfection of *CD3* mRNA together with the cancer-specific *TCR* mRNA, but also by using CD3-engineered NK cells or cell lines such as NK-92 [3], which is a United States Food and Drug Administration approved cell line for clinical use. These cell types have already been investigated in this context, especially for melanoma, and mainly using CARs. However, the use of γδ T cells and NK cells for TCR-T therapy is still to be fully explored. **Combinatorial strategies using cancer-specific TCRs and CARs** are also an

attractive approach to tackle the lack of TCR-T-cell activity [4]. This strategy would target both intracellular (with the TCR) and extracellular (with the CAR) antigens with one single T-cell product. As shown in **Chapter 6**, multiple mRNAs can be co-transfected for expression of different proteins. In the scenario of TCR and CAR co-introduction, mRNA electroporation could be used to deliver either one or both immune receptors. This ensures that TCRs and CARs different tumor antigens can be combined and delivered according to the antigen expression levels and the patient's needs.

## How do we tackle T-cell dysfunction and exhaustion?

One of the most important issues in adoptive T-cell therapy is the upregulation of inhibitory immune checkpoints, directly linked to T-cell exhaustion and senescence [5]. On one hand, T-cell dysfunction in cancer patients hinders the isolation of functioning T cell populations for TCR engineering; on the other hand, even when functioning T cells can be isolated, T-cell exhaustion markers can also be upregulated during ex vivo culture, i.e., when using long-term T-cell expansion protocols, or after administration to the patient. Thus, targeting inhibitory immune checkpoints would reduce the chances of therapy failure due to expression of T-cell exhaustion markers. Such inhibitory immune checkpoints include the well characterized CTLA-4 and PD-1, but also TIM-3 and LAG-3. Traditionally, inhibitory immune checkpoints are targeted using monoclonal antibodies in stand-alone or combinatorial therapies [6,7]. Other investigators have also explored the abrogation of the expression of these molecules in adoptive T-cell therapies making use of CAR-T cells, mostly for solid tumors [8]. However, this line of research has not been fully developed for TCR-T-cell therapies in AML. Several studies have shown that AML blasts upregulate multiple inhibitory immune checkpoint ligands, which has been correlated with the T-cell lack of anti-AML activity [9]. Therefore, this strategy would be interesting to pursue, (i) to know what inhibitory immune checkpoints induce stronger T-cell dysfunction in AML [10,11] and (ii) to improve TCR-T-cell therapies for AML.

## How do we tackle transient TCR expression?

As described in **Chapter 2** and **Chapter 3**, and as used in other chapters of this thesis, mRNA electroporation is a flexible and rapid tool to engineer T cells with multiple proteins. However, its effects are transient. This may in fact be interesting in pre-clinical and early phase clinical trials in which the toxicity of a given immune receptor or combination of introduced proteins needs to be addressed. Nonetheless, in other instances, for example when the immune receptors have been proven to be safe, stable

genetic engineering methods can be applied instead. In this regard, we can take advantage of techniques such as **CRISPR-Cas9**. This system allows targeted genomic modifications and multiplexing without the need of using viral particles, which tend to be more cumbersome in terms of handling and safety precautions. Although CRISPR-Cas9 system can be applied using various delivery vectors (DNA, RNA, and protein complex), following the footsteps taken in this thesis, *Cas9* mRNA can be electroporated with guide RNAs. Native TCR replacement by the introduced TCR using the CRISPR-Cas9 method has already been attempted [12]; however, combinatorial strategies with inhibitory immune checkpoints or in the application of this technique in other unconventional cell types is an expanding field of study.

## *Conclusion*

Although, TCR-T-cell therapy has some challenges to overcome, the clinical trials using this technique described in Chapter 1 warrant its safety and potential efficacy for the treatment of AML. As Voltaire said, "No problem can stand the assault of sustained thinking". Therefore, we just need to keep thinking and, of course, researching.

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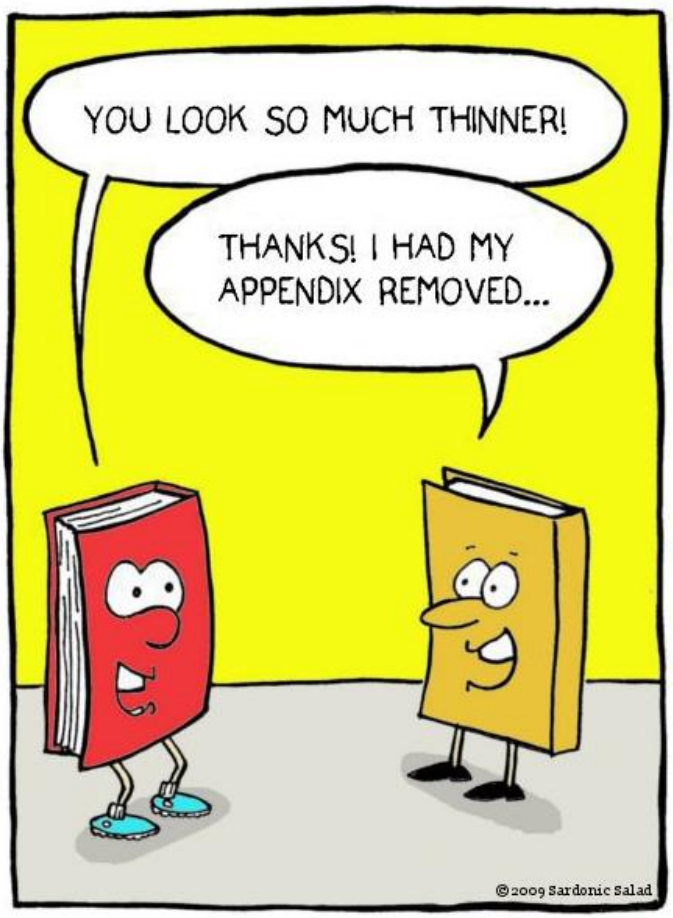


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# Annex I

## Generation of allo-restricted Wilms' tumor 1 (WT1)-specific T-cell receptors (TCR) for TCR-engineering of T cells in adoptive T-cell immunotherapy

Research visit at the:

Laboratory of Professor Yusuke Oji and Professor Fumihiro Fujiki. Department of Cancer Immunotherapy, Osaka University Graduate School of Medicine, Japan.

# Aim of the research visit

The research visit at the laboratory of Prof. Yusuke Oji from the University of Osaka was integrated within my doctoral research project entitled: "Wilms' tumor 1 specific T cell receptor (TCR) RNA-modified T cells for cancer immunotherapy: moving towards clinical application". The aim of the research visit was to gain knowledge on how to isolate, clone and subclone TCRs, especially allo-restricted WT1-specific TCRs derived from HLA mismatched donors. Allo-restricted TCRs are usually of higher avidity and, therefore, are reactive towards tumor cells that express WT1 epitopes at a low density. The TCR sequences were analyzed and cloned into vectors for the posterior subcloning into plasmid vector suitable for the in vitro transcription of messenger RNA (mRNA). These mRNAs containing the sequences of the alpha and beta chains of the WT1-specific TCRs will be used for the engineering of T cells in future projects.

# Added scientific value of the research visit

The field of cancer immunotherapy has opened a new era for the treatment of cancer patients. Efforts on the development of new strategies center on unleashing the breaks of the immune system to attack tumor cells. One of the key pillars in the wave of new approaches is the T-cell receptor (TCR) engineering of T cells. It is based on the introduction of a cancer-specific TCR into T cells to redirect their specificity towards an epitope that is aberrantly or uniquely expressed in tumor cells. Despite the promising results in pre-clinical studies, the outcomes in clinical trials have been modest. One of the reasons behind it is that most of the cancer antigens are also self-antigens. Therefore, cancer-reactive T cells are scarce due to the negative selection of T cells recognizing self-antigens in the thymus. From those T cells that can identify abnormal cells expressing tumor-associated antigens (TAAs), few will exhibit an avidity strong enough to exploit the T-cell functional abilities fully. The term avidity represents the strength of the interaction between a T cell and a tumor cell presenting the cancer antigen. An increasing number of studies show that T cells with high avidity for their cognate epitope perform better and can detect them at lower densities. In many types of malignancies, tumor cells downregulate the expression of major histocompatibility complex (MHC) molecules or have deficiencies in their antigen processing pathways, which negatively impacts the presentation and density of peptides on their surface. High-avidity T cells, compared to T cells of lower avidity, offer an exceptional advantage when downregulation of antigen presentation (hence lower MHC epitope density) would occur.

Wilms' tumor 1 protein (WT1) is a transcription factor that has been described as one of the most prominent TAAs. It is overexpressed in leukemia and many solid tumors, making this antigen an exciting target for T-cell engineering. The purpose of my current doctoral project is to find novel strategies to improve the modification of T cells with WT1-specific TCRs via RNA transfection, for a future clinical application. In this direction, I have developed a novel method to downregulate the expression of the native TCR by the electroporation of Dicer-substrate silencing RNAs (DsiRNAs) targeting the messenger RNA (mRNA) sequences of the TCR alpha and beta constant regions, minimizing de novo expression of native TCR. The effect of the DsiRNAs is combined with the electroporation of DsiRNA-resistant codon-optimized mRNA that encodes a WT1-specific TCR. The results show unprecedented transgenic TCR expression thanks to the marked reduction in TCR mispairing between the native and the introduced TCR. This strategy can be applied to both CD8+ and CD4+ T cells, expanding the possibilities of this technique. Going further, I have also analyzed the

combined electroporation of *TCR* mRNA with mRNA encoding human CD8αβ to increase the levels of this co-receptor in CD8+ T cells, but also to introduce it in CD4+ T cells and gamma-delta (γδ) T cells. The additional expression of CD8 in T cells increases the stability of the interaction between the epitope-specific TCR and the peptide:MHC complex, enhancing the functional avidity of T cells engineered with WT1-specific TCRs of intermediate affinities. However, these modified T cells fail to recognize some tumor cell lines that express low levels of WT1.

For this reason, the transfection of T cells with high-avidity *TCR* mRNAs is a crucial point in our TCR-based lines of research. Therefore, the collaborative project between the Laboratory of Experimental Hematology (LEH) and the group of Prof. Oji of the University of Osaka focused on the generation of high-avidity WT1-specific TCRs for the treatment of hematological malignancies. The group of Prof. Oji already collaborated in my doctoral project by providing the plasmid vectors containing the *TCRs* and *CD8* sequences that I used to produce the *TCR* and *CD8* mRNAs in the experiments mentioned above. For the research stay, I pursued three main personal goals. First, I learned how to generate antigen-specific T-cell clones and to isolate their respective TCRs. Second, I worked on the generation of high-avidity WT1-specific CD8+ T-cell clones that will detect WT1-derived peptides at low concentrations. Third and final, I cloned and subcloned the isolated *TCR* sequences into different vectors, including those suitable for the production of in vitro mRNA. Afterward, these TCRs can be transfected into non-reactive T cells to redirect their specificity towards WT1.

This research stay helped me gain valuable practical skills and made a substantial technical contribution to our laboratory. It also boosted my portfolio and my professional network. In addition to this, it gave me an excellent opportunity to gain first-hand experience on the differences between European and Japanese research environments. This was very appealing to me since, as a Spanish researcher working in Belgium, I understand the positive impact that internationalization can have both on a professional and personal level.

# Motivation of the choice of host institution

The University of Osaka is ranked the 53rd best university in the world and the 3rd best university in Japan in the 2018-2019 list organized by the Center for World University Rankings. It is also part of the Global 30 project together with other Japanese universities. This project of the Japan Society for the Promotion of Science—an organization of the Ministry of Education, Culture, Sports, Science, and Technology—

encourages the internationalization of partnering universities. The objective of this program is to attract, facilitate the arrival of, and provide international students and researchers with high-quality education and research environment.

To support the collaborative use of advanced equipment for life science, the Osaka University Graduate School of Medicine has an extraordinary core facility, the Center for Medical Research and Education. It includes a flow cytometry unit that contains cell-sorting equipment, a gene analysis unit and an optical imaging unit among others. It also hosts the Genome Editing Research and Development Center that aims to improve medical research by providing genomic editing techniques and equipment. These facilities accommodate all the equipment and infrastructure needed for the success of my stay within the group of Prof. Yusuke Oji.

Prof. Oji's Cancer immunology research group was established by Prof. Haruo Sugiyama, who is still actively involved in the daily management of the group. Prof. Sugiyama pioneered the biology of the WT1 protein, which he discovered in 1992. Later on, he became one of the top-leading researchers in WT1 peptide vaccination as cancer immunotherapy. He is listed as the inventor in several granted patents, including a clinical test that detects one leukemic cell in 100.000 peripheral blood cells. This test has been widely used all over the world, helping with the detection of minimal residual disease for many cancer patients with hematological malignancies and solid tumors. For the impact of this invention, Prof. Sugiyama received the 2011 Princess Takamatsu Cancer Research Fund Prize and the Takashi Ogimura Special Award of the Japan Leukemia Research Fund in Heisei 8. Following Prof. Sugiyama's legacy, Prof. Oji has continued bringing the research group at the front of WT1 and cancer research. Prof. Oji has an h-index of 41 with 143 authored publications in peer-reviewed journals, which have been cited over 5000 times.

The collaboration between LEH and Prof. Oji's group was established by Prof. Berneman (head of LEH) and Prof. Sugiyama more than ten years ago. It is worth noting that the Faculty of Medicine and Health Sciences of the University of Antwerp conferred Prof. Sugiyama an Honorary Doctorate for his scientific merit and high-profile international research and clinical programs in the field of Cancer Immunotherapy in 2014. This constructive relationship has contributed to five shared publications in peer-reviewed scientific journals, including top journals such as Blood, Leukemia, and the Proceedings of the National Academy of Sciences of the United States of America in the last eight years. However, despite the long collaborative relationship between the two groups, this was the first time that a student of our group

participated in a research stay in the Japanese laboratory. Therefore, this opportunity opened new ways of collaboration and scientific exchange between LEH and the group of Prof. Oji. For example, it allowed the transfer of know-how from the Japanese group to our group on the generation of antigen-specific T-cell clones, the isolation of their TCRs, and the cloning and subcloning of TCR sequences in different vectors. This practical knowledge played a crucial role in the development of my research and technical skills. For instance, I will be able to isolate and clone TCRs from Belgian and other European individuals with human leukocyte antigen (HLA) types that are not frequent in the Japanese population. Moreover, this opportunity also allowed me to broaden my professional network, and my future career prospects, not only in the cancer research field but in other areas where these techniques are applied.

Finally, the research stay was in line with the current economic and scientific trajectory shared between the European Union and Japan. The European Commission recently signed an arrangement to promote cooperation between European and Japanese researchers, within the frame of the European Union's R&D program called Horizon Europe. Furthermore, in February, the European Union and Japan launched the Economic Partnership Agreement to encourage close collaboration between the two regions. These agreements highlight the attention that European-Japanese cooperative projects are currently attracting.

# Work plan

## Work package 1: Generation of WT1-reactive CD4 T-cell clones

During the first month of the research stay, I trained on how to generate and isolate WT1-reactive T-cell clones. First, I isolated peripheral blood mononuclear cells (PBMCs) from whole blood samples of Japanese donors. Then, the PBMCs were primed with human leukocyte antigen (HLA) class II-restricted $WT1_{332}$ peptide and cultured in the presence of interleukin (IL)-2. $WT1_{332}$-reactive CD4 T cells were monitored and sorted by upregulation of epitope-specific activation marker CD154. I expanded the sorted cells with phytohemagglutinin (PHA), IL-2, and irradiated PBMCs. Next, I screened T-cell clones for cytokine production to confirm $WT1_{332}$ specificity. Finally, I isolated the *TCRα* and *TCRβ* genes from screened T-cell clones by the 5′-RACE PCR method. The isolated genes were inserted into cloning vectors.

## Work package 2: Generation of high-avidity WT1-reactive CD8+ T-cell clones

During the second month, I generated HLA-A2-restricted WT1-specific CD8 T-cell clones with high avidity for their epitope. Therefore, I isolated PBMCs from healthy Japanese donors. Samples were stained with WT1 peptide/HLA-A2 tetramers for $WT1_{187}$, $WT1_{235}$, and $WT1_{239}$ peptide-reactivity. Then, I sorted the tetramer-positive CD8 T cells. I expanded those CD8 T-cell clones using irradiated allogeneic PBMCs in the presence of IL-2 and PHA. The use of allogeneic PBMCs allowed the generation of allo-restricted high-avidity peptide-specific T cells. To confirm the specificity of the T-cell clones, I stained them with WT1/HLA-A2 tetramers. For the validation of their high avidity against the target peptide, I screened the T-cell clones for those capable of reacting towards Raji cells that have been engineered to express HLA-A2 and WT1 proteins. The screening was done by analyzing intracellular levels of cytokines interferon gamma (IFN-$\gamma$) and tumor necrosis factor alpha (TNF-$\alpha$). Finally, as described in Work package 1, the TCR genes of high-avidity WT1-reactive T cells were isolated and cloned into appropriate vectors.

## Work package 3: Sub-cloning of T-cell sequences

In a final step, I developed plasmid vectors with the TCR sequences that will be used at LEH. Briefly, the wild type sequences of the cloned TCR genes were codon optimized. Then, to have both *TCR*$\alpha$ and *TCR*$\beta$ sequences in the same construct, the codon-optimized *TCR* sequences were linked by using the sequence of a self-cleaving 2A peptide. Finally, I subcloned the constructs into the SpeI and XhoI sites of the pST1 plasmid. This plasmid is a suitable vector for the production of in vitro transcribed mRNA for eventual electroporation of T cells.

# Figures from the research visit

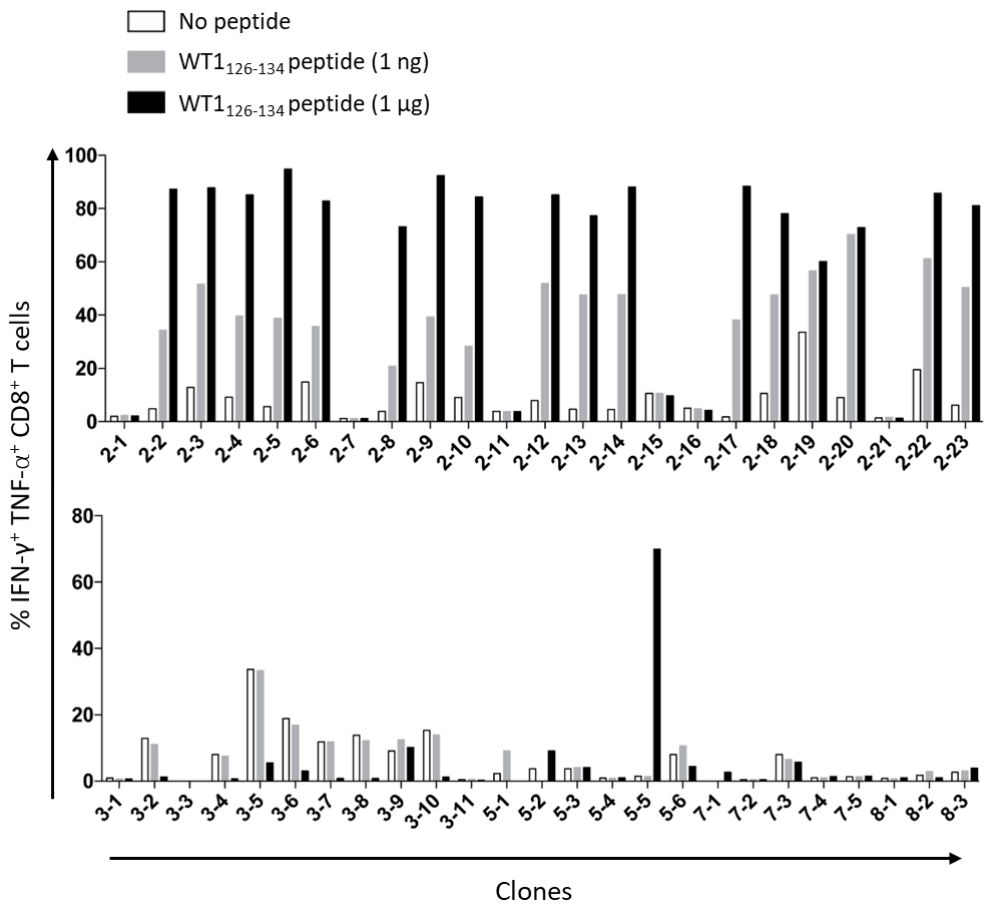


**Figure 1. WT1$_{126\text{-}134}$-reactive T-cell clone screening.**

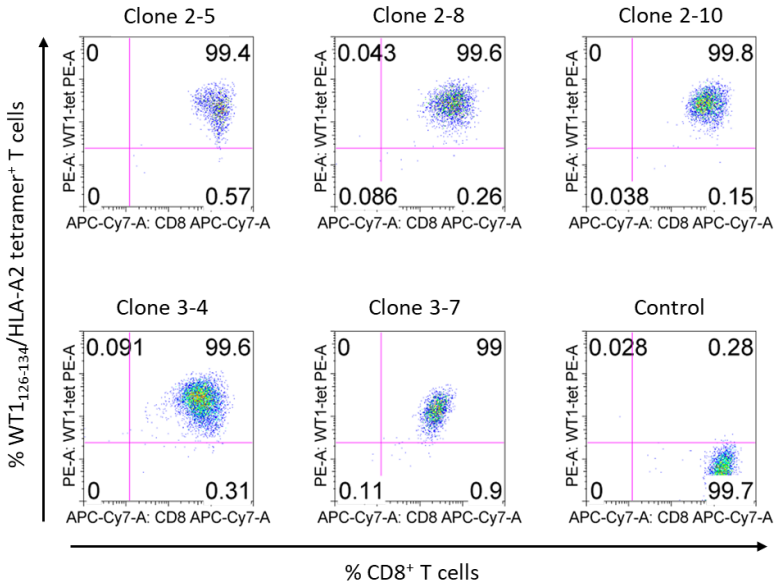


**Figure 2. WT1$_{126-134}$/HLA-A2-specific T-cell clone screening.**

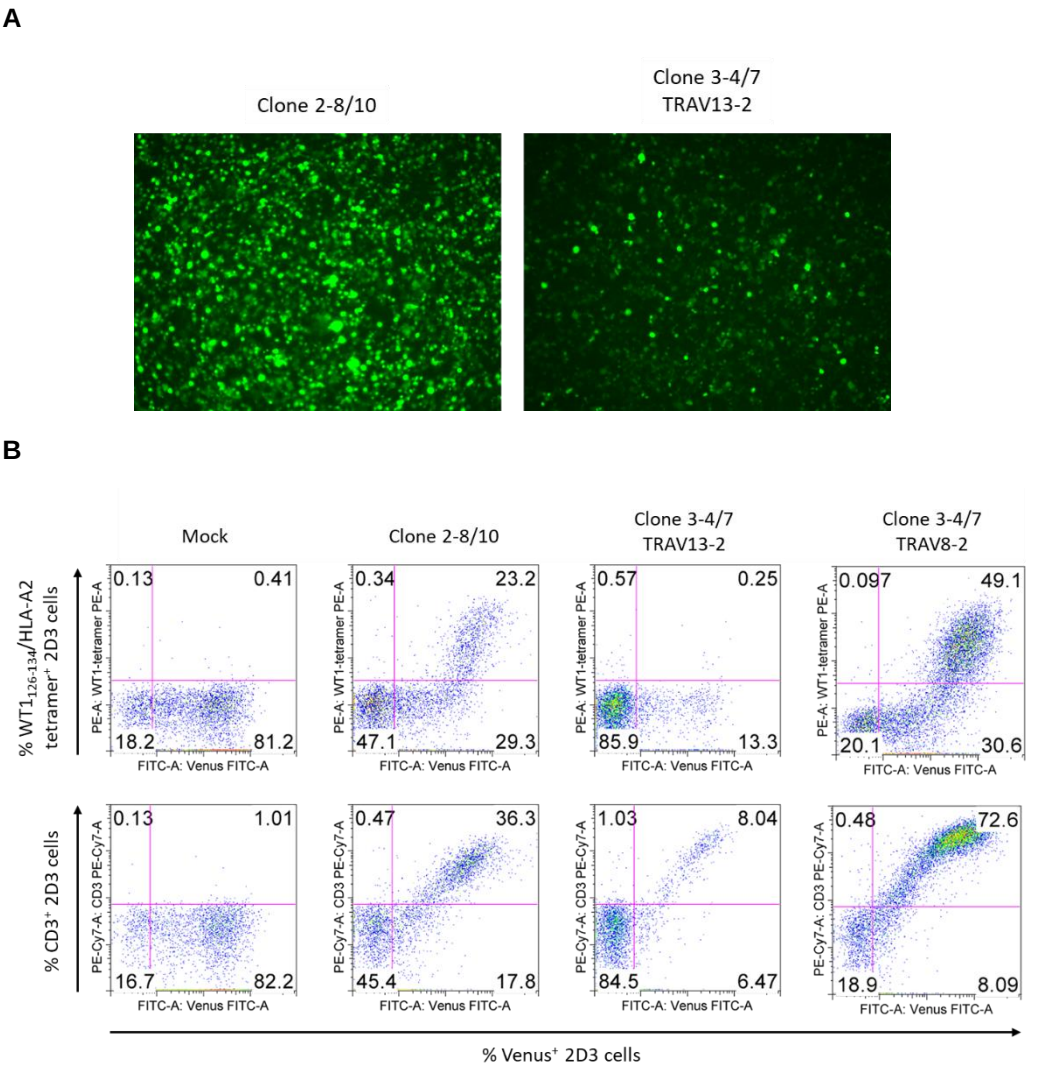


**Figure 3. TCR expression in TCR-transduced Jurkat-derived 2D3 cells.**

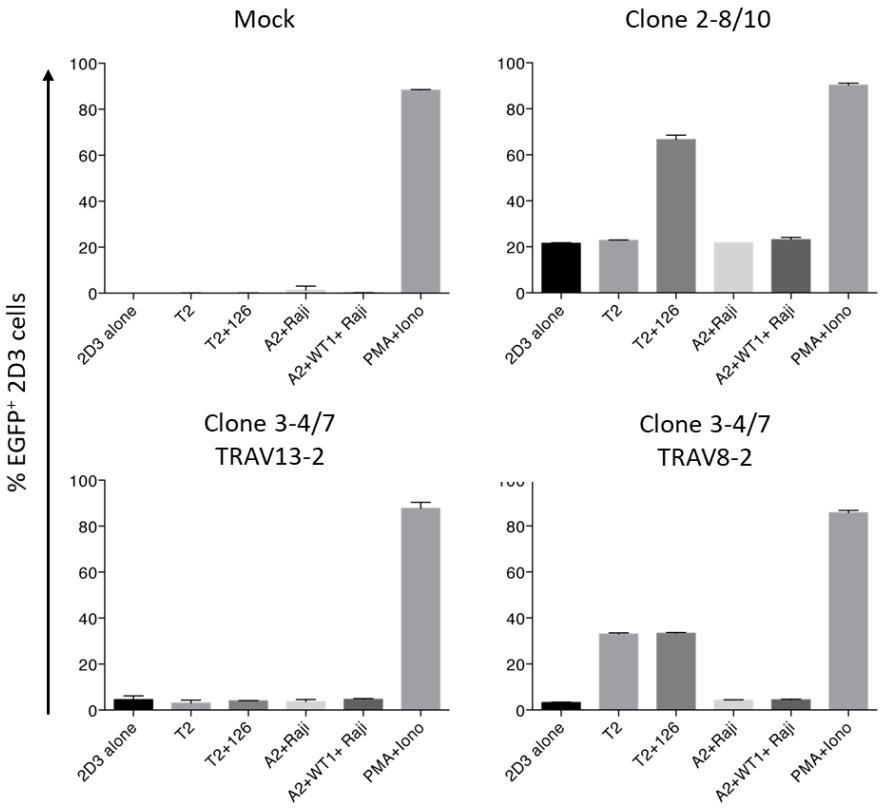


**Figure 4. WT1 specificity of allo-restricted TCR-transduced Jurkat 2D3 cells.**

# Conclusion

As part of my doctoral studies, I wanted to learn an essential technique for my project: how to isolate and clone T-cell receptors that can recognize tumor antigens. The best option to do this was to travel to the laboratory of our collaborators in Japan. They have extensive experience on this topic, and the possibility of learning new techniques and about other working environments was very appealing for me. I also chose the group of Prof. Yusuke Oji because of the international relevance in my field of study and the long-standing collaboration between our laboratories. The partnership was started several years ago by Prof. Zwi Berneman (head of LEH) and Prof. Haruo Sugiyama, former leader of the Japanese group and a pioneering researcher in the biology and the use of Wilms' tumor 1 protein as a cancer vaccine, protein that he discovered in 1992. Moreover, the University of Osaka is ranked amongst the best universities in Japan. Therefore, the research stay was a very attractive opportunity to further enhance our collaboration and to learn different techniques in an outstanding environment.

The research visit focused on the generation of allogeneic and high-avidity TCRs specific for tumor antigens using HLA-mismatched donors. In particular, I learned how to isolate TCRs from T-cell clones that are reactive towards tumor cells that express WT1 epitopes. The WT1 protein is a tumor-associated antigen found in many hematological malignancies. The TCR sequences were analyzed and cloned into vectors for the posterior subcloning into plasmid vectors suitable for in vitro transcription of mRNA. These mRNAs contained the sequences of the TCR $\alpha$ and $\beta$ chains for future engineering of T cells at LEH. This research stay allowed me to learn many techniques that are essential to my field of study. The know-how that I obtained during the research stay will be vital for current and future research projects. Moreover, the techniques and protocols that I learned can also be applied to other antigens and malignancies, allowing me to open additional lines of research in the future, since adoptive transfer of engineered T cells with cancer-specific receptors is currently one of the most promising cancer immunotherapies.

**Success is walking from failure to failure with no loss of enthusiasm.**

— Winston Churchill

# Annex II

## Cellular immunotherapy: A clinical state-of-the-art of a new paradigm for cancer treatment

This annex has been published in:

Rodríguez Pérez A*, Campillo-Davo D*, Van Tendeloo VFI, Benítez-Ribas D.
*Clinical and Translational Oncology* (2020); 22(11):1923-1937.

*These authors contributed equally to this study.

# Abstract

Cancer immunotherapy has opened a new chapter in Medical Oncology. Many novel therapies are under clinical testing and some have already been approved and implemented in cancer treatment protocols. In particular, cellular immunotherapies take advantage of the antitumor capabilities of the immune system. From dendritic cell-based vaccines to treatments centered on genetically engineered T cells, this form of personalized cancer therapy has taken the field by storm. They commonly share the ex vivo genetic modification of the patient's immune cells to generate or induce tumor antigen-specific immune responses. The latest clinical trials and translational research have shed light on its clinical effectiveness as well as on the mechanisms behind targeting specific antigens or unique tumor alterations. This review gives an overview of the clinical developments in immune cell-based technologies predominantly for solid tumors and on how the latest discoveries are being incorporated within the standard of care.

# Introduction

The field of cancer immunotherapy has opened new opportunities for the treatment of cancer patients. The development of new strategies, center on unleashing the breaks of the immune system to attack tumor cells. All these efforts have produced excellent results in preclinical and clinical studies for multiple types of malignancies [1], which have led to the recent approval of some of these therapies by regulatory agencies. One of the most promising types of therapies is the ex vivo modification and transfer of immune cells to either induce the activation of the patient's immune system or to redirect their specificity towards a tumor-associated antigen (TAA) [2]. Generally, TAAs are proteins that are only found in tumors or that are aberrantly expressed in tumors compared to healthy tissues [3]. This fact makes them an attractive tool to specifically target tumor cells (on-target on-tumor recognition), avoiding the attack of normal cells after administration. Immune cell-based therapies are classically divided into two categories, active and passive therapies. In the first case, active approaches take advantage of the potent antigen-presenting capacity of dendritic cells (DCs) to activate the host immune system by loading them with TAAs. Therefore, peptides derived from those TAAs will be presented to circulating T cells, promoting the antitumor activity of tumor-specific T cells. In the second type, T cells are engineered with receptors that specifically target a TAA. In general, autologous DCs or T cells are isolated from the patient by leukapheresis and modified ex vivo before the cells are administered back into the patient in what is referred to as personalized therapy (**Figure 1**). In this review, we discuss the latest clinical advances in immune cell-based therapies with a primary focus on solid tumors and how current discoveries are already being incorporated into standard of care protocols, e.g., in refractory hematological malignancies.

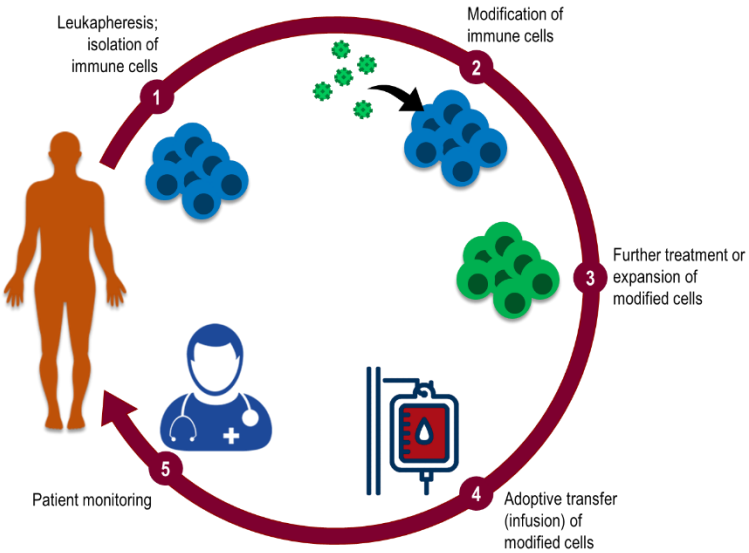


**Figure 1. Basic model of cellular immunotherapies.** In a first step, immune cells from the patient are isolated after Leukapheresis. Second, the immune cells are modified with tumor-associated antigens (TAAs) in the case of dendritic cells (DCs) or receptors specific for those TAAs, in the case of T cells. After modification, the immune cells may be further modified or expanded prior to infusion back into the patient. Finally, after treatment, the patient is monitored for any signs of adverse effects from the therapy.

# Dendritic cell-based cancer vaccines

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that can display TAA derived peptides on both major histocompatibility complex (MHC) class I and class II molecules to CD8-positive and CD4-positive T cells, respectively. DCs have been extensively used as cell-based cancer vaccine due to their exceptional ability to boost antigen- specific immune responses (**Table 1**). This strategy has previously proved effective in different preclinical trials for multiple types of hematological and solid tumors. However, despite showing significant benefit evidence when tested as monotherapy into clinical trials after. Where overall survival was measured as endpoint, minimal or insignificant improvement was shown. Thus, current studies are further exploring DC-based vaccines combined with different standards of care for follicular lymphoma [4], multiple myeloma [5], acute myeloid leukemia [6], melanoma, glioblastoma multiforme, renal cell cancer, and prostate cancer [7, 8] aiming to enhance survival rates [9]. In the meantime, preclinical studies are working on the next generation of DC vaccines, for example, by increasing their immunogenicity to improve the number and function of effector lymphocytes using various maturation cocktails,

by isolating different precursor cells or by modifying products with diverse genetic techniques [10].

## Solid tumors

### Prostate cancer

Prostate cancer (PCa) is one of the leading causes of mortality among the male population worldwide [11]. Among the biomarkers commonly used for the diagnosis of prostate cancer [12], prostatic acid phosphatase (PAP) is a glycoprotein expressed in the epithelial cells of prostate tissue and present in 95% of PCa patients [13]. Taking advantage of the high frequency of PAP-expressing PCas, the first DC-based therapy licensed for cancer treatment in 2008, was designed to test responses against PAP by culturing DCs with a recombinant fusion protein consisting of PAP and the granulocyte-macrophage colony-stimulating factor (GM-CSF). This therapy, called sipuleucel-T (Provenge, Dendreon Corporation), was approved after being tested in trials for the treatment of asymptomatic metastatic castration-resistant prostate cancer (mCRPC) [8, 14, 15]. The latest study, a randomized placebo- controlled phase III study, called Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT), involved multiple centers for a total of 512 patients (NCT00065442, [15]). This study served as the basis for FDA approval in 2010, it showed that patients receiving the DC vaccine had a significant increase in OS of 4 months compared to the placebo control group. In other phase II trials, sipuleucel-T has been evaluated compared to standard of care or in combination with chemotherapy, radiotherapy, or hormone deprivation regiments (NCT01807065, NCT01981122). In more recent combinatorial trials, sipuleucel-T is under study to investigate the added effect of different immune checkpoint inhibitors to the immune response generated with the DC vaccine (NCT01804465).

### Glioma

Gliomas are primary tumors that affect the central nervous system [16]. The exact origin of this type of tumors is still under debate, however, they are thought to be derived from glial or neural precursor cells and have been historically categorized according to their histology, location, differentiation status, and anaplastic features [17]. The high-grade malignant glioblastoma multiforme (GBM), accounts for 60–70% of gliomas and is characterized for its rapid growth and aggressiveness [18]. One meta-analysis, by Polyzoidis and colleagues reviewed data from twenty-two clinical studies [19]. 90% of which, were exploratory trials, where DCs were mainly pulsed with either tumor lysates or peptides. Then administered and analyzed after different standard of care protocols that included surgery, radiation, and chemotherapeutic agents in newly-diagnosed and recurrent GBM patients. The pooled observations showed that, although

DC treatment reported induction of immune responses, it had moderate mean OS advantages, particularly for newly diagnosed patients [19]. To date, there is still at least one phase III trial evaluating the additional effect of autologous vaccination with DCs pulsed with tumor lysates in combination with chemotherapy after standard of care for newly diagnosed glioblastoma patients [20]. An interim analysis of this study revealed that median OS (mOS) was 23.1 months after surgery for the intent-to-treat population, of which around 90% eventually received the DC therapy [20]. Compared to standard of care that supposes approximately a 44% increase in mOS after surgery. Importantly, a proportion of the individuals showed extended survival that was not linked to previously known prognostic factors. However, a definitive analysis after completion of the study will be needed to assess if survival periods could be duly extended. Renal cell carcinoma Renal cell carcinoma (RCC) represents one of the most frequent types of cancer worldwide and the most common type of renal cancer in adults [21]. DC vaccines have been tested together with sunitinib, a tyrosine kinase inhibitor to treat RCC (NCT03226236, [22]). In contrast to glioblastomas, where the relatively good overall response rate (ORR) led to integrate DC vaccines into adjuvant combinatorial treatments, the combination of DC vaccination and sunitinib could not demonstrate clinical benefit after a second interim analysis of a phase III trial [23]. The product called Rocapuldencel-T, DC primed with patient's own tumor cell lysates, was then discontinued. Thus, current strategies mainly focus on the combination of DC-based therapies with immune checkpoint inhibitors, such as pidilizumab.

**Melanoma**

DC therapeutic vaccination has been frequently tested in patients with malignant melanoma [24, 25]. Before biological therapies and new drugs were approved for melanoma, a phase III trial by Schadendorf and colleagues showed that DC vaccination was as efficient as dacarbazine for metastatic melanoma, although there was no evidence of improved OS [24]. Presently, regardless of the huge impact of new cell-based immunotherapies, increased response rates and long survival results did not come without important toxicities, and more importantly, some patients do not show any response. Nevertheless, vaccination with autologous DC will still play a role in melanoma management as a stand-alone therapy, together with other lines of treatments (**Table 1**), or as a complementary therapy after surgery in advanced stages (NCT02718391).

**Other solid tumors**

Different groups are trying to expand DC vaccines and gather some evidence for other tumors such as sarcomas (NCT01883518, [26]), mesothelioma [27], neuroblastoma

(NCT01241162), or colorectal cancer (NCT03152565), alone or combined with systemic therapy. As an added strategy, exploratory trials exist for DC primed against cancer-testis antigens (CTAs) or tumor-associated antigens (TAAs) expressed in different solid tumors including New York esophageal squamous cell carcinoma 1 (NY-ESO-1) or Wilms' tumor 1 (WT1) in an attempt to create off-the-shelf DC vaccines that could target multiple malignancies sharing the same antigen (NCT02387125).

**Table 1. Clinical trials with DC vaccination**

| Intervention | Tumor type | Phase | Clinical trial no. |
|---|---|---|---|
| DC + Sunitinib | mRCC | III | NCT01582672 |
| DC + Dasatinib | Melanoma | II | NCT01876212 |
| Chemoradiation adding or not DC | GBM | III | NCT00045968 |
| DC + chemotherapy | Colorectal cancer | III | NCT02503150 |
| DC + CY adding or not Pidilizumab | mCRPC | RCT:II | NCT01420865 |
| DC + Ipilimumab | mCRPC | RCT:II | NCT01804465 |
| DC + Chemo/Radiotherapy | mCRPC | II | NCT01807065 |
| | | | NCT02793765 |
| DC + Enzalutamide | mCRPC | II | NCT01981122 |
| DC + Pidilizumab | mRCC | II | NCT01441765 |
| DC + Chemoradiotherapy | GBM | II | NCT00323115 |
| DC + boost RT + High dose IL-2 | mRCC | II | NCT03226236 |
| MART-1 TCR + MART-1 DC | Melanoma | II | NCT00910650 |
| DC after SCT + Pidilizumab | Multiple myeloma | II | NCT014417665 |
| DC only | Sarcomas | II | NCT01883518 |
| Adjuvant DC + Pembrolizumab | NHL | II | NCT02677155 |
| MAGE-A1, MAGE-A3, | Sarcomas and neuroblastomas | I | NCT01241162 |
| NY-ESO-1 DC after Dacarbazine | | | |
| NY-ESO-1 DC only | Ovarian melanoma | I | NCT02387125 |
| | NSCLC Sarcomas | | |
| WT1 DC + Chemotherapy | GBM | I/II/III | NCT02649582 |
| | Mesothelioma | | NCT02649829 |
| DC + Adjuvant Pembrolizumab | Melanoma | I | NCT03092453 |
| DC + Avelumab | Colorectal cancer | I/II | NCT03152565 |
| DC + Nivolumab | GBM | I/II | NCT02529072 |

Examples of clinical trials using DC vaccines in simultaneous combination with systemic therapy (target therapy: sunitinib; radiotherapy, chemotherapy, immunotherapy in the form of immune checkpoint inhibitors or adoptive cell transfer), in an adjuvant setting, or in exploratory trials with DC for new different tumor types

## Adverse events

This type of therapy is usually well tolerated and severe adverse effects are normally rare [7]. The most common adverse events involved in the treatment with DC vaccines are related to local reactions at the site of injection. Notably, there is a risk of node

rupture if the therapy is administered intranodally [7]. However, DC vaccines are mainly administered via intradermal injection, where local pain, erythema, pruritus, and irritation are the most frequent side effects. In some cases, dermal reactions can be followed by flu-like symptoms such as myalgia, arthralgia, fever, or malaise. All these adverse effects are reported as grade 1 or 2, while severe grade 3 or 4 events are extremely rare, conferring them an excellent safety profile No severe autoimmunity induction has been seen so far with the use of DC vaccines. Hence, due to the low toxicity, the quality of life is preserved with this form of treatment, which makes it an ideal candidate therapy to be used in combination with other standard of care treatments and other immunotherapies.

# Gene-modified adoptive T-cell therapy

The second form of immune cell-based therapies focuses on redirecting the effector activity of T lymphocytes towards tumor antigens in a specific manner. For this purpose, T cells are stably or transiently engineered with receptors via viral or non-viral methods. Naturally, T lymphocytes express a T-cell receptor (TCR) which confers specificity towards a particular epitope (peptide) of a protein, which is presented on MHC molecules. Via the CD8 or CD4 co-receptors, CD8 + or CD4 + T cell populations are able to discern diverse patterns of peptides bound to class I and class II HLA proteins, respectively. Therefore, TCR therapy can be applied to virtually all types of tumor antigens independent of their cellular localization. However, due to the fact that TCRs are restricted by MHC presentation, this form of therapy can only be used in patients that express a particular type of MHC molecules, limiting its applicability. In other instances, the antigen of interest may be a surface protein expressed only by certain tumor cells. For this type of antigen, T cells are engineered to express a chimeric antigen receptor (CAR). They are formed by a single chain variable fragment (scFv) of a monoclonal antibody that recognizes the surface marker limited to cancer cells. This extracellular domain is linked to a cytoplasmic signaling domain via a transmembrane region. The cytoplasmic domain is mainly formed by the CD3 zeta (ζ) chain of the TCR-CD3 complex in charge of initiating the activation cascade of the T cells upon recognition of the target antigen. Over the years, other co-stimulatory domains have been added to the CD3ζ (e.g., CD28, 4-1BB) to amplify the activation signal, giving rise to different generations of CARs. Currently, several preclinical strategies are being tested to create safer and more effective generations of CAR-T cells [28].

# CAR-engineered T cells

## Hematological malignancies

CAR-T cell therapy offers a completely new approach to cure blood cancers failing to all available therapeutic options. In particular, B-cell malignancies are one of the most common pediatric and adult hematological malignancies. An ideal candidate to target this type of cancer is the B-cell surface antigen CD19, only expressed on the B-cell lineage. Although it is also expressed in normal B cells, it is expressed at high and stable levels in tumor tissues from most patients with B-cell acute lymphoblastic leukemia (B-ALL), non-Hodgkin's lymphoma (NHL), and chronic lymphocytic leukemia (CLL).

### Acute lymphoblastic leukemia

CAR-T cells were first approved by the FDA for commercial use on 30th August 2017 for the treatment of acute lymphoblastic leukemia (ALL). It was indicated for refractory/relapsed patients under the age of 25 years failing several lines of therapy, including hematopoietic stem cell transplantation, for those who refused transplantation or for those patients without a suitable donor [29]. This product, named tisagenlecleucel, targets CD19 and it is manufactured by Novartis under the commercial name of Kymriah®. It was approved after the success of the ELIANA trial, with ORR of nearly 90%. Among the 52 patients enrolled, 34 individuals achieved complete response (CR) for over a year. The durability of the clinical response was associated with persistence of tisagenlecleucel in peripheral blood and with persistent B-cell aplasia. In the last update, a follow-up analysis revealed an ORR of 54% for 115 patients, with mOS of 11.1 months and without reaching a median duration of response [30]. In June 2018, the Committee for Human Medicinal Products (CHMP) of the European Medicines Agency (EMA) recommended the approval of tisagenlecleucel for the same indication [30].

### Non-Hodgkin lymphoma

In October 2017, a second CAR-T therapy against CD19 + B cells, axicabtagene ciloleucel, was approved by the FDA. Later also approved by the EMA, it is manufactured by Kite Pharma under the name of Yescarta ®. This product was developed for the treatment of refractory/relapsed mediastinal B-cell lymphoma, diffuse large B cell lymphoma (DLBCL), and transformed follicular lymphomas [31]. Tested in a multicenter phase II trial called ZUMA-1 involving 101 patients, it showed an ORR of 72% after a single infusion, whereas 54 patients achieved CR. The median duration of response from last report was 11.1 months without an upper interval range estimation. A median overall survival rate has not been reached, but overall survival

rate at 24 months was 50.5% [32]. Nevertheless, long-term 4-year follow-up evaluation of the previous phase I trial showed long-duration CRs [33].

### Other hematological malignancies

Currently, more CAR-T therapies are being developed for other hematological cancers [34]. This is the case in multiple myeloma (MM) [35, 36], with CARs targeting MM antigen BCMA, together with CD138 and SLAMF7. Moreover, anti-CD19 CARs are being tested beyond their approved indications: for ALL patients older than 25 years, for chronic lymphocytic leukemia or other NHLs. Withal, alternative targets are needed when expression of CD19 [34] is absent. In this direction, CD22, CD20 and inactive tyrosine-protein kinase transmembrane receptor ROR1 are under evaluation for B-cell malignancies, as well as CD33 and CD133 for myeloid diseases [37]. Adjuvant checkpoint inhibition with pembrolizumab is under phase I/II study (NCT02649829) for patients not responding to anti-CD19 CAR-T alone. The objective is to evaluate whether the combination therapy can overcome the adaptive resistance induced by tumor cells upregulating programmed death-ligand 1 (PD-L1) [38]. This combination therapy has proved to increase antitumor responses in DLBCL.

## Solid tumors

Several scientific and logistic problems arise from the use of CAR-T cells in solid tumors, mainly related to a suppressive tumor microenvironment, T-cell homing to the tumor site, and survival and persistence of CAR-T cells in the tumor [28, 37]. Preliminary studies have demonstrated interesting antitumor activity and have helped design biomarker-driven trials, with different TAAs expressed in solid tumors under investigation [39] (**Table 2**).

### Gliomas

CAR-T cells have achieved promising results for glioblastoma multiforme (GBM) in clinical trials, despite the absence of pre-conditioning regimens due to the common lymphopenia associated with previous chemoradiotherapy [40, 41]. Two antigens, the active mutant epidermal growth factor receptor (EGFRvIII) and the interleukin-13 receptor $\alpha$2 (IL-13R$\alpha$2) are specifically expressed in GBM cells [40]. Although these antigens are not heterogeneously expressed along the tumor tissue, EGFRvIII is expressed in approximately 25–50% GBM tumors and IL-13$\alpha$2 is present in all GBM. In addition to the target-dependent tumor progression, it has been shown that, after surgical resection, GBM cells develop adaptive resistance mechanisms [42]. Overall, early clinical trials targeting EGFRvIII, IL-13R$\alpha$2, and also the human epidermal growth factor receptor 2 (HER-2), have proved that CAR-T cells against GBM are safe as an adjuvant therapy [40, 41]. Not only these T cells can infiltrate tumors and become

activated but, also, they can exert antitumor activity in few patients. For example, a patient with multiple intracranial and spinal lesions that relapsed after surgical removal of three out of five cerebral lesions was treated with an anti-IL-13Rα2 CAR-T designed to target specifically a patient neoepitope [41], achieving full regression from all lesions. Other trials have studied alternative local delivery routes with good clinical responses, for instance, the intraventricular administration using a ventriculoperitoneal shunt [40, 43]. Finally, diverse first-inhuman trials are focusing on new targets such as ephrin-A2 (EphA2), however, ongoing trials will assess the benefit of next-generation constructs.

**Neuroblastoma**

Early in their development, CAR-T cells exhibited encouraging results for neuroblastoma, a pediatric cancer with a dismal prognosis and few therapeutic options [44, 45]. The main anti-neuroblastoma CAR-T target is disialoganglioside (GD2), an antigen of embryonic origin since these tumors derive from the neuroectoderm [45]. In 2011, 19 patients were treated with the anti-GD2 CART, of which 8 did not exhibit any clinical signs of disease and 3 of these received infusions as consolidation therapy [45]. The remaining patients received standard therapies, including surgery and chemoradiotherapy. This strategy is currently under investigation with next-generation constructs and with pre-conditioning regimens, already providing positive results at 1-year observation follow-up analysis [46]. Other antigens such as neural cell adhesion molecule L1 (L1-CAM) or CD171, that were formerly studied in the first-in-human trial using CAR-Ts for children [44], are also being investigated using 1st, 2nd, and 3rd generation CAR-T products in two phase I trials (NCT00006480, NCT02311621).

**Sarcomas**

Different subtypes of sarcomas, despite their heterogeneity and differences in clinical presentation and biology, express common TAAs or even unique tumor antigens. This allows the development of cellular therapies that target a broad range of sarcomas [47]. The initial promising results originated from targeting HER-2 in different refractory/ metastatic sarcomas [48] (**Table 2**). Likewise, after encouraging clinical data from GD2-targeted therapy in neuroblastoma, clinical trials are also evaluating 3rd generation anti- GD2 CAR-T constructs in sarcoma (NCT02107963). Other CAR-T therapies are exploring alternative targets such as interleukin 11 receptor alpha (IL-11Rα) and natural killer group 2D receptor ligands (NKG2D-L) in osteosarcoma, which have not yet reached clinical evaluation. Other solid tumors CAR-T cells have also been designed to target overexpressed antigens like EGFR in advanced relapsed/metastatic non-small-cell lung carcinoma (NSCLC) and in unresectable or relapsed/metastatic biliary tract cancers [49, 50]. Moreover, anti-HER-2 CAR-T cells have also been explored for biliary

tract and pancreatic cancers [51]. Lastly, the first example of CAR-Ts targeting carcinoembryonic antigen (CEA) came from a phase I trial evaluating intra-arterial infusions for colorectal cancer liver metastases [52]. A definite analysis of a different trial using anti-CEA CAR-T cells, using a systemic infusion for 10 refractory colorectal cancer patients with liver and lung metastases, showed biochemical and radiological responses [53]. Other tumors harbor organ-specific but not cancer-specific antigens. For instance, in ovarian cancer, malignant pleural mesothelioma and pancreatic cancer, mesothelin rises as an attractive tumor antigen that is not commonly expressed in healthy tissues [54]. Another example is prostate membrane antigen (PSMA) which is not only prostate-restricted, but also expressed in genitourinary cancers and tumor neovasculature. The first CAR-T trial against this protein in prostate carcinoma resulted in biochemical responses [55], although this trial was later suspended (NCT00664196). Currently, several trials are being developed using anti-PSMA CAR-T cells for prostate cancer, survival results are expected.

**Table 2. Examples of CAR-T cell therapy for solid tumors**

| Antigen | Disease | CAR preparation | Phase | Clinical trial no. | Clinical outcome |
|---|---|---|---|---|---|
| EGFRvIII | GBM | Neoadjuvant 2nd gen + TMZ | I | NCT02664363 | $n = 9$ 1 MRD > 18 m 7 SR |
| | | 3° gen CD28-41BB | I/II | NCT01454596 | $n > 100$ |
| | | 2nd generation | I | NCT01454596 | $n = 10$ 9 SD all went SR |
| IL-13Rα2 | GBM specific neoepitope | 2nd generation | I | NCT02208362 | $n = 15$ 1 PR, 4SD |
| | | Intraventricular 2nd gen | I | NCT02208362 | $n$:1 1 CR 7.5 m PFS |
| EGFR (HER-1) | Biliary tract > 50% | 2nd generation | I/II | NCT01869166 | $n = 14$ 1 CR 22 m PFS 10 SD |
| | NSCLC > 50% | 2nd generation | I | | $n = 11$ 2 PD 5 SD |
| PDL-1 | NSCLC with > 10% | 2nd generation | I | NCT03330834 | |
| CEA | Liver metastases | Intralesional | I | NCT01373047 | $n = 6$ 4 local OR 1 > 23 m SD |
| | mCRC, breast cancer Lung, GI, breast | 2nd generation | I | NCT00673322 | $n = 10$ mCRC –> 7 SD |
| | | 2nd generation | I | NCT02349724 | |
| | Adenocarcinomas | 2nd generation | II | NCT01723306 | |
| Mesothelin | MPM/ pancreatic Pancreas, ovarian | 2nd generation/ intrapleural | I | NCT01355965 | $n = 4$ 1 short PR, 1 SD |
| | | 2nd generation | I | NCT02159716 | |
| GD 2 | Neuroblastoma Soft tissue sarcomas and neuroblastoma | 1st generation EBV/GD2 39 | I | NCT00085930 | $n = 19$ 3CR > 6 w, 21 m, 60 m |
| | | 3 ° gen CD28-OX40 | I | NCT01822652 | 3 PR 1SD mOS: 329 d |
| | | 4th generation (4SCAR) 28 | II | NCT02765243 | $n = 34$ 13 SD 5 PR |
| | | 4th generation (4SCAR) | I | NCT02992210 | |
| HER 2 | Sarcoma: OS, ES, DS, NES, RMS, SS metastasized breast GBM | 1st /2nd generation | I/II | NCT01935843 | $n = 9$ 4 SD: 1 local OR. 1 CR |
| | | Intracerebral 3rd genHER2/ CMV | I | NCT00902044 | (RMS), 2 SD. mOS: 10.3 m |
| | | | I | NCT00924287 | $n = 17$ 1 PR, 7 SD |
| | Lung, GI, ovarian Biliary tract, pancreas | 2nd generation | I | NCT01109095 | $n = 11$ 1 PR (CC), 5 SD |
| | | 2nd generation | I/II | NCT02349724 | |
| | | | I | NCT01935843 | |
| PSMA | mCRPC and vessels Bladder cancer | 2nd generation | I | NCT01140373 | $n = 5$ 2 PR, 1 SD |
| | | 4th generation (4SCAR) | I/II | NCT03185468 | |
| MUC 1 | Carcinomas, gliomas | 2nd generation, intralesional | I | NCT02617134 | |
| MUC 16 (CA125) | Ovarian carcinoma Serosal cancers | Armored-TRUCK (4th generation) | I | NCT02498912 | |
| FR-α | | | I | NCT02159716 | |
| | | 2nd generation | | | |
| NKG2D-L | Ovarian, colorectal, pancreas, bladder TN breast cancers AML, MM | NKR-2 T cells | I/II | NCT03018405 | |
| VEGFR2 | Metastatic melanoma mRCC | Not found | I | NCT01218867 | |

Other targets for development of CAR-T: FAP, CD133, CAIX, GPC3, L1-CAM, EphA2, ROR1

The total number of CAR-T clinical trials including blood cancer: 245. 105 studies are Phase I/II. Main candidate targets are shown. Regarding CAR preparation, it refers to the most relevant data about CAR injection, being generation or route of delivery when intralesionally given (tested for avoiding systemic toxicity), or sequence in treatment such as neoadjuvant. Phase of clinical trial and number of identification are also shown, for some piece of example trials. Most of the trials are still undergoing, for those we have end results, clinical outcome as radiological responses are shown

## Adverse events

CAR-T therapy is accompanied by severe adverse events, which in some cases can be life-threatening for patients (**Table 4**). With regard to hematological malignancies, preconditioning chemotherapy or irradiation are needed to reduce an immunosuppressive environment and to create an empty T-cell compartment to host the CAR-T cells in the lymphoid organs [34]. Most of the studies testing CAR-Ts for blood cancers use a non-myeloablative pre-conditioning regimen with or without IL-2 infusion, allowing the engraftment and proliferation of the infused T cells, which are responsible for the long-term sequelae. One of the major adverse events is an immune reaction called cytokine release syndrome (CRS). It is caused by overactivation of CAR-T cells upon recognition of their target. CAR-T cells, together with monocytes [56],

release high amounts of pro-inflammatory products such as IL-6 and tumor necrosis factor alpha (TNF-$\alpha$). These molecules induce pyrexia, tachycardia, hypotension, myalgia, pruritus among other symptoms [32, 33]. The average time to CRS onset is around three days, with a duration of one week [57]. Usually, this secondary systemic reaction can be treated with monoclonal antibodies against the IL-6 receptor (tocilizumab) and supportive care, without affecting CAR-T effect [58, 59]. Steroids are only given if refractory symptoms occur after Tocilizumab treatment [58]. Additionally, the sudden destruction of numerous cells by CAR-Ts can cause what is known as tumor lysis syndrome (TLS). It is a metabolic condition resulting from the bloodstream release of cell detritus that generates hyperuricemia, hypocalcemia, hyperkalemia, and hyperphosphatemia [58, 59], which it is treated with prophylaxis and supportive treatment. The second most common adverse event after CRS is neurological events that are limited in time in the form of transient confusion, delirium, aphasia, and seizures. This neurotoxicity, termed CAR-T cell-related encephalopathy syndrome (CRES), is thought to be in part related to CRS [59]. Researchers identified granzyme, cell detritus levels and serum ferritin as good biomarkers to predict these symptoms [60], concluding that CRES is caused by the release of cytotoxic products into the bloodstream, changes in the blood–brain barrier (BBB) permeability to them, and intracranial immune activation [59–61]. CRES average onset occurs within the first 4 to 10 days and lasts up to two weeks. These side effects, more commonly seen in NHL than ALL [31–33], are managed depending on the severity with palliative care using high dose steroids and antiepileptic drugs, without residual consequences nor significant impairment of CAR-T antitumor activity [59, 60]. Tocilizumab is not useful for the management of CRES because it cannot pass the BBB, therefore it is only used if concurrent CRS appears [59]. In the future, clinical trials to investigate the use of anti-TNF-$\alpha$ and IL-1 blockade drugs are needed for the correct management of CRS and CRES [61]. Finally, in the case of anti-CD19 CARs, the depletion of healthy B cells expressing CD19 may cause collateral hypogammaglobulinemia, leading to lifetime passive immunization with gamma globulins for these patients [31, 33]. Moreover, a Yescarta risk evaluation reported two deaths due to the disruption of the BBB, the presence of CAR-T in the central nervous system and cerebral edema [32, 33]. For this reason, it cannot be used in the presence of cerebral lymphoma. Additionally, a hemophagocytic syndrome, an extreme adverse event, was reported in less than 1% of the patients [59]. Even though its adverse events, the risk–benefit assessment was favorable for its approval by the FDA and EMA. Nevertheless, the life-threatening acute toxicities of these cellular therapies demand intensive monitoring and early management, especially during the first weeks of treatment. Cytokine release syndrome, tumor lysis syndrome or neurotoxicity are common adverse events when

applying CAR-T therapy to blood cancers, but are not so common when treating solid tumors [45, 48, 50, 53]. Severe grade 4 effects (according to US National Cancer Institute-Common Terminology Criteria) have not been reported, only grade 3 CRS [58]. Most notably, when testing CAR-T for GBM, none of these side effects appeared [40]. A great concern for solid tumors is the destruction of normal tissues caused by inefficient cancer-specific target selection. Given the fact that there is a limited number of tumor antigens that we can address with CAR-Ts and that the majority are not unique to cancer cells, on-target but off-tumor toxicities may emerge as an autoimmune phenomenon. This fact highlights the need for an accurate target selection when developing cell immunotherapies, based on two main principles: the expression of the antigen in most of the cancer cells and absence in main vital tissues. First-in-human studies are now focused on the safety, the kinetics, and the administration route of the modified T cells to better understand this technology and exploit it in future cancer treatments.

# TCR-engineered T cells

## Solid tumors

Among the different options of cell immunotherapy to tackle solid cancers, TCR-modified T cells play an attractive role [62] (**Table 3**). TCRs can potentially target any tumor antigen, including intracellular tumor antigens, as opposed to surface antigens which are targetable by CARs. Moreover, TCRs emerge as a potential precision immunotherapy tool able to recognize unique tumor antigens, virally encoded antigens in oncovirus-driven cancers and even neoantigens as a result of the patient's own mutanome with the ability to target random somatic point mutations [38, 63, 64].

### Melanoma

First clinical trials using T cells genetically modified with TCRs were developed against melanoma [65, 66]. They targeted differentiation antigens that were identified in previous successfully treated metastatic melanoma patients with tumor infiltrating lymphocytes isolated from tumor samples, expanded ex-vivo and re-infused back into the patient [62, 67, 68]. For example, the glycoprotein 100 (gp100), a melanoma antigen involved in melanosome maturation, and the melanoma antigen recognized by T cells 1 (MART-1) are both melanoma-melanocyte differentiation antigens. The redirection of lymphocytes towards these antigens together with a lymphodepleting regimen was evaluated in metastatic melanomas that failed to previous therapies [66]. In this study, tumors regressed in multiple organs, brain, liver, lungs, and lymph nodes. Most advanced results showed that the survival of MART-1 TCR-engineered T cells could benefit from vaccination with MART-1 peptide- pulsed DCs. Proving that DC

supported in vivo T-cell expansion and boosted T-cell responses [38]. A different family of TAAs, the cancer-testis antigens, have the New York esophageal squamous cell carcinoma 1 (NY-ESO-1) as one of their best representatives for TCR-engineered T cells. NY-ESO-1 has a different pattern of tissue distribution and is expressed in around 25% of melanoma patients. This led to the first-in-human trial using TCR-T-cell therapy against NY-ESO-1 for refractory metastatic melanoma and synovial cell sarcoma patients [69]. The durable results observed on cancer regression moved forward this strategy. Currently, NY-ESO-1 is under investigation in melanoma in different phase I/II trials including anti-NYESO- 1 TCR-modified T cells combined with other immunotherapies to improve their antitumor efficacy and to extend the treatment options of solid tumors [70].

**Synovial cell sarcoma**

Synovial cell sarcoma represents around 20% of soft tissue sarcomas. This group of solid tumors is highly heterogeneous, involving fat tissue, muscle, vessels, nerves, and connective tissue. Nonetheless, specific antigens are extensively expressed in these tumors. Specific translocations characterize some sarcomas and, once these genes are translated, these cancers show unique fusion-protein antigens [47]. For instance, the SYT-SSX gene fusion in synovial cell cancer or EWL-FLI1 in Ewing sarcoma [26]. However, the generation of fusion protein-specific T cells by cloning naturally-occurring TCRs in clinical trials is still pending. Completed and ongoing clinical trials have shown promise when it comes to preliminary antitumor activity of TCR-modified T cells in sarcoma. Particularly, targeting NY-ESO-1, expressed in more than 70% of synovial cell sarcomas. Different trials that enrolled patients with progressive metastatic disease after extensive multi-modality treatments exhibited high response rates and long duration of cancer regression and proved a significant reduction of multiple lung and bone metastases [69, 71]. Additional TAAs have been identified in synovial sarcoma, including preferentially expressed antigen in melanoma (PRAME) which is expressed in all synovial cell sarcomas, but is yet to be evaluated [47].

**Other solid tumors**

Solid tumors are being included in biomarker- designed trials targeting different tumor antigens. Although NY-ESO-1 is the most frequently used tumor antigen in published or ongoing TCR-T clinical trials, especially for MHC class I epitopes, other groups are exploring the use of CD4 + helper T cells modified to express TCRs that recognize cancer peptides restricted MHC class II molecules [72]. Other studies focus on TCR-modified T cells against Wilms' tumor 1 (WT1) or the melanoma antigen gene (MAGE) family of cancer-testis antigens, which have obtained good results even after the failure of prior

antigen-specific TCR therapies [73]. Several other tumor-specific antigens, viral antigens and neoepitopes specific from common driver mutations or patient-specific mutations are also being targeted and are currently under early clinical evaluation for many different solid tumors (**Table 3**).

## Adverse events

CRS related to the adoptive cell transfer of TCR-modified T cells has been observed when targeting NY-ESO-1 in synovial cell sarcoma, melanoma, ovarian carcinoma, and myeloma [74] (**Table 4**). Nonetheless, the adverse effects of this type of therapy appear to be less frequent and less severe compared to CAR-T therapy [58, 74–76]. Previous data on safety reported that off-target side effects may occur due to TCRs that can cross-recognize epitopes from different antigens. T cell cross-reactivity for other targets is unpredictable at the moment. Therefore, first-in-human trials must carefully evaluate these off-target-related adverse events during the first infusion and perform cautious dose-escalation. In this direction, early-phase trials using RNA-transfected T cells may benefit from this transient modification of the cells for evaluating the toxicity of the introduced receptor [77]. Eventually, giving high doses of steroids may be a solution if any form of immune autoreactivity against vital tissues is suspected.

**Table 3. Update of clinical trials using TCR-engineered T cells (NIH clinical trials database)**

| Target antigen | Cancer type | Phase | Clinical trial no. |
|---|---|---|---|
| HLA-A*0201 WT1 | Advanced NSCLC and MPM 1/6 PR 1/6 SD Refractory AML, MDS *n* = 8 5 SD-> 4/5 >12m | I/III/II | NCT02408016<br>NCT02550535 |
| HLA-A*0201 HA-1 | Relapsed/refractory AML, ALL | I | NCT03326921 |
| HLA-A*0201 PRAME | Relapsed/ refractory AML, MM and MDS | I/II | NCT02743611 |
| HLA-A*0201 gp100 | Malignant melanoma 1 CR, 3 PR; n= 21 | II | NCT00509496 |
| HLA-A*0201 MART-1 | Malignant melanoma + MART1 DC vax 9/ 13 (69%) showed tumor regression results | II | NCT00910650 |
| HLA-A*0201 NY-ESO 1 | Malignant melanoma 11/20 objective response<br>4 CR 24, 40, >54, >58 m 7 PR (3-28 m)Synovial cell sarcoma 11/18 OR --> 1 durable<br>1 CR 17 m and OS > 4y. 10 PR (3-18 m) Adaptimmune®: ovarian, melanoma, NSCLC,<br>Synovial cell sarcoma: n=28, cohort 1 5 out of 12 had >2 y OR.<br>Various tumors: NSCLC, HCC, breast cancer | III<br>III<br>II | NCT00670748<br>NCT01967823<br>NCT01892293<br>NCT01343043 |
| HLA-A*0201 NY-ESO 1 | Multiple myeloma: 16/20 OR mPFS 19.6 m | II | NCT01697527 |
| HLA-A*0201 + NY-ESO-1 DC<br>HLA-A*0201 + NY-ESO-1 DC | 4 SS, 2 MM, 1 OS, 1 LS, 1 MPNS. 2/4 OR+ IPILIMUMAB. 4/6 OR. 1 CR > 3y | I | NCT02070406 |
| NY-ESO1 + Nivolumab | NY-ESO1 + solid tumors | I | NCT02775292 |
| HLA-DPB*0401 MAGE-A3<br>HLA-DPB1*0401 MAGE-A3/A6<br>KITE-718 T cells<br>HLA-A*0201 MAGE-A3<br>HLA-A*0201 MAGE-A3/1255 | CD 4+ cells, *n* =17 various solid cancer metastatic cervical cancer: 1 complete response esophageal cancer: 1 partial short response 4 m urothelial cancer: 1 partial durable response > 19m osteosarcoma: 1 partial short response 4m<br>45MAGE-A3/A6 + cancers Bladder, Renal, Breast cancer, Melanoma, Melanoma synovial sarcoma esophageal | I<br>I<br>I/II<br>I/II | NCT02111850<br>NCT03139370<br>NCT02153905<br>NCT01273181 |
| HLA-A*2402 MAGE-A4<br>HLA-A*0201 MAGE-A4$^{c1032}$ | Esophageal, H&N, melanoma, ovarian same as above including gastric, NSCLC | I<br>I | NCT02096614<br>NCT03132922 |
| HLA-A*0201 MAGE-A10<br>HLA-A*0201 MAGE-A10$^{c796}$ | Urothelial, H&N, melanoma NSCLC | I<br>I | NCT02989064<br>NCT02592577 |
| HLA-A*0201 Ag-007 Immatics ® | SCC NSCLC and H&N | I | NCT03247309 |
| HLA-A*0201 HPV-16 E6 | HPV-16 cancer: anal vaginal vulvar penis H&N | I/II | NCT02280811 |
| HLA-A*0201 E7 HPV adding or not Pembrolizumab | HPV-16 cancers | I | NCT02858310 |
| HLA-A*11:01 HERV-E | Metastatic clear cell RCC | I | NCT03354390 |
| HLA-A*0201 TGFβRII | MSI + mCRC | I/II | NCT03431311 |
| Mutated Neoantigen each n HLA | GBM, breast cancer, NSCLC, GI, GU | II | NCT03412877 |
| HLA-A*11:01 KRAS G12V | Pancreatic, mCRC, gastric, other GI | I/II | NCT03190941 |
| HLA-A*0201 AFP$^{c332}$ | HCC | I | NCT03132792 |

MHC haplotype restriction and all clinically developed antigen specificity is shown in first column along with some trial examples. Following columns address which cancers are included, the phase of clinical trial and identification number. Results of trials are shown if available

*WT1* Wilms' tumor 1, *MPM* malignant pleural mesothelioma, *AML* acute myeloid leukemia, *MDS* myelodysplastic syndrome, *SD* stable disease, *MOS* median overall survival, *OR* objective response, *NSCLC* non-small cell lung cancer, *SCC* squamous cell cancer, *HCC* hepatocellular carcinoma, *MSI+mCRC* microsatellite instability metastatic colorectal cancer, *mPFS* median progression-free survival, *H&N* head and neck cancer, *SS* synovial sarcoma, *MM* malignant melanoma, *OS* osteosarcoma, *LS* liposarcoma, *MPNS* malignant peripheral nerve sheath tumor, *HPV* human papillomavirus, *HCC* hepatocellular carcinoma, *GI* gastrointestinal, *GU* genitourinary. Nivolumab and Pembrolizumab are checkpoint inhibitors blocking PD1 and PDL1 respectively. Each *n* HLA: selection of patients regardless one specific HLA, and patient´s tailored products. AFP$^{c332}$ alpha-fetoprotein neoepitope

**Table 4. Description of antigen-derived toxicities due to autoimmune destruction of normal healthy tissues (on-target off-tumor) or antigen-unrelated reactions (off-target) by modified T lymphocytes in solid tumors**

| Target antigen | On-target off-the-shelf/ off -target adverse events: |
|---|---|
| EGFR | Grade 1–2: mucosal and skin toxicities oral mucositis, skin rash, GI hemorrhage, pruritus |
| PSMA | No antigen derived toxicities reported |
| Mesothelin | No antigen derived toxicities reported |
| EGFRvIII, IL-13Rα2 | No antigen derived toxicities reported |
| GD2 | Grade 1–2: skin rash and peripheral nerve pain |
| HER-2 | 1 episode Acute respiratory failure: high avidity scFv<br>1 episode GI hemorrhage due to metastases destruction |
| CEA | CAR-T no toxicities reported |
| | TCR-engineered: severe transient colitis |
| CAIX | Secondary cholangitis |
| NY-ESO-1 | No antigen derived toxicities reported |
| WT1 | No antigen derived toxicities reported |
| Gp100 | Skin rash, vitiligo, anterior uveitis, and tinnitus |
| MART-1 | Respiratory distress due to lung metastases immune infiltration |
| MAGE-A3 cross-recognition (off-target caused by molecular mimicry) | 2 episodes cardiac arrest due to cardiac titin antigen reactivity<br>2 episodes neurotoxicity deaths neural MAGE-A12 epitope reactivity |

*EGFR* epidermal growth factor receptor, *GI* gastrointestinal, *PSMA* prostate specific membrane antigen, *IL-13Rα2* interleukin 13 receptor alpha-2 chain, *GD2* disialoganglioside, *HER-2* human epidermal growth factor receptor 2, *scFv* single-chain variable antibody fragment, *CEA* carcinoembryonic antigen, *CAR-T* chimeric antigen receptor T cell, *TCR* T-cell receptor, *CAIX* carbonic anhydrase IX, *NY-ESO-1* New York esophageal squamous cell carcinoma-1, *WT1* Wilms' tumor protein 1, *Gp100* glycoprotein 100, *MART-1* melanoma associated antigen recognized by T cells 1, *MAGE-A3* melanoma associated antigen 3, *MAGE-A12* melanoma associated antigen 12

# Discussion

The field of cellular immunotherapy for cancer has witnessed great progress in the last decade. From antigen-loaded DC to receptor-modified T cells, multiple cell-based therapies have been developed and new tumor antigens have been discovered and targeted to fine-tune patient treatments (**Table 5**). Dendritic cell vaccination is challenged as monotherapy, giving very few benefits compared to its manufacturing scalable cost. Accordingly, the current approach is to include this technique into the medical oncologist toolbox, by combining DCs with different systemic therapies for malignancies where it showed previous survival benefit or as consolidation/adjuvant therapies for other tumors. While the potential impact of such procedures is recognized, an optimal regimen sequence has still to be found. One of the key pillars in the wave of new cellular immunotherapies is CAR-T cells, mainly for hematological malignancies, but also for solid tumors. It offers a completely new path to cure blood cancers failing

to all available therapeutic options, including allogeneic hematopoietic stem cell transplantation (allo-HSCT). It also offers an alternative to patients for which suitable donors are not available. CAR-T therapy was considered "approval of the year" by the US FDA and cancer "advance of the year" by the American Society of Clinical Oncology (ASCO), which demonstrates the revolutionary clinical importance of CAR-T cells in hematological tumors. This therapy, especially against CD19, is being rapidly developed for refractory/relapsed blood cancer, showing unprecedented clinical outcome. However, clinical responses to CAR-T cell treatment of solid tumors are generally far from satisfactory, regardless of the huge preclinical evidence in favor of this form of immunotherapy. This is probably caused by little migration and infiltration together with limited tumor-killing activity due to an immunosuppressive microenvironment in this type of tumors. Concerning clinical practice in medical oncology, there is room for cell immunotherapy to be introduced, particularly in those cases where few advances in treating solid tumor metastases have been made. Glioblastomas, sarcomas, and mesotheliomas are malignancies where new immune cell therapies do not demonstrate to be beneficial. Most of all, modest progress has been made regarding second line treatment and survival results have not recently improved over the last decade. In any case, highly specific tumor antigens are extensively expressed on these tumors, which should allow the rapid development of T-cell therapies for these tumors. Glioblastomas, sarcomas, and mesothelioma, among other solid malignancies, also have in common that current non-cellular immunotherapy drugs appear to have little efficacy. For example, immune checkpoint inhibitors may be effective for tumors that contain a high mutational load (so-called "hot" tumors) and an immune-active stroma, but they fail for the aforementioned cancer types. The latter tumors have been called "cold tumors" because they are characterized by an immunoevasive stroma and the absence of pre-existing antitumor immunity. Cellular immunotherapy alone, and most probably in combination with other forms of therapy, has the potential to boost the modest response rates of approved immunotherapies and to expand the use of immune checkpoint inhibitors for cold tumors. On the other side of the cancer-specific receptor-engineering spectrum, TCR-modified T cells can target a great diversity of epitopes compared to CAR-T cells. However, due to the fact that TCRs are restricted by MHC presentation, this form of therapy can only be used in patients that express a particular type of MHC molecules, limiting its applicability. Current ongoing trials are mostly recruiting or evaluating HLA-A*02:01-positive patients since this type of MHC class I is expressed in 20 to 40% of Caucasian population and around 10–20% in other populations, limiting the treatment options for patients with other types of MHC. Despite the promising results in very few patients, the outcomes in clinical trials have been modest. One of the reasons behind this is that most of the cancer

antigens are self-antigens. Therefore, cancer-reactive T cells are scarce due to the negative selection of T cells recognizing self-antigens in the thymus. From those T cells that can identify abnormal cells expressing TAAs, few will exhibit an avidity strong enough to fully exploit the T-cell functional abilities. Moreover, TCR-modified T cells may mispair the alpha and beta chains of the introduced and the native TCR, limiting the product activity and becoming a potential safety risk due to the unknown off-target recognition by the mispaired TCRs. Another limitation related to cellular immunotherapies is the immune escape responses from the tumor cells. In many types of malignancies, tumor cells may downregulate the expression of MHC molecules or have deficiencies in their antigen processing pathways, avoiding detection by T cells. In other cases, the eradication of the antigen-positive tumor cells allows the proliferation of those that do not express it. Finally, T-cell persistence and energy in the tumor bed is another issue that must be addressed in future clinical trials. In the meantime, this problem can be solved by administering initial high doses of T cells, by selecting certain populations of T cells like memory cells or by repeated infusions. Altogether, many different cellular immunotherapies are being developed and are under evaluation to tackle the challenges that arise for each type of malignancy. Ultimately, the main objective is to provide clinicians with better tools to address the treatment of both hematological and solid malignancies in a more specific, safe, and efficacious way.

# Conclusion

There is growing momentum in oncology for the advent of novel cellular immunotherapy involving different types of immune cells, genetic modifications, and techniques. Understanding the unique properties of each cell product, from dendritic cells to T cells, is crucial for pharmaceutical formulation and clinical evaluation, and could provide insights as to whether they could work in a synergistic and/ or complementary fashion. Regarding therapeutic DC vaccines, they have previously proved statistically significant outcomes, but small survival benefit when tested alone in phase III trials for some solid tumors. Research is now focused on integrating this therapeutic modality inside current standard of care, especially for renal cell cancer, glioblastoma multiforme, prostate cancer and melanoma. CAR-T cells currently stand out as one of the most promising cellular immunotherapies. They are being approved for some refractory/relapsed blood cancers and introduced into clinical practice in reference centers. In solid tumors, they are under early clinical evaluation and preliminary clinical evidence shows that they can have strong antitumor effects. Regarding TCR-modified T cells, first-in-human clinical trials have shown proof of

concept. They are being analyzed in phase II trials, with efficacy as an endpoint, enrolling around twenty patients or more and already showing some promising data in metastatic melanoma and synovial cell sarcomas. Altogether, these cellular therapies will provide clinicians with new mechanisms in the search for the best therapeutic solutions for cancer patients.

**Table 5. Comparison of autologous adoptive cell cancer therapies**

| | DC vaccine | TCR-engineered T cells | CAR-T cells |
|---|---|---|---|
| Type of therapy | Active | Passive | Passive |
| Aim of the technique | Boost an immune reaction against a tumor antigen | Redirect T-cell specificity against an intracellular tumor antigen | Redirect T-cell specificity against an extracellular tumor antigen |
| General techniques to prime against target antigen | Passive uptake or pulsing of peptide from target antigen or tumor cell lysates | Ex vivo genetic engineering of peripheral T cells with tumor-specific TCR | Ex vivo genetic engineering of peripheral T cells with tumor-specific CAR |
| | Viral transduction with target antigen | Viral transduction with target antigen-specific TCR | Viral transduction with target antigen-specific CAR |
| | Transfection of DNA/mRNA encoding target antigen | Transfection of DNA/mRNA encoding target antigen-specific TCR | Transfection of DNA/mRNA encoding target antigen-specific CAR |
| Antigen restriction | No MHC nor superficial restrictions when using full proteins or tumor lysates. MHC restriction for particular peptides | HLA restriction → only specific peptides presented by individual MHC haplotypes | Only superficial, cellular membrane antigens (about 10% of TAA) → no intracellular |
| | Different antigen kinetics depending on the technique used | Most TCRs investigated are HLA*0201-restricted HLA type present in 35 % of Caucasian population).<br>Other HLA:<br>0101: 30% Caucasians<br>0301: 26–28% Caucasians | No MHC epitopes → epitopes recognized by antibodies |
| | Protein/peptide: MHC processing.<br>Viral: ↓availability, ↑ priming success<br>mRNA: transient expression 3–5 days | | Need for accurate antibodies against target molecule |
| Tumor escape mechanism | Tumor-induced T-cell exhaustion | Loss of MHC expression by tumor cells | Loss of antigen expression by tumor cells |
| | | Endogenous TCR chains contamination | Tumor microenvironment in solid cancers |
| Optimization | More immunogenic DC protocols | Cloning of new epitope specific TCRs | Safer and better 4th generation CARs |
| | | Gene edited avid TCR → difficult for autoantigens | |
| Side effects | Local dermal reaction in the place of injection | Lower risk of off-target reactions and toxicity due to MHC and specific neoepitopes | Off-target reactions |
| | If intranodal injection → risk of lymph node rupture and vaccination failure | CRS, TLS | CRS, TLS.<br>On-target reactions → Hypogammaglobulinemia with anti-CD19 CAR-T |
| Promising for | Prostate, AML → only adjuvant DC | Melanoma, sarcoma (phase II) | Refractory blood cancer |
| | GBM, melanoma → combined strategies | Refractory multiple myeloma | Exploratory for solid tumors |

*TCR* T-cell receptor, *CAR* chimeric antigen receptor, *MHC* major histocompatibility complex, *HLA* human leukocyte antigen, *DC* dendritic cell, *AML* acute myeloid leukemia, *GBM* glioblastoma multiforme, *CRS* cytokine release syndrome, *TLS* tumor lysis syndrome

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Transferred NY-ESO-1 (c259)T Cells in Synovial Sarcoma. *Cancer Discov* **2018**, *8*, 944-957, doi:10.1158/2159-8290.CD-17-1417.

77. Campillo-Davo, D.; Fujiki, F.; Van den Bergh, J.M.J.; De Reu, H.; Smits, E.; Goossens, H.; Sugiyama, H.; Lion, E.; Berneman, Z.N.; Van Tendeloo, V. Efficient and Non-genotoxic RNA-Based Engineering of Human T Cells Using Tumor-Specific T Cell Receptors With Minimal TCR Mispairing. *Front Immunol* **2018**, *9*, 2503, doi:10.3389/fimmu.2018.02503.

**You can tell a lot about a person from his biography.**

— Phil Dunphy (*Modern Family*)

# Curriculum vitae

# Diana CAMPILLO DAVÓ

Date of Birth: 14th October 1987

Nationality: Spanish

E-mail: Diana.CampilloDavo@uantwerpen.be

## Languages

Spanish: native language

Valencian: native language

English: C2 level, Cambridge Proficiency Test (2019)

French: A2+/Level 3, Linguapolis-University of Antwerp (2017)

Dutch: A2+ level/Nederlands voor anderstaligen Threshold 2.1, CVO Meise-Jette (2021)

## Education

| | |
|---|---|
| Jan 2015 – Nov 2021 | Ph.D. degree in Biomedical Sciences. Faculty of Medicine and Health Sciences, University of Antwerp (Belgium).<br><br>Dissertation: "Advancing RNA-based T-cell receptor redirection of lymphocytes to improve antitumor responses in adoptive T-cell immunotherapy for acute myeloid leukemia". |
| Feb – May 2019 | Post-graduate Interdisciplinary Program in Healthcare Innovation. Institute for Interdisciplinary Innovation in healthcare, Université libre de Bruxelles (Belgium). Grade: 86.75%.<br><br>Case study: "CARTEU: a public-private partnership initiative to improve access to CAR-T therapies in Europe". Best student award. |
| Oct 2011 – Dec 2012 | M.Sc. degree in Research and Progress in Molecular and Cellular Immunology. Faculty of Medicine, University of Granada (Spain). Grade: 9.69 out of 10.<br><br>Dissertation: "Regulation of the cytotoxic action of DNA methylation inhibitors in combination therapies on leukemia T cells". Grade: 10 out of 10. |
| Sep 2005 – Sep 2011 | Licenciatura in Biology (5-Year B.Sc. degree). Faculty of Science, University of Alicante (Spain). Grade: 7.18 out of 10. |

## Professional experience

| | |
|---|---|
| Nov 2021 – Present | Postdoctoral researcher at the Laboratory of Experimental Hematology, Faculty of Medicine and Health Sciences, University of Antwerp, Belgium.<br><br>Project: Genetic engineering of T lymphocytes for cancer immunotherapy. |
| Jan 2015 – Nov 2021 | Predoctoral researcher at the Laboratory of Experimental Hematology, Faculty of Medicine and Health Sciences, University of Antwerp, Belgium.<br><br>Project: Genetic engineering of T lymphocytes for cancer immunotherapy. |
| Mar – Jul 2020 | European Commission Blue book trainee at the Life Sciences Unit of the European Research Council Executive Agency, Belgium. |
| May – Jul 2019 | Visiting Research Scholar at the Laboratory of Cancer immunotherapy, Osaka University Graduate School of Medicine, Japan. |
| Jan – Dec 2014 | Predoctoral researcher at the Institute of Parasitology and Biomedicine "López-Neyra", Spanish National Research Council, Spain and the Faculty of Medicine, University of Granada, Spain.<br><br>Project: Genetic basis of autoimmune diseases. |
| Dec 2012 – Sep 2013 | Laboratory technician at Bioarray, S.L., a Spanish technology-based company specialized in genetic testing for both clinical and research purposes. |

## Grants and awards

| | |
|---|---|
| 2021 | MeToYou Grant 2021 (€26.910). Awarded by Stichting MeToYou (Belgium).<br><br>Gilead Cell Therapy Grant (€25.000). Awarded by Gilead Sciences, Kite Pharma, and the Belgian Hematology Society. |
| 2019 | International Research Visit Travel Grant (€2.600) at the Department of Cancer Immunotherapy of the Osaka University. Awarded by the European Association for Cancer Research (EACR).<br><br>International Research Visit Travel Grant (€1.200) at the Department of Cancer Immunotherapy of the Osaka University. Awarded by the OJO call of the University Research Fund of the University of Antwerp.<br><br>Best student award for the case study entitled "CARTEU: a public-private partnership initiative to improve access to CAR-T therapies in Europe" at the |

Interfaculty and interdisciplinary program in Healthcare Innovation organized by the Free University of Brussels.

2018 Travel Grant (free registration and €500) for the 23rd Congress of the European Hematology Association. Awarded by the European Hematology Association.

2016 Travel Award (free registration and €300) for the 14th Annual Meeting of the Association for Cancer Immunotherapy. Awarded by the Association for Cancer Immunotherapy.

2015 ImmunoTools Special Award 2015 consisting of a kit of 25 antibodies and cytokines. Awarded by ImmunoTools.

2014 DOCPRO4 Ph.D. fellowship granted by the University Research Fund (BOF) of the University of Antwerp. Reference number 29639.

2013 Ph.D. fellowship granted by the Spanish Ministry of Economy and Competitiveness. Reference number BES-2013-063592 associated with project number SAF2012-34435.

**Publications (Scopus *h*-index: 9)**

| | All | Since 2016 |
|---|---|---|
| Citations | 277 | 277 |
| *h*-index | 10 | 10 |
| *i10*-index | 10 | 10 |

*Google Scholar, 19th October 2021*

1. **Campillo-Davo D**, Flumens D, Roex G, Versteven M, Van Acker HH, Fujiki F, Sugiyama H, Berneman ZN, Van Tendeloo V, Anguille S, Lion E. RNA-based co-transfer of human *CD8αβ* with WT1-specific *TCRαβ* redirects antileukemic activity of CD4 and γδ T cells towards MHC class I-restricted WT1 epitopes and boosts CD8 T-cell responses in combination with *CD3* mRNA. Under review in *Frontiers in Immunology*. JIF 2020: 7.561.

2. Versteven M, Flumens D, **Campillo-Davo D**, De Reu H, Van Bruggen L, Peeters S, Van Tendeloo V, Berneman Z, Dolstra H, Anguille S, Hobo W, Smits E, Lion E. Anti-tumor potency of short-term interleukin-15 dendritic cells is potentiated by in situ silencing of programmed-death ligands. Under review in *Frontiers in Immunology*. JIF 2020: 7.561.

3. **Campillo-Davo D**, Roex G, Versteven M, Flumens D, Berneman ZN, Van Tendeloo VFI, Anguille S, Lion E. Advances in cellular cancer immunotherapy using messenger RNA electroporation for versatile gene transfer. Under review in *Molecular Therapy – Nucleic Acids*. JIF 2020: 8.886.

4. Janssens I, **Campillo-Davo D**, Van den Bos J, De Reu H, Berneman ZN, Wens I, Cools N. Clinical-grade manufacturing of mRNA-engineered regulatory T cells. Under revision in *Cytotherapy*. JIF 2020: 5.414.

5. **Campillo-Davo D**, Anguille S, Lion E. Trial watch: Adoptive TCR-engineered T-cell immunotherapy for acute myeloid leukemia. *Cancers* (2021);13(18):4519. DOI: 10.3390/cancers13184519. JIF 2020: 6.639.

6. Elst J, Maurer M, Sabato V, Faber MA, Bridts CH, Mertens C, van Houdt M, van Gasse AL, van der Poorten MLM, de Puysseleyr LP, Hagendorens MM, Van Tendeloo VFI, Lion E, **Campillo-Davo D**, Ebo DG. Novel insights on MRGPRX2-mediated hypersensitivity to neuromuscular blocking agents and fluoroquinolones. *Frontiers in Immunology* (2021);12:668962. DOI: 10.3389/fimmu.2021.668962. JIF 2020: 7.561.

7. **Campillo-Davo D**, Roex G, Versteven M, Flumens D, Berneman ZN, Van Tendeloo VFI, Anguille S, Lion E. The ins and outs of messenger RNA electroporation for physical gene delivery in immune cell-based therapy. *Pharmaceutics* (2021);13(3):396. DOI: 10.3390/pharmaceutics13030396. JIF 2020: 6.321.

8. Elst J, Sabato V, Faber MA, Bridts CH, Mertens C, Van Houdt M, Van Gasse AL, Hagendorens MM, Van Tendeloo V, Maurer M, **Campillo-Davo D**, Timmermans JP, Pintelon I, Ebo DG. MRGPRX2 and immediate drug hypersensitivity: Insights from cultured human mast cells. *Journal of Investigational Allergology and Clinical Immunology* (2021);31(6). DOI: 10.18176/jiaci.0557. JIF 2020: 4.333.

9. Roex G, Timmers M, Wouters K, **Campillo-Davo D**, Flumens D, Schroyens W, Chu Y, Berneman ZN, Lion E, Luo F, Anguille S. Safety and clinical efficacy of CAR-T-cell therapy in multiple myeloma: a systematic review and meta-analysis. *Journal of Hematology & Oncology* (2020);13(1):164. DOI: 10.1186/s13045-020-01001-1. JIF 2020: 17.388.

10. **Campillo-Davo D**, Flumens D, Lion E. The quest for the best: How TCR affinity, avidity, and functional avidity affect TCR-engineered T-cell antitumor responses. *Cells* (2020);9(7):1720. DOI: 10.3390/cells9071720. JIF 2020: 6.600.

11. van Ens D, Mousset CM, Hutten TJA, van der Waart AB, **Campillo-Davo D**, van der Heijden S, Vodegel D, Fredrix H, Woestenenk R, Parga-Vidal L, Jansen JH, Schaap NPM, Lion E, Dolstra H, Hobo W. PD-L1 siRNA-mediated silencing in acute myeloid leukemia enhances anti-leukemic T cell reactivity. *Bone Marrow Transplantation* (2020);55(12):2308-2318. DOI: 10.1038/s41409-020-0966-6. JIF 2020: 5.483.

12. Rodríguez A*, **Campillo-Davo D***, Van Tendeloo VFI, Daniel Benítez-Ribas D. Cellular immunotherapy: a clinical state-of-the-art of a new paradigm for cancer treatment. *Clinical and Translational Oncology* (2020);22(11):1923-1937. DOI: 10.1007/s12094-020-02344-4. JIF 2020: 3.405. *These authors contributed equally to this study.

13. **Campillo-Davo D**, Versteven M, Roex G, Reu H, Heijden SV, Anguille S, Berneman ZN, Tendeloo VFIV, Lion E. Rapid assessment of functional avidity of tumor-specific T-cell receptors using an antigen-presenting tumor cell line electroporated with

full-length tumor antigen mRNA. *Cancers* (2020);12(2):256. DOI: 10.3390/cancers12020256. JIF 2020: 6.639.

14. Jessy Elst J, Sabato V, Faber M, Mertens C, Hagendorens M, De Clerck L, **Campillo-Davo D**, Van Tendeloo V, Ebo D. RNA Silencing: a model to explore the MRGPRX2-pathway in cultured human mast cells. *The Journal of Allergy and Clinical Immunology* (2019);145(2):AB249. Conference abstract. DOI: 10.1016/j.jaci.2019.12.104. JIF 2019: 10.228.

15. Timmers M, Roex G, Wang Y, **Campillo-Davo D**, Van Tendeloo VFI, Chu Y, Berneman ZN, Luo F, Van Acker HH, Anguille S. Chimeric antigen receptor-modified T cell therapy in multiple myeloma: beyond B cell maturation antigen. *Frontiers in Immunology* (2019);10:1613. DOI: 10.3389/fimmu.2019.01613. JIF 2019: 5.085.

16. Versteven M, Damoiseaux D, **Campillo-Davo D**, Van Acker H, De Reu H, Anguille S, Berneman ZN, Smits EL, Van Tendeloo VF, Lion E. Abstract B137: Preclinical evaluation of a Wilms' tumor protein 1-targeted interleukin-15 dendritic cell vaccine: T-cell activity and batch production. *Cancer Immunology Research* (2019);7(2):B137. Conference abstract. DOI: 10.1158/2326-6074.CRICIMTEATIAACR18-B137. JIF 2019: 8.728.

17. Van Acker HH, Versteven M, Lichtenegger FS, Roex G, **Campillo-Davo D**, Lion E, Subklewe M, Van Tendeloo VF, Berneman ZN, Anguille S. Dendritic cell-based immunotherapy of acute myeloid leukemia. *Journal of Clinical Medicine* (2019);8(5):579. DOI: 10.3390/jcm8050579. JIF 2019: 3.303.

18. **Campillo-Davo D**, Fujiki F, Van den Bergh JMJ, De Reu H, Smits EL, Goosens H, Sugiyama H, Lion E, Berneman ZN, Van Tendeloo VFI. Efficient and non-genotoxic RNA-based engineering of human T cells using tumor-specific T cell receptors with minimal TCR mispairing. *Frontiers in Immunology* (2018);9:2503. DOI: 10.3389/fimmu.2018.02503. JIF 2018: 6.429.

19. **Campillo-Davo D,** Roex G, Van Acker HH, Berneman ZN, Lion E, Van Tendeloo VFI. Generation of Wilms' Tumor 1 TCR-redirected CD4 and gamma-delta T cells by RNA electroporation and co-transfer of CD8 mRNA. *Human Gene Therapy* (2018);29(11):ICLE8-0029. Conference abstract. DOI: 10.1089/hum.2018.29071.abstracts. JIF 2018: 3.855.

20. Versteven M, Van den Bergh JMJ, Broos K, Fujiki F, **Campillo-Davo D**, De Reu H, Morimoto S, Lecocq Q, Keyaerts M, Berneman Z, Sugiyama H, Van Tendeloo VFI, Breckpot K, Lion E. A versatile T cell-based assay to assess therapeutic antigen-specific PD-1-targeted approaches. *Oncotarget* (2018);9(45):27797-27808. DOI: 10.18632/oncotarget.25591. JIF 2016: 5.168.

21. Van Acker HH, **Campillo-Davo D**, Roex G, Versteven M, Smits EL, Van Tendeloo VF. The role of the common gamma-chain family cytokines in γδ T cell-based anti-cancer immunotherapy. *Cytokine & Growth Factor Reviews* (2018);41:54-64. DOI: 10.1016/j.cytogfr.2018.05.002. JIF 2018: 5.458.

22. Bossini-Castillo L*, **Campillo-Davo D***, López-Isac E*, Carmona FD, Simeon CP, Carreira P, Callejas-Rubio JL, Castellví I, Fernández-Nebro A, Rodríguez-Rodríguez L, Rubio-Rivas M, García-Hernández FJ, Madroñero AB, Beretta L, Santaniello A, Lunardi C, Airó P, Hoffmann-Vold AM, Kreuter A, Riemekasten G, Witte T, Hunzelmann N, Vonk MC, Voskuyl AE, de Vries-Bouwstra J, Shiels P, Herrick A, Worthington J, Radstake TRDJ, Martin J; Spanish Scleroderma Group. An MIF promoter polymorphism is associated with susceptibility to pulmonary arterial hypertension in diffuse cutaneous systemic sclerosis. *Journal of Rheumatology* (2017);44(10):1453-1457. DOI: 10.3899/jrheum.161369. JIF 2017: 3.470. *These authors contributed equally to this study

23. Lopez-Isac E, Bossini-Castillo L, **Campillo-Davo D**, Carmona FD, Simeon CP, Carreira P, Callejas-Rubio JL, Castellvi I, Fernandez-Nebro A, Rodriguez-Rodriguez L, Rubio Rivas M, Garcia Hernandez FJ, Madronero AB, Beretta L, Santaniello A, Lunardi C, Airo P, Hoffmann-Vold A-M, Kreuter A, Riemekasten G, Witte T, Hunzelmann N, Vonk MC, Voskuyl AE, Bouwstra JDV, Shiels P, Herrick A, Worthington J, Radstake TRDJ, Martin J. AB0003 A MIF promoter polymorphism is associated with the susceptibility to pulmonary arterial hypertension in diffuse cutaneous systemic sclerosis patients. *Annals of the Rheumatic Diseases* (2017);76:1047-1048. Conference abstract. DOI: 10.1136/annrheumdis-2017-eular.1907. JIF (2017): 12.350.

24. Ruiz-Magaña MJ, Martínez-Aguilar R, Lucendo E, **Campillo-Davo D**, Schulze-Osthoff K, Ruiz-Ruiz C. The antihypertensive drug hydralazine activates the intrinsic pathway of apoptosis and causes DNA damage in leukemic T cells. *Oncotarget* (2016);7(16):21875-86. DOI: 10.18632/oncotarget.7871. JIF 2016: 5.168.

25. López-Isac E, **Campillo-Davo D**, Bossini-Castillo L, Guerra SG, Assassi S, Simeón CP, Carreira P, Ortego-Centeno N, García de la Peña P; Spanish Scleroderma Group., Beretta L, Santaniello A, Bellocchi C, Lunardi C, Moroncini G, Gabrielli A, Riemekasten G, Witte T, Hunzelmann N, Kreuter A, Distler JH, Voskuyl AE, de Vries-Bouwstra J, Herrick A, Worthington J, Denton CP, Fonseca C, Radstake TR, Mayes MD, Martín J. Influence of TYK2 in systemic sclerosis susceptibility: a new locus in the IL-12 pathway. *Annals of the Rheumatic Diseases* (2016);75(8):1521-6. DOI: 10.1136/annrheumdis-2015-208154. JIF 2016: 12.811.

26. **Campillo-Davo D**, Fujiki F, Van den Bergh JMJ, Smits EL, Sugiyama H, Van Tendeloo VFI, and Berneman ZN. Electroporation of Dicer-substrate siRNA duplexes targeting endogenous TCR enhance tumor killing activity of Wilms' tumor 1 (WT1)-specific TCR-redirected cytotoxic T cells. *Blood* (2016);128(22):813. Conference abstract. DOI: 10.1182/blood.V128.22.813.813. JIF 2016: 13.164.

27. **Campillo-Davo D**, Martín-Ibáñez J (2014). La genética de la artritis reumatoide contribuye a entender su biología y descubrir potenciales fármacos para su tratamiento. [Peer commentary on "Genetics of rheumatoid arthritis contributes to biology and drug discovery" by Y. Okada, D. Wu, G. Trynka, *et al.* Nature, 2014]. *Cuadernos de Autoinmunidad de la Asociación Andaluza de Enfermedades Autoinmunes* (2014);7(2):41-42. http://aadea.es/cuaderno-autoinmunidad-ano-7-volumen-2.

28. García Irles M, Marco de la Calle F, de la Sen Fernández ML, **Campillo Davó D**, Martínez Peinado P, Sempere Ortells JM. Redes de investigación docente universitaria: innovaciones metodológicas: Elaboración de las Guías Docentes de las asignaturas del área de Inmunología, en el marco de los nuevos planes de estudio del EEES. Universidad de Alicante (2011). ISBN 978-84-695-1151-0:350-369. http://hdl.handle.net/10045/20537.

**Participation in conferences and other scientific meetings**

36th General Annual Meeting of the Belgian Hematology Society. 29-30/01/2021. Virtual. Organization: Belgian Hematology Society.

EHA-SWG Scientific Meeting on Immunotherapy for Hematological Disorders. 19-20/11/2020. Organization: European Hematology Association.

Virtual 8th International mRNA Health Conference. 9-10/11/2020. Virtual. Organization: Interplan AG.

"Science & Bières" event for the dissemination of Science. 19/11/2019. Louvain La Neuve, BELGIUM. Organization: Association of Spanish Scientists in Belgium. Oral presentation (presenting author).

2nd International Conference on Lymphocyte Engineering. 13–15/09/2019. London, UK. Organization: Israeli Society of Gene and Cell Therapy.

10th International Symposium on the Clinical Use of Cellular Products Cellular Therapy 2019. 22–23/03/2019. Erlangen, GERMANY. Organization: Universitätsklinikum Erlangen. Poster presentation (not presenting author).

1st European CAR T Cell Meeting. 14–16/02/2018. Paris, FRANCE. Organization: European Hematology Association (EHA) & European Society for Blood and Marrow Transplantation (EBMT). Oral presentation (not presenting author).

4th CRI-CIMT-EATI-AACR International Cancer Immunotherapy Conference. 30/09–03/10/2018. New Your, USA. Organization: CRI-CIMT-EATI-AACR. Poster presentation (presenting author).

1st International Conference on Lymphocyte Engineering. 13–15/09/2018. Madrid, SPAIN. Organization: Israeli Society of Gene and Cell Therapy. Oral presentation (presenting author).

9th International Conference on WT1 in Human Neoplasia. 29–30/06/2018. Berlin, GERMANY. Organization: Charité Comprehensive Cancer Center. Oral presentation (not presenting author).

23rd Congress of the European Hematology Association. 14-17/06/2018. Stockholm, SWEDEN. Organization: European Hematology Association. Poster presentation (presenting author).

2nd Research Day on "Tumor Immunology and Immunotherapy". 14/06/2018. Leuven, BELGIUM. Organization: KU Leuven. Oral and poster presentation (not presenting author).

9th International Symposium on the Clinical Use of Cellular Products. Cellular Therapy 2017. 16-17/03/2017. Erlangen, GERMANY. Organization: Universitätsklinikum Erlangen. Poster presentation (presenting author).

58th Annual Meeting & Exposition of the American Society of Hematology. 3-6/12/2016. San Diego, USA. Organization: American Society of Hematology. Oral presentation (not presenting author).

Immunity for Health 2016. 20-21/10/2016. Gent, BELGIUM. Organization: Flanders Vaccine. Oral presentation (presenting author).

Annual BSAC meeting 2016. 21/10/2016. Gent, BELGIUM. Organization: Belgian Society for the Advancement of Cytometry. Oral presentation (presenting author).

LKI Symposium "Tumor Immunology & Immunotherapy: harnessing the immune system to fight cancer". 12-14/09/2016. Leuven, BELGIUM. Organization Leuven Cancer Institute, KU Leuven, University of Ghent, and University of Antwerp. Poster and oral presentation. Oral and poster presentation (presenting author).

14th CIMT Annual Meeting 2016: Mechanisms of efficacy in cancer immunotherapy. 10-12/06/2016. Mainz, GERMANY. Organization: CIMT - Association for Cancer Immunotherapy. Poster presentation (presenting author).

8th International Conference on WT1 in Human Neoplasia. 19-20/11/2015. Kyoto, JAPAN. Organization: Osaka University Graduate School of Medicine. Oral presentation (not presenting author).

## Student mentoring

### Ph.D. theses

Donovan Flumens (2020 – Present): Development of a combinatorial multi-epitope T-cell receptor (TCR), chimeric antigen receptor (CAR) and immunosuppressive immune checkpoint (IICP)-disrupted (MulTplex) adoptive T-cell therapy against leukemia. Faculty of Medicine and Health Sciences, University of Antwerp, Belgium.

Gils Roex (2018 – Present): Optimization of chimeric antigen receptor (CAR) design for improved cellular immunotherapy of hematological diseases. Faculty of Medicine and Health Sciences, University of Antwerp, Belgium

### M.Sc. thesis

Thomas Huybrechts (2021 – 2022): CD200-CD200R axis in leukemia: development of a CD200-specific non-signaling immune receptor to enhance leukemia-specific TCR-T-cell therapies. M.Sc. in Biomedical Sciences: Molecular mechanisms of

diseases. Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Belgium

Gils Roex (2018): Generation of TCR-redirected T cells and characterization of their antitumor activity. M.Sc. in Bioscience Engineering: Cellular and genetic engineering, Faculty of Bioengineering Sciences. Katholieke Universiteit Leuven (KU Leuven), Belgium.

**B.Sc. thesis**

Valerie Gladines (2016): Adoptive T-cell immunotherapy: successfully engineering CD8 T cells. B.Sc. in Biochemistry and Biotechnology, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Belgium.

**Professional B.Sc. thesis**

Amber Dams (2021): Improving immunotherapies against leukemia through genetically engineering T cells with immune receptors. Course: Biomedical Laboratory Technology – Pharmaceutical and Biological Laboratory. Artesis Plantijn University College of Antwerp (AP Hogeschool Antwerpen), Belgium.

## Scientific memberships

- Association for Cancer Immunotherapy (CIMT)
- Association of Spanish Scientists in Belgium (CEBE)
- Belgian Hematology Society (BHS)
- European Association for Cancer Research (EACR; also, Research ambassador)
- European Hematology Society (EHA)
- Society for Immunotherapy of Cancer (SITC)

**Oh, it's quite simple. If you are a friend, you speak the password, and the doors will open.**

— Gandalf (*The Lord of the Rings: The Fellowship of the Ring*, J.R.R. Tolkien)

**I'm glad to be with you, Samwise Gamgee…here at the end of all things.**

— Frodo (*The Lord of the Rings: The Return of the King*, J.R.R. Tolkien)

# Acknowledgements

This adventure started in 2015. Before that, I had never imagined that one day I would be moving to Belgium to do a Ph.D., but here I am at the end of it in 2021, in a country that I proudly call my second home and that welcomed me with open arms. As there is no "I" in "Team", a Ph.D. thesis is not a colossal effort of just one individual. From a kind word, a bit of chocolate (always welcome), a laugh, a piece of advice, mental support, cooking meals (physical support), to letting me vent when things did not go as planned, many many people have helped me during this journey of almost seven years.

# Members of the jury

Dear Prof. Filip Lardon, Prof. Steven Van Laere, Prof. Mirjam Heemskerk, Prof. Niels Schaft, and Dr. Sébastien Wälchli, thank you for thoroughly reviewing this thesis and for providing constructive feedback.

# Promotors/Inspirators

Viggo, I still remember the first day I came to Belgium for the job interview. At the reception of UZA, I asked for Prof. Viggo Van *Tendeluu* and the receptionist looked right at me, dead serious, and said "You mean Van *TendelOO*, right?" (over the years I have seen that I am not the only one, i.e., international conferences!). My first introduction to the Flemish language! In contrast to that lady, you were absolutely welcoming and made the transition between countries, labs, and cultures much easier. Your fascination for Spain and Spanish language and culture was always a highlight of our informal conversations, sometimes sparkled by the books you had in your drawer about slang words in Mexican Spanish ("Essseeeee"). I am immensely grateful for giving this Spanish girl one the greatest opportunities of her life, for going the extra mile when I needed help, for giving me the space to try new things, follow a gazillion of courses and conferences, and for guiding me in such kind, fun, and exceptional way. Eva, I will be forever grateful for, mid Ph.D., accepting being my co-promoter and for always being there whenever I needed your help (working together until 1 AM who?). Your enthusiasm, creativity, positivity, encouragement, and reminders to celebrate achievements have been an immense flow of energy when things got difficult. Prof. Berneman, you have been a great inspiration during my entire Ph.D. and a perfect example of a researcher passionate for science and dedicated to finding new ways of helping patients (and with an unmatched sharp eye for typos when proofreading manuscripts). Your insight, advice, and encouragement have been incredibly significant to me.

# UAntwerp colleagues & friends

My dearest **TIGrs**, each and every one of you have taught me so many different things both on a professional and personal level and, for that, I cannot thank you enough. Yannick and Johan, you were my mentors at the very beginning, when I was a total

newbie with regard to electroporation. You showed me that working hard was not at odds with having fun in the lab and that pranks were a way to bond and to enjoy the long hours in the lab. Sébastien, I have always been amazed by your work ethic and the brilliant researcher and clinician that you are, thanks for bringing some fun during the conferences in Germany and, especially, in Japan. Heleen, one the hardest workers I have ever met, you showed me that a higher level of precision and effectiveness in the lab was possible. It was great to have you as desk mate and have chit-chats about trips and holidays, research, and just a little bit of everything. George, when I knew you were coming to the lab I thought, "Finally! A second international Ph.D. student at LEH!":-P. You soon became a friend with whom I could vent my frustrations and share curiosities about Belgian, Spanish, and Syrian culture, but also with whom I could take scientific discussions to a more philosophical and abstract level. Thanks for always being there! Gils, the young padawan that soon turned into a Jedi Master. You were the first master student I had to guide, so thanks for being so patient with me when I was kind of a mess with the organization of your experiments, etc. Guiding you, both in your master as well as in your Ph.D., was and is a pleasure! Donovan, together with Gils, you were always there when I needed a hand in the lab, ready to help, especially during the pandemic. It has been a pleasure guiding you through the marvelous would of T cells and TCR engineering in the last couple of years. Now you are a TCR-T cell and CRISPR master! Maarten, during these years, you were always ready to give feedback and to engage in scientific discussion but, more importantly, you showed me that I should not forget to enjoy the pleasures of life and that, yes, work is important, but a glass of wine (or even better, champagne) always helps to see the world through rose-colored spectacles a bit more (especially when drinking rosé :-P). Philip and Laurens, the most recent additions to the group, I foresee a brilliant future for you two! Laura, the Superwoman of mRNA synthesis, and Stefanie, the Superwoman of EC dossiers, your kindness and willingness to help goes beyond words. Thank you so much for all your support in the lab, especially at the end of my Ph.D. Hans, we started in the lab at the same time, and that was a blessing for me. You are the MacGyver of the lab, the Superman of flow cytometry, and you are always ready for pranks and to be creative for gifts. Above all, you are always ready to listen when I had a problem, and to help whenever you were needed Thank you for your help all these years. My PhD years without you would have been more difficult and less fun. As a proud TIGr, nothing describes the resilience and will to succeed that I have witnessed and learned from all you during these seven years better than the words sang by Katy Perry:

*I got the eye of the tiger, a fighter*
*Dancing through the fire*
*'Cause I am a champion, and you're gonna hear me roar*
*Louder, louder than a lion*
*'Cause I am a champion, and you're gonna hear me roar*

All the other colleagues from the UZA containers from the **Laboratory of Experimental Hematology** (Marjolein, thank you sooo much with all the thesis defense-related stuff, you have been a lifesaver; **IRIS**: Nathalie, Inez, Ibo, Mats, Jasper, Yousra, Amber, Coloma, Tamara, Wai Ping (you are one of the kindest and most honest persons I know), Maxime (it is always such a pleasure working with you!), Judith (thank you for all your help with protocols and in the lab, especially at the beginning), Megha (I loved our little talks, also together with Ho Wa and George, in the kitchen!), Ann; **ECTG**: Peter, Alessandra, Deby, Elise, Tamariche, Jasmijn), **CCRG** (Griet, Tine, Sandy, Carole, Jolien, Kim De Rycke, Kim Caluwaerts, Chantal), and—now ex-UZA-containers colleagues—**OHK** (Bert (you are a role model for young and not-so-young researchers), Eline (I loved all your creativity and handmade crafts in your office and our boardgame sessions), Michel (it was always fun to be around you and you lighted up the lab), Sara, Steffi, Nadia) thanks/bedankt/gracias/merci/شكرا (George, I hope this is written correctly!)/शुक्रिया (Megha, I hope this is written correctly!)/grazie for all your support all these years. I have deeply enjoyed our work and not-related-to-work discussions, lunches (whenever I joined you at that weeeird 12 PM lunch time :-P), beers in the LEH lounge terrace (outside office hours!), pranks and challenges, and overall, spending almost 7 years of my life with you guys (That long?? Gosh!). The colleagues-from-another-lab (read it as brothers-from-another-mother :-P) from **CORE**: Evelien (one of the smartest persons I know, with an incisive eye for science and research), Jorrit, Jonas, and Elly (you three are great researchers with great minds and kind and generous people with which I have had the pleasure to collaborate and I hope to continue collaborating ;-) ) Ho Wa (you are the bestest in the whole world!!), Sanne, Astrid, Julie, and so many others that I have had the pleasure to work with or collaborate or just share some thoughts: Thanks! 😊

## Osaka colleagues & friends

Prof. Sugiyama, Prof. Oji, Prof. Fujiki, Morimoto-san, and the many wonderful colleagues from the Department of Cancer Immunology of the Osaka University Graduate School of Medicine, thank you for the fruitful collaboration that has been vital for the completion of this thesis, for providing the TCRs and other sequences, and for the warm welcome and marvelous time that I spent in your laboratory (and Japan in general). どうもありがとうございます (Dōmo arigatōgozaimasu).

## Friends

Bego, Connie y Sabina, mis tres fantásticas, qué haría yo sin vosotras. Como dice Sam en el Señor de los Anillos "I can't carry it for you, but I can carry you", y vosotras me habéis ayudado a levantarme cuando estaba abajo, me habéis escuchado, me habéis guiado y me habéis aconsejado durante todos estos años sin importar lo lejos que estuviéramos. A mis queridos teatreros españoles (Rafa, Maite G. Manzano, Jaime-Jalbano, Jaime-Jaimilo, Esther, Patrick, Ángel, Vicente, Maite de Sola, Barbolla y alguno

más que seguro me dejo en el tintero); and my dear non-Spanish thespians (Johanna, Jorge, Kristina, Marianne, Peter, Steve, Joanna, and many more), sois mi "familia Bruselense elegida". Gracias por conseguir que desconectase durante unas horas a la semana del trabajo y me lo pasara tan bien que no me importase el ir corriendo a los ensayos, con el estrés de los trenes y el cansancio de llegar tan tarde a casa. Los ratos con vosotros han hecho que no perdiera la cabeza estos años y que disfrutase de mi otra pasión más allá del laboratorio. Luciano, Caterina, Michele, Eleonora, Daniele, Sarah, you are also part of my "chosen family" from Brussels. As "university" people, you knew how difficult a Ph.D. could be and you supported and encouraged me during all these years. Thanks for our boardgame nights, our movie nights, and our dinners!

# Family

Tía Angelita, Tía Nena y Tío Antonio - Santiago y Ana - Marga, Diego, Gabriel – Demás familia (¡sois muchos para escribir todos vuestros nombres! 😊): muchas gracias por todos vuestros ánimos y todas vuestras muestras de cariño. Nuria, Dani y las peques, qué haría yo sin vosotros. Siempre que he viajado de vuelta a casa, lo primero que habéis hecho ha sido preguntarme cómo va la tesis, me habéis dado consejos y, simplemente, habéis estado ahí siempre que lo necesitaba. Nuria, eres todo un ejemplo que seguir, en particular para mi tesis doctoral, y estoy muy muy orgullosa de todo lo que has conseguido y cómo me enseñas cuál es el camino a seguir. Papá y mamá, sois mis rocas, sois mis guías. Soy lo que soy y lo que he conseguido en mi vida es gracias a vosotros. Pese a que ha sido muy duro estar lejos de casa todos estos años, sabíais que era lo mejor para mí y me habéis apoyado en cada paso del camino, no solo durante el doctorado. Sabía que, si caía, estaríais ahí para levantarme, y eso no tiene precio. Sois los padres más maravillosos del mundo y estoy inmensamente orgullosa de ser vuestra hija. Esta tesis os la dedico a vosotros. Carlos, mi *pixuxo*, |\/|/\. Habría sido imposible hacer esta tesis sin tu ayuda, así de simple y claro. Has soportado años de estrés por mi parte, de jornadas de doce horas en el laboratorio, de vernos casi nada entre semana, de fines de semana en el laboratorio, y tantas otras cosas que simplemente diré que te adoro, que mil gracias y que te quiero.

# Varia

It may seem as an odd acknowledgment; however, I must leave written record that the British music band Bastille made a big contribution to this dissertation by helping me find the motivation to write it during the—what I will name *à la* Harry Potter as—"*that-which-must-not-be-named*" (a.k.a. 2020 pandemic). Also, to my cat Maddie, for being always ready for cuddles and for playing whenever I needed a break.

To all of you I say, **thank you from the bottom of my heart**. **¡Mil gracias!** 😊

> **Dobby is free.**
>
> — Dobby (*Harry Potter and the Chamber of Secrets*, J.K. Rowling)