Designing and combining CARs for improved treatment of hematological malignancies

Gils Roex

DESIGNING AND COMBINING CHIMERIC ANTIGEN RECEPTORS

FOR IMPROVED CELLULAR IMMUNOTHERAPY OF HEMATOLOGICAL MALIGNANCIES

Thesis submitted for the degree of doctor in Medical Sciences at the University of Antwerp to be defended by

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Laboratory of Experimental Hematology (LEH)
Antwerp, 2024

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Ontwerp en combinatie van chimerische antigeenreceptoren voor verbeterde cellulaire immunotherapie van hematologische aandoeningen

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List of abbreviations

Roman	
αMEM	alpha minimum essential medium
Numerical	
7-AAD	7-aminoactinomycin D
A	
AA	amino acids
ABD	antigen-binding domain
ACT	adoptive cell transfer
AE	adverse event
ALL	acute lymphoblastic leukemia
alloSCT	allogeneic stem cell transfer
APC	allophycocyanin
ASCO	American Society of Clinical Oncology
ASCT	autologous stem cell transplantation
ASH	American Society of Hematology
ASTCT	American Society for Transplantation and Cellular Therapy
ATCC	American Type Culture Collection
autoSCT	autologous stem cell transplantation
axi-cel	axicabtagene ciloleucel
B	
B-ALL	B-cell acute lymphoblastic leukemia
BCMA	B-cell maturation antigen
BFP	blue fluorescent protein
BMMNC	bone marrow mononuclear cells
BOF	special research fund
brexu-cel	brexucabtagene autoleucel
BSA	bovine serum albumin
BV	brillian violet
C	
CAR	chimeric antigen receptor
CDR	complementary determining region
CFP	cyan fluorescent protein
CHOP	cyclophosphamide, doxorubicin, vincristine, and prednisolone
CI	confidence interval
cilta-cel	ciltacabtagene autoleucel
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CR	complete response
CRES	CAR-T-cell-related encephalopathy syndrome
CRISPR	clustered regularly interspaced short palindromic repeats

CRS	cytokine release syndrome
CSD	co-stimulatory domain
Cy	cyclophosphamide
D	
DLBCL	diffuse large B-cell lymphoma
DOR	duration of response
E	
E:T	effector-to-target
EBMT	European Group for Blood and Marrow Transplantation
EFS	event-free survival
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
EGFRt	truncated epidermal growth factor receptor
EHA	European Hematology Association
EMA	European Medicines Agency
EU	European Union
F	
FACS	Fluorescence-assisted cell sorting
FBS	fetal bovine serum
FDA	US Food and Drug Administration
FITC	Fluorescein isothiocyanate
FL	follicular lymphoma
Flu	fludarabine
FWO	Research Foundation – Flanders
G	
Gen	generation
GFP	green fluorescent protein
GPRC5D	G protein–coupled receptor class C group 5 member D
GvHD	graft-versus-host disease
H	
HD	hinge domain
HDR	homology-directed repair
HLA	human leukocyte antigen
HP	hinge pool
HTS	high throughput screening
HvGD	host-versus-graft disease
I	
i.v.	intravenous
ICANS	immune effector cell-associated neurotoxicity syndrome
ICE	immune effector cell-associated encephalopathy
ICOS	inducible T cell co-stimulator
ide-cel	idecabtagene vicleucel

IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL2R	interleukin-2 receptor
IMDM	Iscove's Modified Dulbecco's Medium
iPSC	induced pluripotent stem cells
IR	infrared
IRES	internal ribosomal entry site
ISD	intracellular signaling domain
ITAM	immunoreceptor tyrosine activation motif
IVT	in vitro transcription
K	
KO	knock-out
KotK	Kom op tegen Kanker
L	
LB	lysogeny broth
LD	lymphodepleting
LeY	Lewis Y
liso-cel	lisocabtagene maraleucel
M	
mAbs	monoclonal antibodies
MCL	mantle cell lymphoma
MGUS	monoclonal gammopathy of unknown significance
MHC	major histocompatability complex
MM	multiple myeloma
MOI	multiplicity of infection
mOS	median overall survival
mPFS	median progression-free survival
MRD	minimal residual disease
MSLN	mesothelin
N	
NC	negative control
NFAT	nuclear factor of activated T cells
NGFR	nerve-growth-factor receptor
NHL	non-Hodgkin lymphoma
NK	natural killer
NKG2D	natural killer group 2, member D
NT	neurotoxicity
NY-ESO-1	New York esophageal squamous cell carcinoma 1
O	
ORR	overall / objective response rate
OS	overall survival

OTR	oxygen transfer rate
P	
PBMC	peripheral blood mononuclear cell
PCA	principle component analysis
PCR	polymerase chain reaction
PD-1	programmed death-1
PE	phycoerythrin
PEI	polyethyleneimine
PFS	progression-free survival
PI	propidium iodide
PMBCL	primary mediastinal B-cell lymphoma
PR	partial response
R	
R/R	relapsed/refractory
rh	recombinant human
S	
scFv	single-chain variable fragment
sCR	stringent complete response
SLAMF7	Signaling lymphocytic activation molecule family member 7
T	
TACI	transmembrane activator and CAML interactor
TALEN	transcription activator-like effector nuclease
TB	terrific broth
TCR	T-cell receptor
TFL	transformed follicular lymphoma
tisa-cel	tisagenlecleucel
TMD	transmembrane domain
TRUCK	T cells redirected for universal cytokine-mediated killing
TU	transducing units
U	
uBC/HD	unique barcodes per hinge domain
UCB	umbilical cord blood
V	
VGPR	very good partial response
VH	variable heavy chain
VL	variable light chain

English summary

With almost 20 million new yearly cases, cancer remains the primary cause of premature death in the world. Hematological malignancies collectively account for 1.27 million of those cases, of which the large majority are of B-cell origin. In **Chapter 1**, a brief overview of the main types of B-cell hematological malignancies and their current treatments are discussed. The therapeutic armamentarium has grown considerably over the years, involving combinations of surgery, radiation therapy, chemotherapy, stem cell transplants, small molecules, immunomodulatory compounds and immunotherapy. Unfortunately, many patients still experience relapse with disease that becomes refractory to the standard of care. Chimeric antigen receptor (CAR-) T-cell therapy, in which T cells are armored with a synthetic tumor-specific receptor, has been able to provide hope for many of these patients. This chapter also provides a primer on the historical development of CARs, the importance of the modular CAR composition, and the current commercially available CAR-T-cell products for B-cell hematological malignancies.

PART 1 – Combining multiple target antigens for CAR therapy of B-cell hematological malignancies

The majority of CAR-T-cell therapies are directed towards the B-cell lineage marker CD19 and the plasma cell-associated marker B-cell maturation antigen (BCMA). In **Chapter 2**, we provide a 2020 summary on the clinical outcomes of the pivotal trials of the most advanced CD19 CAR-T-cell products for use in relapsed/refractory (R/R) diffuse large B-cell lymphoma (DLBCL) and R/R B-cell acute lymphoblastic leukemia (B-ALL), including axicabtagene ciloleucel (axi-cel), tisagenlecleucel (tisa-cel) and lisocabtagene maraleucel (liso-cel). Despite the heavily pretreated patient cohorts, 52-83% of patients responded to those therapies, with around 50% reaching complete response (CR). Furthermore, 1-year overall survival (OS) was 49% and 58% for tisa-cel and liso-cel, respectively, and 2-year OS for axi-cel was 50.5%. Based on these convincing results, in 2017 and 2018, axi-cel and tisa-cel were approved by the US Food and Drug Administration (FDA) for R/R DLBCL. In addition, tisa-cel also received approval for use against R/R B-ALL after demonstrating high efficacy with a CR of 81% and a 1-year OS of 76%. For all products, therapy-related toxicities, such as cytokine release syndrome (CRS) and neurotoxicity (NT), were common but generally manageable. Despite the early phase character of the trials, outcomes of BCMA CAR-T-cell therapies in R/R multiple myeloma (MM) were already promising with ORR (CR) generally ranging from 85-95% (45-82%).

In **Chapter 3**, we systematically reviewed efficacy and safety data on BCMA CAR-T cells for R/R MM in 27 studies comprising 640 patients. A meta-analysis revealed a pooled ORR of 80.5% and CR of 44.8%. CAR-T-related toxicity was frequent with 80.3% and 10.5% of patients experiencing any grade of CRS and NT. By performing a subgroup analysis, we discovered that patients with more than five prior lines of treatment (19.1% vs 2.8%) and patients older than

60 (20.5% vs 6.4%) had a higher frequency of NT. Product-specific characteristics also had a notable impact on therapeutic potency and safety. In fact, alpaca/llama-based constructs had a pooled CR rate of 71.9% and median progression-free survival (mPFS) of 19.9 months, exceeding those of human- and murine-based CARs. However, this potency was associated with significantly higher CRS rates (91%). Similarly, 4-1BB costimulation improved mPFS (12.2 months) compared to CD28 (8.0 months), but also had more reports of NT (12.8% vs 3.4%). In terms of CAR-T-cell production, it was discovered that studies using lentiviral instead of retroviral particles showed higher CR (50.6% vs 18.0%) and mPFS (12.8 vs 4.3 months) without a significant differences in safety. These findings could support the design of future BCMA CAR-T-cell products and clinical trials.

Although BCMA CAR-T-cells provide considerable improvements over conventional treatments for R/R MM, PFS curves do not present a plateau towards the end as is the case with CD19 CAR-T-cell therapies. One relapse mechanism is related to the loss of BCMA expression following CAR-T-cell therapy, which has spurred the search for additional MM-specific antigens. In **Chapter 4**, we reviewed preclinical and clinical studies evaluating target antigens other than BCMA, such as CD138, CD38, SLAMF7, GPRC5D and CD19. Of particular interest is the combined targeting of BCMA with CD19, as CD19 is expressed on a drug-resistance and disease-promoting stem cell-like subset of MM cells. It has been shown that the BCMA/CD19 dual targeting strategy deepens the response of MM patients who had autologous hematopoietic stem cell transplantation. Whether combined BCMA/CD19 targeting has similar success as a stand-alone therapy remained to be investigated.

For this reason, we investigated a BCMA/CD19 dual-CAR strategy to overcome antigen escape in **Chapter 5**. Interestingly, BCMA expression has also been described on subsets of $CD19^+$ cells in B-cell leukemia and lymphoma patient samples, expanding the potential scope of the therapy from MM alone. As product quality, and manufacturing cost and length of autologous CAR-T cells are suboptimal, we chose to use the FDA-approved NK-92 cell line as a homogeneous, readily available cell source to test our dual-CAR strategy (dual-CAR NK-92). Electroporation of BCMA- and CD19-CAR encoding mRNA resulted in dual-CAR expression in 88.1% of cells. Dual-CAR NK-92 were highly potent, lysing 80.1% of $BCMA^+CD19^+$ target cells, and 75.2% and 56.5% of $BCMA^-CD19^+$ and $BCMA^+CD19^-$ target cells, respectively. Importantly, for safety, NK-92 cell require sublethal irradiation prior to administration. We demonstrated that 10 Gy irradiation completely halts dual-CAR NK-92 proliferation, but did not meaningfully affect their CAR expression or anti-tumor capacity. In aggregate, we demonstrated efficient production of off-the-shelf BCMA/CD19 dual-CAR NK-92 that show potency against antigen escape tumor models.

PART 2 – Exploring the molecular design of chimeric antigen receptors

The CAR consists of four major domains – the antigen-binding domain (ABD), the hinge domain (HD), the transmembrane domain and the intracellular signaling domain (ISD) – which are commonly derived from natural protein sequences. This modularity theoretically allows quasi-infinite combinations of CAR domains, yet CARs currently used in the clinic are composed of a limited set derived from IgG, CD8α, 4-1BB, CD28 and CD3ζ. Hypothesis-driven, low throughput exploration of novel domains is resource-intensive and time-consuming. High throughput screening methods can serve as a more unbiased approach to simultaneously assay a large number of potential CAR domain candidates. We compiled a generalized workflow and overview of methodologies for functional library screening of CAR domains, including DNA library construction, cellular library construction, library selection and lead identification in **Chapter 6**. In addition, we comprehensively reviewed studies discovering novel CAR domains in high throughput screening campaigns and discuss important considerations, such as single integration events to prevent co-selection of dysfunctional library elements, functional properties that are relevant to the domain in question, multiple selection rounds to reach sufficient enrichment of lead candidates, and computational solutions to practical library size limitations.

In **Chapter 7** we presented unpublished data of an exploratory pilot screening campaign of a medium-size HD library by applying the CARPOOL workflow developed at MIT. Thus far, the field has largely focused on the optimization of the ABD and ISD of CARs because they directly influence CAR performance through antigen binding or antigen-dependent signaling, respectively. However, the functional role of structural CAR components is starting to be elucidated. For instance, the HD facilitates proper binding of the ABD to its antigen and can modulate antigen sensitivity through CAR multimerization. In this preliminary research, we incorporated a library of more than 100 novel HDs in a CD19 CAR backbone. Surprisingly, while we detected enrichment and depletion of individual HDs, there does not seem to be a trend towards specific HD length or subdomain groups in the context of this CD19 CAR. It also appeared that the magnitude of activation-based selection of HD was smaller than for ISDs, which implies that more rounds of selection are required.

Two limitations of pooled high throughput screening approaches are paracrine signaling between library elements, introducing confounding effects, and limited functional information per library element. Arrayed screening allows for multiparametric functional evaluation of library elements completely isolated from the others. In **Chapter 8,** we showed data of an ongoing pilot study for the development of such arrayed screen. Due to downscaling of the culture size to 96-well plate format, extensive optimization is required. We found that the use of rich Terrific Broth medium was required to achieve sufficient viral transfer plasmid yields in small culture volumes. Subsequent viral particle production resulted in titers of 1-4x10^6 TU/mL, but appeared to be dependent on HD length. Upon viral transduction, CAR-expression levels of

around 40% could be achieved in primary T cells. This could be further enriched to >80% with puromycin selection, but had a detrimental impact on CAR-T cell viability, affecting downstream applications. Although additional optimization is required, we believe this method can provide complementary data to those obtained in Chapter 7.

Finally, in **Chapter 9**, we summarize the clinical developments of CAR-T-cell therapy against CD19, BCMA and other MM antigens until 2024. Since the publication of Chapter 2 and 3 in 2020, these therapies expanded to other B-cell neoplasms and moved up in treatment order. In fact, phase 3 randomized trials versus the current standard-of-care are underway. To conclude, we address four key outstanding questions to unlock the full therapeutic potential of CAR therapy for cancer.

Nederlandse samenvatting

Met bijna 20 miljoen sterfgevallen per jaar blijft kanker de hoofdoorzaak van vroegtijdig overlijden. Hiervan worden 1.27 miljoen gevallen veroorzaakt door hematologische aandoeningen, waarvan een grote meerderheid zijn oorsprong vindt in B-cellen. **Hoofdstuk 1** geeft een kort overzicht van de meest voorkomende types van B-cel hematologische kankers en hun behandelingen voorgesteld. Over de jaren heen werd het scala aan therapieën sterk uitgebreid, gebruik makend van een combinatie van chirurgie, radiotherapie, chemotherapie, stamceltransplantatie, *small molecules*, immunomodulatorische geneesmiddelen en immuuntherapie. Helaas hervallen veel patiënten nog steeds waarbij hun ziekte ongevoelig wordt aan de standaardbehandelingen. Chimerische antigen receptor (CAR-)T-celtherapie – waarbij T-cellen uitgerust worden met een kunstmatige tumor-specifieke receptor – geeft terug hoop aan deze patiënten. Dit hoofdstuk voorziet ook een inleiding tot de ontwikkeling van CARs, het belang van hun modulaire structuur, en de CAR-T-celproducten tegen B-cel hematologische kankers die vandaag op de markt zijn.

DEEL 1 - Combineren van meerdere doelwitantigenen voor CAR-therapie tegen B-cel hematologische kankers

Het grootste deel van CAR-T-celtherapieën zijn gericht tegen de B-celspecifieke merker CD19 en de plasma cel-geassocieerde merker B-cell maturation antigen (BCMA). In **Hoofdstuk 2** geven we een samenvatting van pivotale klinische studies van de meest geavanceerde CD19 CAR-T-celproducten tegen recidief/refractair (R/R) diffuus grootcellig B-cellymfoom (DLBCL) en acuut lymfoblastische leukemie (B-ALL) in 2020, waaronder deze van de producten axicabatagene ciloleucel (axi-cel), tisagenlecleucel (tisa-cel) en lisocabtagene maraleucel (liso-cel). Ondanks de zwaar voorbehandelde patiënten, reageerden 52-83% van de patiënten op deze therapie, met rond 50% van de patiënten die een complete respons (CR) vertonen. Daarnaast behaalden tisa-cel en liso-cel een 1-jarige overleving van respectievelijk 49% en 58%, en axi-cel een 2-jarige overleving van 50.5%. Op basis van deze overtuigende resultaten werden axi-cel en tisa-cel in 2017 en 2018 goedgekeurd door de *US Food and Drug Administration* (FDA) voor R/R DLBCL. Verder ontving tisa-cel ook goedkeuring voor gebruik in R/R B-ALL nadat 81% van de patiënten een CR hadden en een 1-jarige overleving van 76% bereikt werd. Voor alle producten werden gewoonlijk bijwerkingen zoals cytokine release syndroom (CRS) en neurotoxiciteit (NT) vastgesteld, die over het algemeen goed te behandelen waren. Ondanks de vroege fase van de klinische studies rond BCMA CAR-T-celtherapieën, bedroeg de algemene responsgraad (ORR) en CR graad respectievelijk 85-95% en 42-82%.

In **Hoofdstuk 3** presenteren we een systematische review van effectiviteits- en veiligheidsdata van BCMA CAR-T-cellen tegen R/R multiple myeloom (MM) over 27 studies met 640 patiënten. Een meta-analyse van deze data gaf een algemene ORR van 80.5% en CR van 44.8%. CAR-T-gerelateerde neveneffecten waren frequent (80.3%) en 10.5% van de patiënten ervaarden CRS

en NT. Subgroepanalyse onthulde dat patiënten met vijf of meer voorgaande behandelingen (19.1% vs 2.8%) en patiënten ouder dan 60 (20.5% vs 6.4%) meer NT ervoeren. Product-specifieke eigenschappen hadden ook een opmerkelijke impact op de therapeutische kracht en veiligheid van CAR-T-cellen. Alpaca/llama-gebaseerde CARs hadden een gecombineerde CR van 71.9% en een mediaan progressievrije overleving (mPFS) van 19.9 maanden, wat de resultaten van humane- en muis-gebaseerde CARs overtrof. Dit ging echter gepaard met een significant hogere frequentie van CRS (91%). Costimulatie met 4-1BB verbeterde de mPFS ook (12.2 maanden) ten opzichte van CD28 (8.0 maanden), maar induceerde ook meer NT (12.8% vs 3.4%). In termen van CAR-T-celproductie toonden we aan dat het gebruik van lentivirale ten opzichte van retrovirale partikels voor de productie van CAR-T-cellen voor een hogere CR (50.6% vs 18.0%) en mPFS (12.8 vs 4.3 maanden) zorgden zonder significante verschillen in nevenwerkingen. De bevindingen kunnen het ontwerp van toekomstige BCMA CAR-T-celproducten en klinische studies ondersteunen.

Ondanks de aanzienlijke verbeteringen in vergelijking met conventionele behandelingen voor R/R MM, vertonen de PFS curves van BCMA CAR-T-cellen geen plateau naar het einde toe, zoals wel het geval is voor CD19 CAR-T-celtherapieën. Eén potentieel hervalmechanisme is gerelateerd aan het verlies van BCMA expressie (zogenaamde "*antigen escape*") na CAR-T-celtherapie. Deze ontdekking zette de zoektocht naar andere MM-specifieke antigenen in. In **Hoofdstuk 4** worden preklinische en klinische studies naar alternatieve target antigenen voor BCMA gereviewd, zoals CD138, CD38, SLAMF7, GPRC5D en CD19. Van bijzonder belang is het onderzoek naar het simultaan targetten van BCMA en CD19 aangezien CD19 aanwezig is op drug-resistente en ziekte-bevorderende stamcelachtige subsets van MM cellen. Eerder werd aangetoond dat dubbele targeting van BCMA en CD19 de respons verbeterde van MM patiënten die eerder een autologe stamceltransplantatie ondergingen. Of gecombineerde BCMA/CD19 targeting een gelijkaardig resultaat kan leveren als alleenstaande therapie, bleef een onbeantwoorde vraag.

Om die reden onderzochten we in **Hoofdstuk 5** een BCMA/CD19 duale-CAR strategie om *antigen escape* tegen te gaan. Het is merkwaardig dat BCMA ook op subsets van $CD19^+$ cellen in B-celleukemie werd gedetecteerd, waardoor het toepassingsgebied verder reikt dan MM alleen. Aangezien productkwaliteit, productiekost en productietijd van autologe CAR-T cellen suboptimaal zijn, kozen we om de FDA-goedgekeurde NK-92 cellijn te gebruiken als homogene, snel beschikbare bron van cellen te gebruiken om onze dual-CAR ("dual-CAR NK-92") strategie te testen. Elektroporatie van BCMA- en CD19-CAR coderend mRNA leidde tot dual-CAR expressie in 88.1% van de cellen. Dual-CAR NK-92 lyseerden 80.1% van de $BCMA^+CD19^+$ target cellen, en 75.2% en 56.5% van respectievelijk de $BCMA^+CD19^-$ en de $BCMA^-CD19^+$ cellen. Om de veiligheid te waarborgen moeten de NK-92 voor toediening een sublethale stralingsdosis krijgen. We toonden aan dat een stralingsdosis van 10 Gy voldoende was om celdeling van de dual-CAR NK-92 volledig te stoppen, terwijl de CAR expressie en

anti-tumoractiviteit niet werden aangetast. Alles tezamen toonden we de efficiënte productie van kant-en-klare BCMA/CD19 dual-CAR NK-92 die zeer potent zijn tegen modellen van *antigen escape*.

DEEL 2 – Verkennen van het moleculair ontwerp van chimerische antigeen receptoren

De CAR bestaat hoofdzakelijk uit vier domeinen – het antigen-bindend domein (ABD), het scharnierdomein (HD), het transmembranair domain en het intracellulaire signaaldomein (ISD) – dewelke gewoonlijk worden afgeleid van natuurlijke eiwitsequenties. Deze modulariteit stelt ons in staat theoretisch een quasi oneindig aantal combinaties van CAR domeinen te maken. Toch bestaan de CARs die momenteel in het ziekenhuis gebruikt worden slechts uit een beperkt aantal domeinen afgeleid van IgG, CD8α, 4-1BB, CD28 en CD3ζ. Hypothese-gedreven zoektochten naar nieuwe domeinen met *low throughput* technieken is kostelijk en traag. *High througput* screening methoden kunnen een meer onbevooroordeelde manier bieden om simultaan een groter aantal potentiële CAR domeinen te testen. We stellen een algemene workflow voor en geven een overzicht van methoden voor functionele library screening van CAR domeinen, inclusief het samenstellen van de DNA en cellulaire library, en library selectie en identificatie van lead kandidaten in **Hoofdstuk 6**. Daarnaast reviewen we de studies die nieuwe CAR domeinen ontdekten met behulp van *high throughput* screening campagnes. We bediscussiëren ook de belangrijkste overwegingen zoals de nood aan singuliere virusintegraties om co-selectie van disfunctionele library-elementen te voorkomen, de functionele eigenschappen die relevant zijn voor het CAR domein in kwestie, en computationele oplossingen voor praktische limieten in termen van de grootte van de library.

In **Hoofdstuk 7** presenteerden we niet-gepubliceerde data van een pilootstudie waarin we een HD library van gemiddelde grootte screenen met behulp van de CARPOOL workflow ontwikkeld aan MIT. Tot nu toe werd de focus voornamelijk gelegd op de optimalisatie van het ABD en het ISD van CARs omdat zij de performantie van de CAR direct beïnvloeden via respectievelijk antigenbinding of antigen-gedreven signaling. De functionele rol van structurele CAR onderdelen wordt echter steeds duidelijker. Het HD faciliteert correcte binding tussen het ABD en het antigen, en kan antigengevoeligheid moduleren via CAR multimerisatie. In dit preliminair onderzoek incorporeren we meer dan 100 nieuwe HDs in een CD19 CAR backbone. Terwijl we doorheen de selectierondes veranderingen in abundantie van individuele HDs konden waarnemen, waren er geen trends naar een bepaalde lengte of subgroep van HDs in de context van deze CD19 CAR. Het leek er ook op dat de omvang van de activatie-gebaseerde selectie kleiner was voor HDs dan ISDs, wat impliceert dat er meer selectierondes nodig zijn.

Twee beperkingen van gepoolde *high througput* screening methoden zoals besproken in hoofdstuk 7 zijn paracriene interacties tussen library elementen die elkaar kunnen beïnvloeden en de beperkte functionele informatie die per element verkregen wordt. Daarentegen laat

arrayed screening toe een multiparametrische functionele evaluatie van de library elementen volledig los van elkaar uit te voeren. In **Hoofdstuk 8** toonden we data van een lopende pilootstudie van de ontwikkeling van zulke *arrayed* screen. Uitgebreide optimalisatie is nodig door het schalen van de cultuurvolumes tot 96-well formaat. We vonden dat het gebruik van nutriëntenrijk Terrific Broth medium nodig was om voldoende opbrengst van het virale transfer plasmide te krijgen in kleine cultuurvolumes. Daaropvolgende productie van viruspartikels resulteerde in een titer van 1-4x10^6 TU/mL en leek afhankelijk van HD lengte. CAR-expressie van 40% in primaire T cellen kon met virale partikels bereikt worden. Door middel van puromycine selectie kon dit verrijkt worden tot >80%, al had dit een negatieve impact op CAR-T-cel viabiliteit met gevolgen voor verdere toepassingen. Hoewel verdere optimalisatie vereist is, geloven we dat deze methode complementaire data aan hoofdstuk 7 kan leveren.

Ten slotte vatten we in **Hoofdstuk 9** de klinische ontwikkelingen van CAR-T-celtherapieën tegen CD19, BCMA en andere MM antigenen tot 2024 samen. Sinds de publicatie van hoofdstukken 2 en 3 in 2020 werden deze behandelingen uitgebreid naar andere B-celtumoren en werden ze naar voor geschoven in de behandelingsvolgorde. Ondertussen zijn er fase 3 gerandomiseerde studies ten opzichte van de huidige standaardbehandelingen opgezet. Om af te sluiten behandelen we nog vier belangrijke openstaande vragen om het volledige potentieel van CAR therapie tegen kanker te bereiken.

Research aim and outline of the thesis

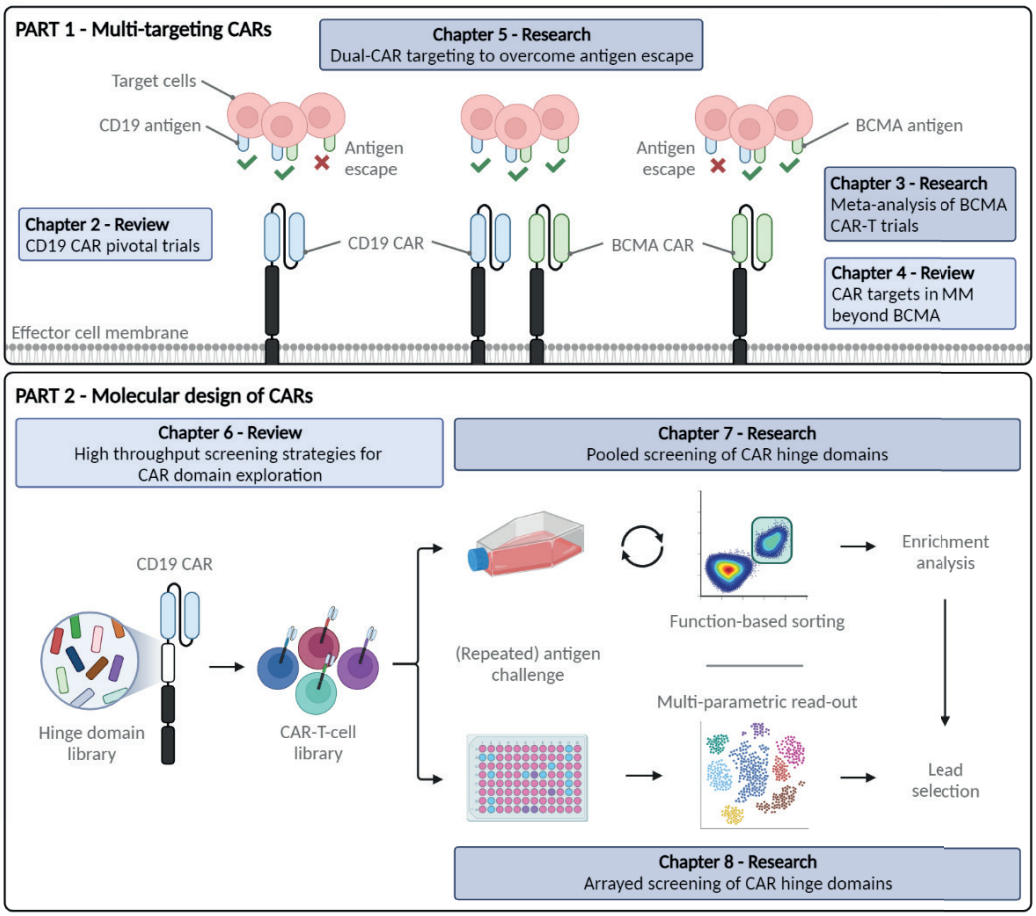


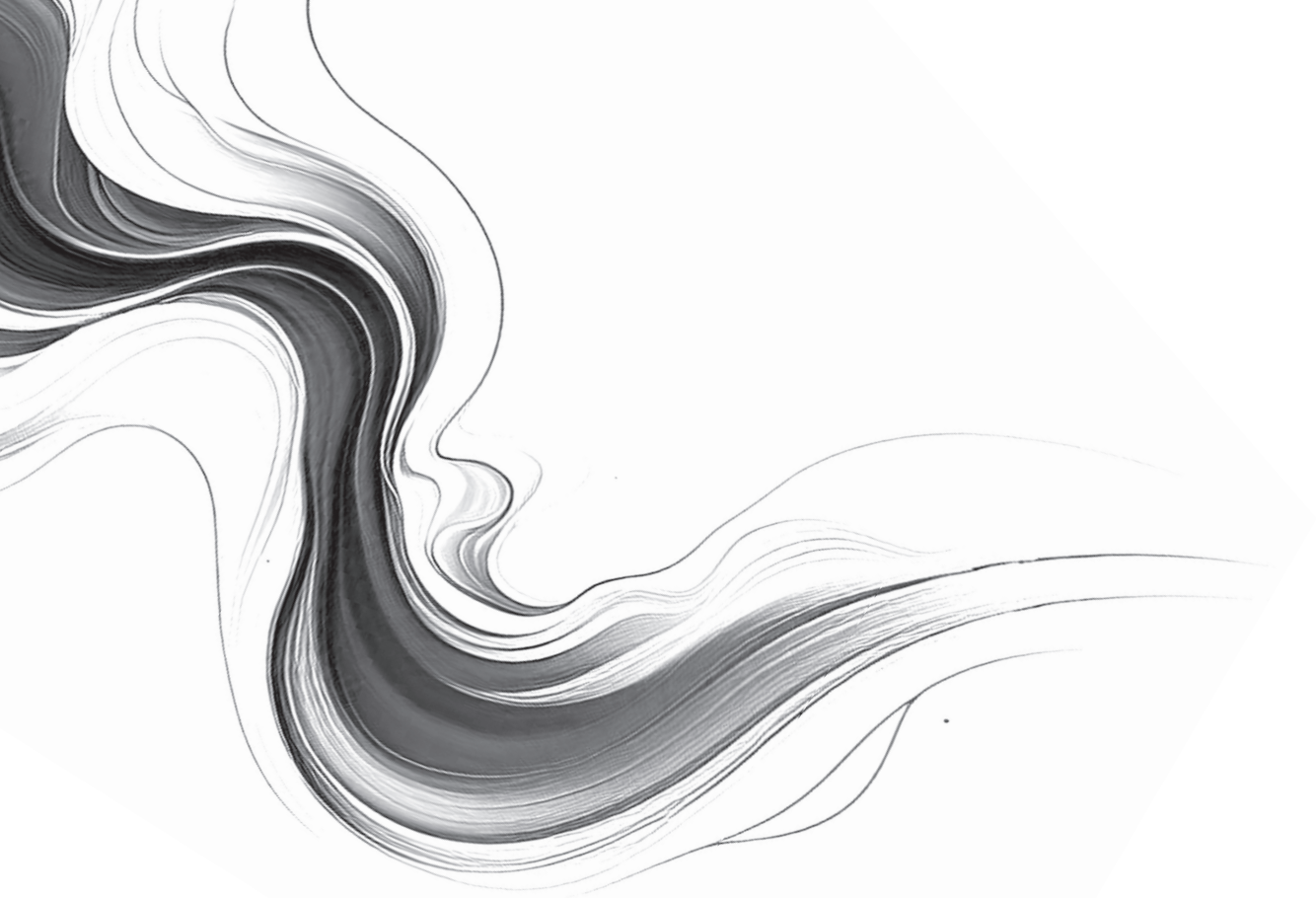
Figure 1. Graphical abstract of thesis.

The overall objective of this doctoral research is to develop a novel chimeric antigen receptor (CAR)-based cellular immunotherapy for the treatment of B-cell hematological malignancies by improving multiple aspects of the therapeutic product. We hypothesize that combining multiple target antigens addresses the issue of tumor antigen escape seen in clinical settings (part 1) and that comprehending the behavior of protein domains within a CAR framework facilitates the rational design of CARs (part 2).

In the *first part* of this thesis, particular attention is given to CAR-mediated targeting of tumor associated antigens. **Chapter 1** provides the necessary context and basic concepts required for understanding the rest of the manuscript. It discusses the human and economic impact of the rising incidence of cancer around the world and in Belgium. I highlight the shortcomings of current state-of-the-art treatments of B-cell malignancies and showcase the need for improved,

more advanced therapeutics such as CAR-T cells. An introduction to CAR design allows the reader to appreciate subtle differences in therapeutic CAR products, such as those covered in **Chapter 2**. There, I review the clinical efficacy and toxicities reported in pivotal clinical trials of CD19 CAR-T-cell therapies in relapsed/refractory B-cell malignancies. The main achievements on efficacy, but also the challenges on relapses and toxicity, and how they are being addressed are discussed. The chapter also briefly touches upon B cell maturation antigen (BCMA)-targeted CAR-T-cell products used in multiple myeloma (MM). Given the developing clinical landscape of BCMA CAR-T-cell therapies with a large diversity in clinical trial designs, we questioned which patient- and product-specific characteristics would have an impact on therapeutic potency and safety. To investigate this, I performed a systematic review and meta-analysis of BCMA CAR-T-cell therapy in MM in **Chapter 3**. The analysis highlighted aspects such as prior lines of treatment, patient age, CAR composition and CAR modification method were influential on the observed outcome and side effects. It also clearly demonstrates the high initial clinical activity of BCMA CAR-T-cell therapies, even in heavily pretreated patients. Yet, many patients relapse, partly due to the loss of target antigen. This raises the question: Which antigens can be used as an alternative to or in combination with BCMA in CAR-T-cell therapy against MM? Therefore, in **Chapter 4** I review the expression profile, function and current use as CAR-target of MM-associated antigens under preclinical and clinical investigation. I also discuss dual-targeting of BCMA with CD19 for MM. Interestingly, due to the developmental relationship between B cells and plasma cells, some overlap exists between CD19 and BCMA expression in those cell types. Subsequent to this observation, we aimed to leverage this BCMA/CD19 co-expression to combat antigen escape in both B-cell and plasma cell malignancies. Indeed, in **Chapter 5**, I demonstrate that a BCMA/CD19 dual-targeting approach is capable of lysing models of singular antigen loss in B-cell malignancies and MM. In addition, with the NK-92 cell line as a cellular vehicle, we show that this approach can serve as an off-the-shelf, safe and highly effective treatment strategy for B-cell lymphoma, leukemia and MM.

The *second part* zooms in on CAR design and the influence of adjusting individual building blocks. Quite recently, high throughput screening strategies have found their way to CAR development. **Chapter 6** provides a synthesis of current high throughput methodologies and considerations, and summarizes novel CAR domains these studies have uncovered. In this study, we discovered a paucity in screening efforts for structural CAR domains. Hence, we aimed to develop screening methodologies for the discovery of novel CAR hinge domains. **Chapter 7** shows the first results on the application of a bulk high throughput screening campaign in collaboration with the Birnbaum Lab at the Massachusetts Institute of Technology (Boston, USA). Building further on these findings, **Chapter 8** describes the ongoing optimization of the methodology for an arrayed screening protocol to enable multi-parametric readouts per library member that could be used to power predictive machine learning algorithms. Finally, in **Chapter 9**, I take a broader look at CAR-T-cell therapy in the context of B-cell hematological malignancies and discuss future perspectives and the challenges that lie ahead.



1

GENERAL INTRODUCTION

Roex G. 2024

The human and economic burden of cancer

The human body is a complex system of trillions of cells[1], each designed to fulfill specific functions within tissues and organs. Among the processes that orchestrate cellular function, cell division stands out as a tightly regulated process that depends on the cell type and environment. For example, neurons, heart cells and red blood cells do not have the ability to divide, whereas stem cells in the bone marrow and skin are capable of continuous division and differentiation into new blood cells and skin cells, respectively. Internal and external feedback loops play pivotal roles in maintaining cellular equilibrium and preventing excessive cell growth. However, disruption to this delicate balance caused by genetic, environmental, age-related, and lifestyle factors can lead to uncontrolled cell proliferation and the development of cancer.

Cancer, alongside cardiovascular diseases, stands as the primary cause of premature death in the world[2]. In 2020, approximately 19.3 million new cancer diagnoses and nearly 10 million cancer deaths were being reported[3]. Projections indicate a significant increase in annual cancer diagnoses over the next two decades, reaching 28.4 million cases, largely fueled by improved socio-economic conditions and increased life-expectancy in low and medium human development index countries[3]. Breast, lung, colorectal, prostate, and stomach cancer collectively account for nearly 50% of all cancer cases, with hematological malignancies ranking fourth with 6.6% (1.27 million) global cases.

The economic burden associated with cancer is immense. The anticipated global economic cost of cancer from 2020 to 2050 is estimated at $25.2 trillion (~€23.3 trillion; at 2017 constant prices)[4]. For Belgium the projected healthcare cost related to cancer over the same period is $120 billion (~€111 billion), translating to approximately $332 (~€307) per person per year[4]. However, this estimate may be conservative given that the calculated per capita cost in 2018 was already €577[5].

B cell malignancies and their treatment

Hematological malignancies are a collective term for cancerous disorders of blood cells. According to the World Health Organization the most frequent forms are Hodgkin (HL) and non-Hodgkin lymphomas (NHL), multiple types of leukemia, myeloproliferative neoplasms and myelodysplastic syndromes[6]. The focus of this thesis will be on B cell hematological malignancies as the majority of blood cancers are from lymphoid origin and, in turn, 75-95% of those cases originate from B cells[7, 8]. Below, several major B cell hematological malignancies and their frontline treatments are highlighted.

Lymphomas are characterized by an abnormal growth of lymphocytes in the lymphatic system. NHL comprises the majority of the lymphoma cases with tumors originating from mature B cells accounting for up to 90% of all NHL cases[9, 10]. Typically, NHLs are subdivided into aggressive lymphomas, such as diffuse large B cell lymphoma (DLBCL), and indolent lymphomas, such as follicular lymphoma (FL). Lymphomas can manifest locally or can be disseminated, making surgical resection, radiation therapy, chemotherapy and immunotherapy all viable options, often used in combination[9, 10]. For example, standard frontline treatment of DLBCL typically consists of immunotherapy with the anti-CD20 monoclonal antibody rituximab, combined with a chemotherapeutic cocktail. Patients receiving this treatment have a 5-year survival rate of 55-60%, but up to half of them relapse[11]. Fit relapsed patients can subsequently undergo salvage chemotherapy in combination with autologous stem cell transplantation (autoSCT), yet only 40% will achieve a lasting response[11]. The prognosis of patients not eligible for salvage therapy is even grimmer with only 20-30% responding to subsequent lines of therapies and a median overall survival (mOS) of approximately 6 months[12].

The basic subclassification for leukemia is based on the rate of disease progression (acute or chronic) and the leukocyte lineage (lymphoblastic or myeloid), forming four main types of leukemia: acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia and chronic myeloid leukemia. The incidence of B-cell ALL (B-ALL) is skewed towards children and young adults (median age of onset of 27 years[13]) whereas the majority of B-CLL diagnoses are found in older adults (median age of onset 70 years[14]. B-ALL is an aggressive form of leukemia, generally requiring an intense multidrug chemotherapeutic treatment[15]. Other approaches, such as tyrosine kinase inhibitors for patients with Ph-positive ALL and allogeneic stem cell transplantation for high-risk cases are recommended. Response rates for these therapies are generally high with 98% of pediatric patients achieving complete remission (CR) and 80-90% of adults showing some form or response[15, 16]. Unfortunately, whereas pediatric patients stay in long-term remission, the majority of adult patients relapse. In contrast to ALL, CLL is a slow progressive form of leukemia that is combated with a less aggressive treatment modalities. As of 2022, the oral non-chemotherapy-based combination treatment with ibrutinib and venetoclax is approved for the frontline treatment of CLL, showing progression-free survival (PFS) rates of over 74% after 42 months, far exceeding the prior standard of care[17].

Multiple myeloma (MM) is a malignancy of plasma cells that accumulates in the bone marrow. Plasma cells are terminally differentiated B cells that are specialized in the production of antibodies. There are three anti-myeloma drug classes which are commonly used in combination, including proteasome inhibitors (e.g., bortezomib), immunomodulatory drugs (e.g., lenalidomide) and anti-CD38 monoclonal antibodies (e.g., daratumumab). Although they form an improvement over prior therapies, MM patients still frequently suffer from relapse. With each relapse, drug resistance is acquired and the duration of responses to subsequent

therapies shorten. The prognosis of patients that are triple class-exposed and/or refractory is poor with a median PFS of around four months[18].

Chimeric antigen receptor (CAR)-T cell therapy

A brief history on CAR-T cells

Chimeric antigen receptors (CARs) are artificial proteins comprising of several domains derived from naturally occurring receptors (Figure 2). Their modularity is nicely illustrated by the different steps in the development of the current CARs. Arguably, the first chimeric receptors that resemble CARs were invented by two independent groups at the end of the 1980's through the fusion of immunoglobulin (Ig)-derived variable regions to the constant regions of the T cell receptor (TCR) under the name "T-bodies", offering T cells the ability to recognize cells in an MHC-independent manner[19, 20]. In 1993, Eshhar and colleagues took the design further by utilizing a single-chain approach: the variable heavy (VH) and variable light (VL) chains of an antibody were linked together forming a single-chain variable fragment (scFv)[21], retaining antigen-specificity in the process, which was subsequently fused to the CD3ζ signaling domain of the TCR complex, forming a first generation CAR[22]. T-cell signaling via CD3ζ was sufficient to induce lytic activity, but the cells were found to become anergic and stop proliferating due to the lack of a co-stimulatory signal in the tumor micro-environment[23]. To resolve this, researchers incorporated co-stimulatory signaling with a CD28 co-stimulatory domain (CSD)[24, 25], and later also a 4-1BB CSD[26], to form the second generation of CARs. Third generation CARs include multiple CSDs in an attempt to further enhance persistence and potency, though preclinical and clinical in vivo data are ambiguous on their superiority over second generation CARs[27]. As the field's molecular toolbox expanded, the focus of next-generation CAR therapeutic strategies moved away from tweaking the CAR construct itself more downstream in the signaling cascade. As such, fourth generation CARs couple antigen-specific recognition to the expression of transgenes, such as cytokines, enzymes and other immunomodulatory proteins, in the form of genetic circuits[28]. Some of these strategies already combine the three signals of T-cell activation (TCR, co-stimulation and cytokine support) into one cellular vehicle, though they often still require additional interactions between the payload (e.g., cytokines) and its target (e.g., cytokine receptors). Finally, and somewhat in contrast to the fourth generation, fifth generation CARs integrate all three T-cell activation signals in the CAR through the addition of the intracellular domain of a cytokine receptor to a second generation CAR enhancing proliferation and in vivo persistence, and reducing terminal differentiation[29].

The structure of a CAR decomposed

While it is clear from this brief history discussion on 35 years of CAR development that modifications of the CAR design can heavily impact therapeutic outcome, only in the past decade researchers have increased their efforts in dissecting the function of each of the CAR domains (Figure 2).

Antigen-binding domain

The antigen-binding domain (ABD) facilitates recognition of the target cell surface protein and usually comprises an scFv. Murine-derived scFvs, such as the anti-CD19 scFv FMC63 used in commercial CAR-T-cell products, were found to be immunogenic, though the clinical implications on therapeutic efficacy and safety are still unclear[30]. In addition, instabilities in the murine frameworks can promote self-aggregation and antigen-independent signaling (also referred to as tonic signaling)[31]. Humanization of the framework regions reduces immunogenicity and the tendency to self-aggregate while maintaining the antigen specificity[31]. Similarly, the length of the linker connecting the VH and VL chains should be carefully considered as it can result in antigen-independent CAR multimerization and tonic signaling leading to impaired clinical efficacy[32]. Other ABD formats such as nanobodies[33], natural receptor[34], ligands[35] or short peptides[36] have been developed and can overcome some of the abovementioned shortcomings of scFvs. The ABD is also responsible for the binding kinetics of the CAR, mostly expressed in the form of an affinity metric, which is not only a determining factor of the antigen sensitivity[37], but also affects the therapeutic safety and potency[38]. Anno 2024, there are more than 64 different targets under investigation in the context of both hematological and solid tumors of which the most common are CD19, BCMA, CD22, CD20, CD123, mesothelin, GD2 and HER2[39].

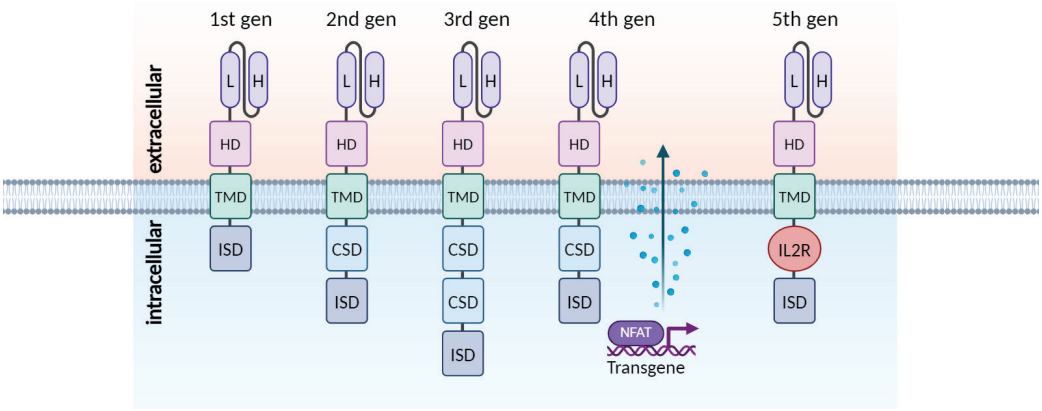


Figure 2. Composition of the different generations of chimeric antigen receptors. Abbreviations: CSD, co-stimulatory domain. Gen, generation. HD, hinge domain. IL2R, interleukin-2 receptor. ISD, intracellular signaling domain. NFAT, nuclear factor of activated T cells. TMD, transmembrane domain. VH, variable heavy chain. VL, variable light chain. Created with BioRender. Adapted from Jolien Op de Beeck.

Hinge domain

The hinge domain (HD) is also present in the extracellular part of the CAR, where it connects the ABD to the transmembrane domain (TMD). With its length, its primary function is to provide sufficient reach and freedom of movement to the ABD to allow for unobstructed access to the target epitope[40, 41] and immunological synapse formation[42]. Furthermore, dimerization of

CARs through the HD can facilitate efficient formation of the immune synapse and make CARs more sensitive to low density target antigens[43, 44]. Where there is an abundance in ABDs, there is a scarcity in described HDs. Commercially available CAR-T-cell products as well as most preclinical studies utilize HDs derived from CD8α, CD28 and IgG[27, 45]. In the past few years, the variety of HDs was slightly expanded with domains derived from IgD, CD34, LNGFR and other Siglec family members[41, 46-48].

Transmembrane domain

Connecting the extracellular region to the intracellular region of the CAR is the TMD. The domain plays a role in signal transduction in cis as well as in trans by interacting with, for example, the endogenous TCR-CD3 complex[49] or CD28 co-stimulatory proteins[50]. CAR expression and homodimerization mediated by the TMD also modulate antigen sensitivity[51]. The origin of the TMD usually matches that of the domains flanking it, again confining the state-of-the art domains to a set consisting of CD8α, CD28, CD3ζ and inducible T-cell co-stimulatory (ICOS) [45, 52]. Interestingly, the TMD is sufficiently short (~20 amino acids [AA]) and simple that computational modelling can be applied to create de novo TMDs with predictable valency and endogenous protein interactions, allowing for tight control over the lytic capacity and cytokine secretion of CAR-T cells[50].

Co-stimulatory domain

The intracellular region of the CAR converts the binding of the CAR to the target antigen into a downstream signal in the effector cell. Two parts can be discriminated, the co-stimulatory domain (CSD) and the intracellular signaling domain (ISD). As natural co-stimulatory receptors for T cells, CD28 and 4-1BB were obvious choices as the first CSDs and are consequently the only CSDs found in current commercial CAR-T-cell products[45]. Signaling through these domains has a noticeable effect on the phenotypical and functional responses of T cells, presumably because of metabolic reprogramming[53]. For instance, CD28 induces a shift towards aerobic glycolysis and an effector phenotype, whereas 4-1BB signaling favors fatty acid oxidation and memory formation. The former will therefore result in a powerful but relatively short response and the latter a weaker initial response, yet long-lasting persistence; something that has been repeatedly observed experimentally and in the clinic[54]. This discrepancy in performance illustrates the profound impact that CSDs can have and has fueled the search for alternative domains. CARs with domains derived from CD27, CD40, ICOS, OX40 and MyD88, among others, have useful properties such as enhanced cytotoxicity, reduced exhaustion, strong proliferation and extended persistence[55].

Intracellular signaling domain

The ISD is almost exclusively derived from the TCR-CD3 complex, initiating the signaling cascade upon ligand binding. Immunoreceptor tyrosine activation motif (ITAM) multiplicity and membrane proximity have been found to be important factors driving T-cell responses.

Indeed, the TCR-CD3 complex carries ten ITAMs while monomeric and homodimeric CARs carry three and six, respectively, leading to differences in activity[56]. Hence, adjusting the number and position of ITAMs have been shown to be successful strategies to finetune T-cell responsiveness towards varying antigen densities[44, 57, 58].

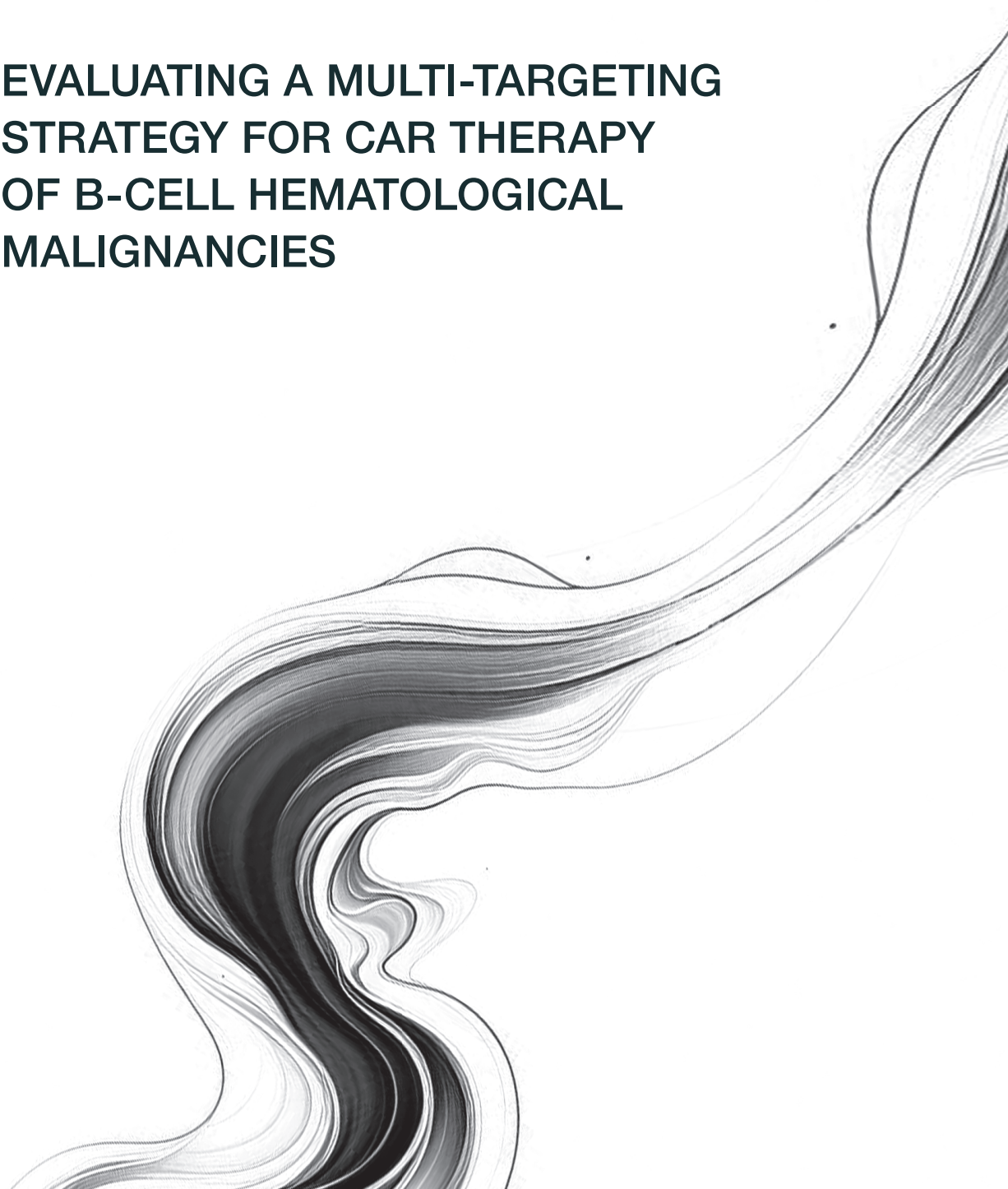
CAR-T-cell therapy against B-cell malignancies

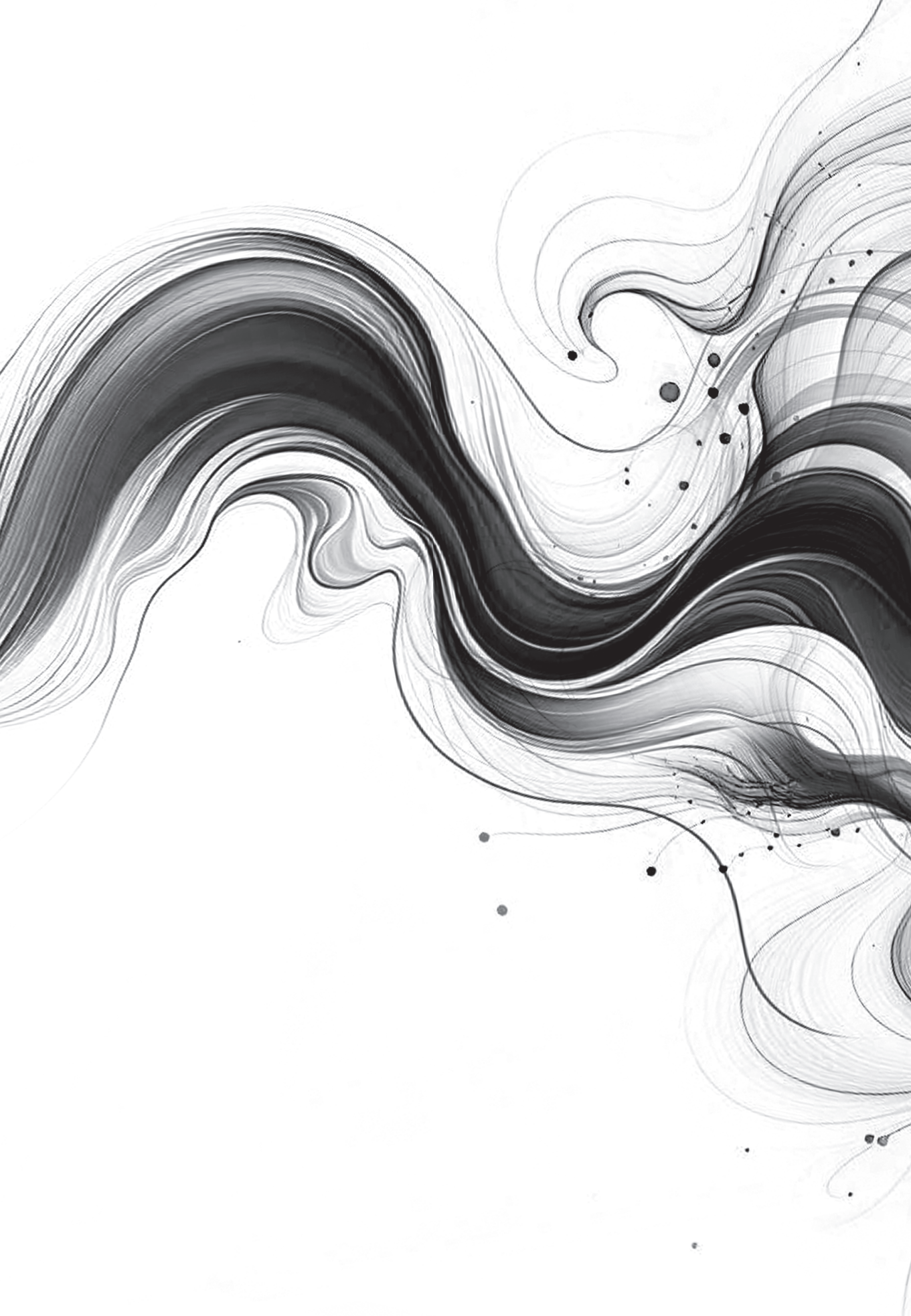
The standard-of-care for B-cell malignancies has made considerable progress over the past decades, but – as discussed above – still does not provide a long-lasting cure for the majority of patients. Patients with refractory of relapsed disease, in particular, often face a grim prognosis with minimal treatment options. Therefore, the development of CAR-T-cell therapy has given these groups a new perspective. Indeed, even in these difficult to treat groups, long-term follow-up clinical studies show that commercial CAR-T-cell products are able to induce responses in up to 92%, 82% and 97% of lymphoma, leukemia and MM patients, respectively[45, 59]. More importantly, in many cases, patients obtain long-lasting responses with median PFS frequently exceeding 2 years in NHL and pediatric B-ALL, and 1 year in MM[45, 54, 59]. **Chapter 2** and **Chapter 3** contain a more in-depth discussion of these results.

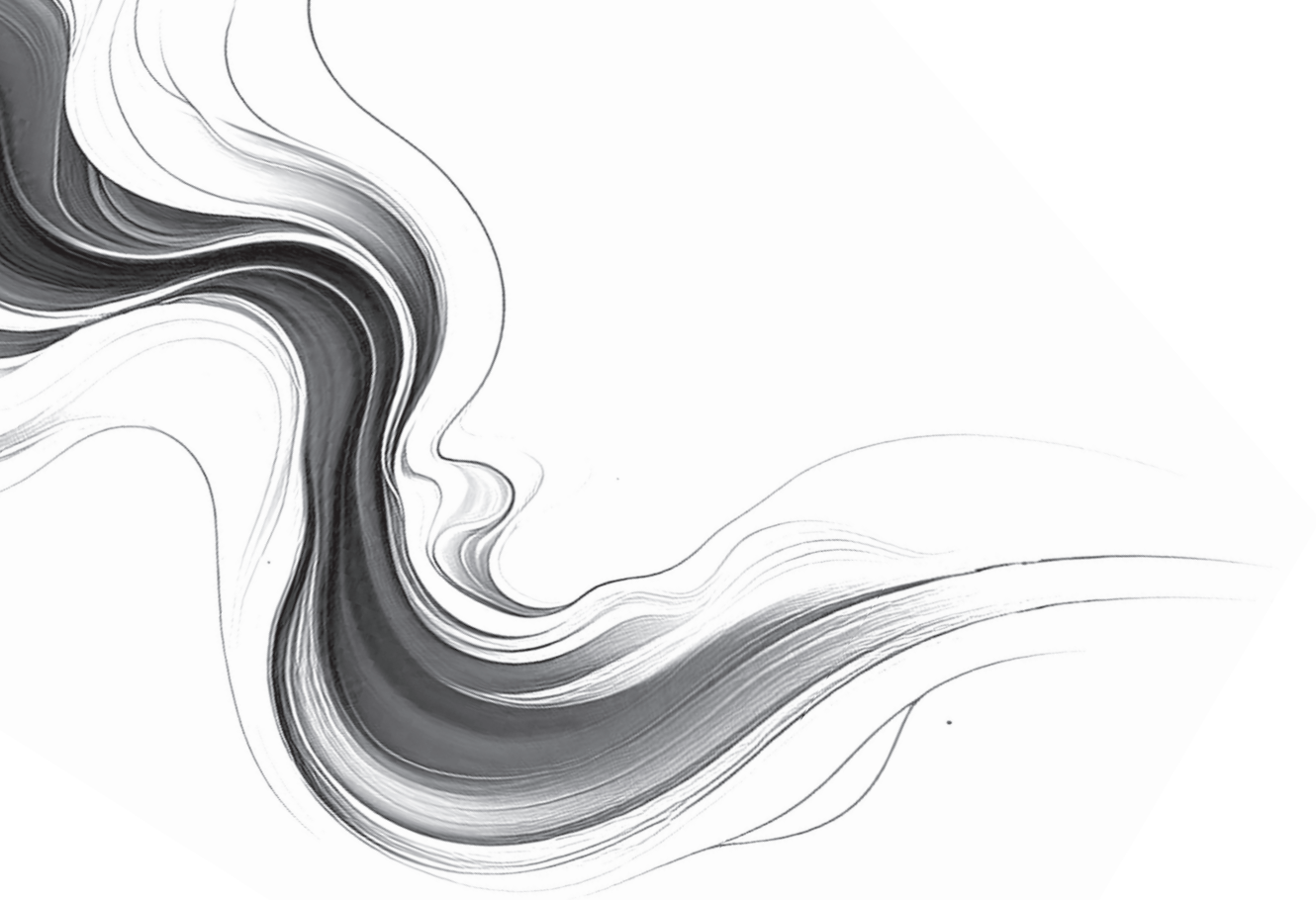
As of May 2024, six CAR-T-cell therapies have been approved for commercial use by the US Food and Drug Administration and the European Medicines Agency: four for B-cell lymphoma (axicabtagene ciloleucel [axi-cel], brexucabtagene autoleucel [brexu-cel], tisagenlecleucel [tisa-cel] and lisocabtagene maraleucel [liso-cel]) two for B-cell leukemia (brexu-cel and tisa-cel) and two for MM (idecabtagene vicleucel [ide-cel] and ciltacabtagene autoleucel [cilta-cel])[45]. Despite the impressive clinical data, more than half of patients treated with CAR-T cells eventually relapse. Two main mechanisms are thought to be at the basis of these relapses: poor CAR-T-cell performance and diminished target antigen expression. Historically, CAR-T-cell therapy has mainly been a late-stage cancer treatment. Indeed, heavily pre-treated patients rely on CAR-T-cell therapy as last resort with NHL patients that have received at least two prior lines of treatment, and MM patients with at least five prior lines of treatment[45]. As a result, the leukapheresis product used might be of poor quality, leading to worse in vivo persistence and therapeutic potency. Notably, encouraging clinical results have supported the FDA-approval of axi-cel (April 2022), liso-cel (June 2022) and cilta-cel (April 2024) as second line treatments for DLBCL and MM [45, 60, 61]. Furthermore, favorable data from the ZUMA-12 clinical trial, evaluating axi-cel as part of first-line DLBCL treatment, has paved the way for the first phase 3 randomized clinical trial evaluating axi-cel versus the standard of care[62, 63]. Data from this trial could support the notion that higher quality products of treatment-naïve patients result in greater clinical potency. A second mechanism behind relapses is the downregulation or complete loss of the targeted antigen, a phenomenon that has been reported in 20-28% and 16-68% of B-cell lymphoma and ALL, respectively, and to a lesser extent in MM[45]. Strategies to increase target antigen expression[64] or to simultaneously target multiple antigens are actively being explored[45, 65-67].

PART 1

EVALUATING A MULTI-TARGETING STRATEGY FOR CAR THERAPY OF B-CELL HEMATOLOGICAL MALIGNANCIES







2

CHIMERIC ANTIGEN RECEPTOR-T-CELL THERAPY FOR B-CELL HEMATOLOGICAL MALIGNANCIES: AN UPDATE OF THE PIVOTAL CLINICAL TRIAL DATA

Roex G, Feys T, Beguin Y, Kerre T, Poiré X, Lewalle P, Vandenberghe P, Bron D, Anguille S.

Pharmaceutics. 2020 Feb 24;12(2):194. DOI: 10.3390/pharmaceutics12020194

Abstract

Chimeric antigen receptor (CAR)-T-cell therapy is an innovative form of adoptive cell therapy that has revolutionized the treatment of certain hematological malignancies, including B-cell non-Hodgkin lymphoma (NHL) and B-cell acute lymphoblastic leukemia (ALL). The treatment is currently also being studied in other B-cell neoplasms, including multiple myeloma (MM) and chronic lymphocytic leukemia (CLL). CD19 and B-cell maturation antigen (BCMA) have been the most popular target antigens for CAR-T-cell immunotherapy of these malignancies. This review will discuss the efficacy and toxicity data from the pivotal clinical studies of CD19- and BCMA-targeted CAR-T-cell therapies in relapsed/refractory B-cell malignancies (NHL, ALL, CLL) and MM, respectively.

Introduction

For decades, the treatment of hematological malignancies was dominated by systemic chemotherapy, radiation therapy, and stem cell transplantation. More recently, new insights in the genetic and molecular basis of these malignancies paved the way for the development of targeted therapies, while the increased understanding of the interplay between the patient's immune system and cancer cells led to the development of several innovative immunotherapies. One of these immunology-based treatment strategies that recently generated much excitement is chimeric antigen receptor (CAR)-T-cell therapy [68]. This type of adoptive cell therapy (ACT) already proved to be a real breakthrough in the treatment of certain non-Hodgkin lymphoma (NHL) types and B-cell acute lymphoblastic leukemia (ALL), and is currently also being evaluated in other hematological malignancies, including multiple myeloma (MM) and chronic lymphocytic leukemia (CLL) [68].

Exploiting the immune system to attack cancer cells is not a new concept. In fact, the development of allogeneic stem cell transplantation (alloSCT) has first highlighted the potential of T cells to eliminate cancer cells. In this respect, Kolb et al. showed that donor lymphocyte infusions can induce long-lasting remissions in patients with relapsed chronic myeloid leukemia (CML) [69]. With ACT, immune cells are collected from a patient or a donor after which they are manipulated and/or expanded ex vivo and reinfused to the patient [68]. The success of ACT mainly depends on the presence of an adequate amount of effector cells in the patient, which in turn requires precursors with either natural anti-tumor recognition, or engineering of T cells to provide this recognition [68]. Therefore, researchers have developed several strategies to improve the tumor recognition of adoptively stimulated cells. Genetic engineering of novel receptors (i.e., CARs) led to the development of molecules that can both recognize proteins present on the surface of tumor cells and provide T-cell activation, proliferation, and memory [70]. CAR constructs are hybrid molecules; the extracellular part is based on the structure of a monoclonal antibody and responsible for surface antigen recognition. This recognition occurs in a major histocompatibility complex (MHC)-independent manner. The intracellular part is based on the structure of the T-cell receptor (TCR) coupled with one or more co-stimulatory domains, allowing to transduce the antigen recognition into T-cell activation [70].

CAR-T-Cell Design

In general, CARs are composed of three major domains: an ectodomain, a transmembrane domain, and an endodomain. The ectodomain or extracellular portion of the CAR typically consists of heavy and light chains derived from an antibody in single-chain variable fragment format, and a hinge region. It redirects the specificity of the receptor to recognize antigens on the cell surface independently of MHC molecules. CD19 has been most frequently chosen as

target antigen in B-NHL, B-ALL, and B-CLL for several reasons: its frequent and high-level expression in these malignancies, with a broader and higher expression relative to other potential targets like CD20 or CD22, and its restriction to the B-cell lineage in healthy tissue. The transmembrane domain of the CAR construct primarily plays a role in stabilizing the CAR, while the intracellular endodomain provides the necessary signals to activate the T cells after antigen recognition [70].

The design of CARs considerably evolved over the years. First-generation CARs were designed similarly to the endogenous TCR complex. In these initial constructs, the intracellular component usually consisted of CD3ζ, which was linked to an extracellular antigen-recognition domain that allowed for direct, MHC-independent recognition of antigens on the tumor cell surface [71]. Importantly, these first-generation designs did not include co-stimulatory domains and, as such, did not provide a second signal for full T-cell activation. As a result, these first-generation CAR-T cells were more prone to apoptosis and had limited in vivo expansion potential, resulting in poor cytotoxicity [71]. The addition of co-stimulatory signaling domains (e.g., CD28, 4-1BB) in second-generation CARs resulted in improved T-cell activation, enhanced survival capabilities, and a more effective expansion of the modified T cells in vivo [24, 71]. These second-generation receptors form the basis of the currently approved CAR-T-cell therapies. It is now becoming increasingly clear that each type of co-stimulatory domain has specific roles in CAR signaling; for example, CD28-based CAR-T cells exhibit more potent effector cell functions but limited persistence, whereas 4-1BB tends to drive the CAR-T cells towards a central memory phenotype resulting in improved persistence [55, 72]. Third-generation CAR-T cells combine the signaling potential of two co-stimulatory domains (e.g., both CD28 and 4-1BB). The anti-tumor activity of fourth-generation CARs, including T cells redirected for universal cytokine-mediated killing (TRUCKs), is even further enhanced by additional genetic modifications, for example by the addition of transgenes for cytokine secretion (e.g., IL-12) [73, 74].

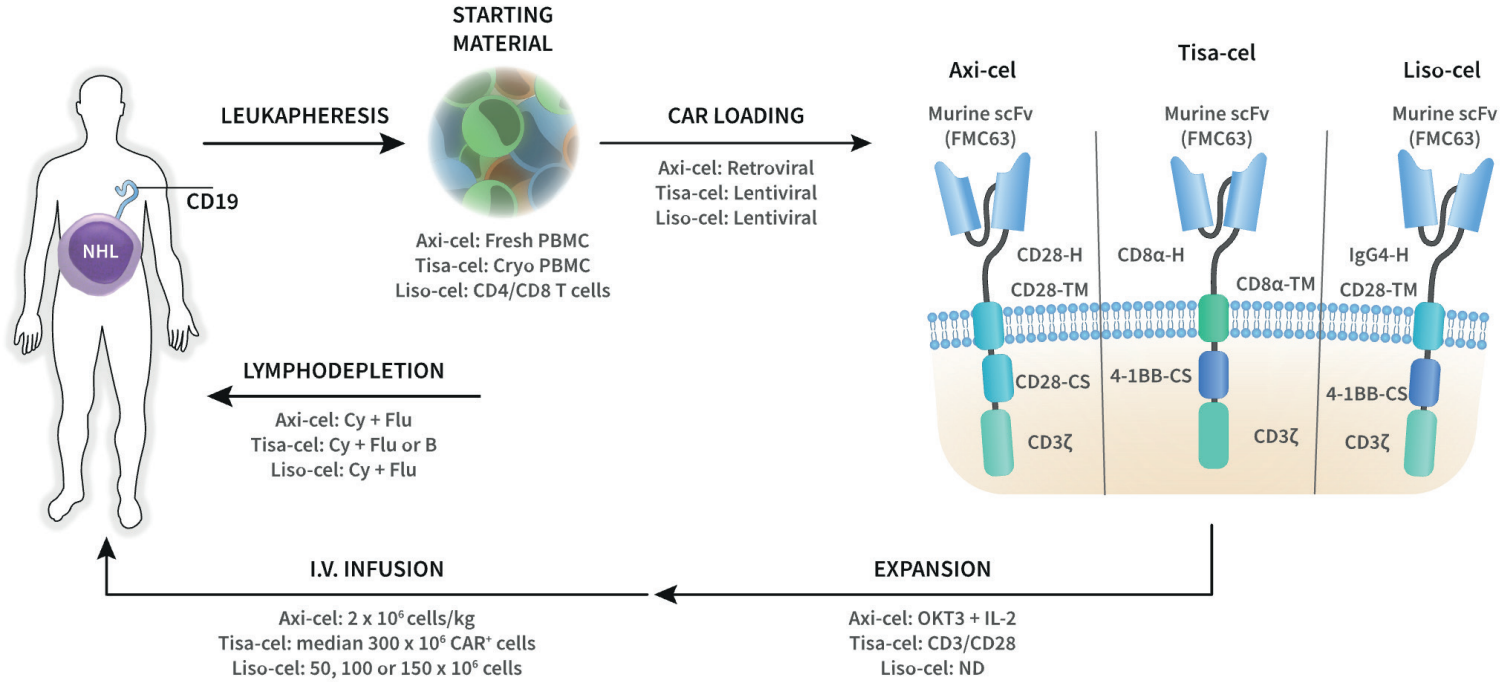


Figure 3. Overview of CD19-targeted chimeric antigen receptor (CAR)-T-cell therapies axicabtagene ciloleucel (axi-cel), tisagenlecleucel (tisa-cel), and lisocabtagene maraleucel (liso-cel) in CD19⁺ non-Hodgkin lymphoma (NHL). T cells are collected from the patient by leukapheresis (**1**) after which they are loaded with the CD19 CAR gene by means of lentiviral or retroviral transduction (**2**), and ex vivo expanded (**3**). The resultant CAR-T cells are then administered back to the patient by intravenous (i.v.) infusion (**4**). Lymphodepleting chemotherapy is usually administered prior to CAR-T-cell infusion in order to promote in vivo CAR-T-cell expansion and persistence. Axi-cel, tisa-cel, and liso-cel are second-generation CARs, of which the intracellular part contains the T-cell receptor ζ chain (CD3ζ) and a co-stimulatory (-CS) domain (CD28 or 4-1BB). The intracellular part is linked by the transmembrane domain (-TM) with the extracellular part of the CAR which is composed of the hinge and the antigen-recognition domain. The three constructs bear a different hinge (-H) but share the same murine FMC63-derived single chain variable fragment (scFv) as antigen-binding domain. B, bendamustine; CD3/CD28, anti-CD3/CD28 microbeads; Cy, cyclophosphamide; Flu, fludarabine; IL-2, interleukin-2; ND, no data; OKT3, anti-CD3 monoclonal antibody; PBMC, peripheral blood mononuclear cells.

CAR-T-Cell Manufacturing and Administration

Although allogeneic CAR-T cells have been used, the production of CAR-T cells typically starts with the collection of peripheral blood mononuclear cells (PBMCs) from the patient (autologous) using a large volume leukapheresis procedure (Figure 3). The cells are then transferred to a cell-processing facility where they are loaded with the CAR, usually by incubating them with CAR-encoding viral vectors, which enter the T cells and introduce the CAR RNA (Figure 3). This CAR RNA is then reverse transcribed into DNA, which recombines into the T-cell genome, resulting in permanent CAR gene incorporation. Both lentiviral and, to a lesser extent, gamma-retroviral vectors have been used for CAR gene transduction of primary T cells (Figure 3) [75].

The CAR gene-modified T cells are then expanded ex vivo and prepared as a pharmaceutical intravenous infusion product. The cells are usually administered as single infusion. The median time from leukapheresis to CAR-T-cell administration is 4–5 weeks and the entire process from referral to infusion can take up to 2 months [76]. Therefore, physicians often perform bridging chemotherapy to avoid rapid disease progression and to maintain the patient's general condition during the CAR-T-cell production period. Lymphodepleting (LD) chemotherapy, such as fludarabine and cyclophosphamide, is often administered prior to the infusion of the CAR-T cells (Figure 3) [77]. LD chemotherapy decreases the number of T cells in vivo, including regulatory T cells, and consequently upregulates cytokines such as IL-7 and IL-15 [77]. These cytokines promote T-cell expansion and augment the anti-tumor activity of the CAR-T cells.

Efficacy and Toxicity of CAR-T-Cell Therapy in B-Cell Malignancies

CAR-T-cell therapy has emerged rapidly over the last few years, ultimately leading to the approval of the first two CAR-T-cell medicines, tisagenlecleucel (tisa-cel) and axicabtagene ciloleucel (axi-cel) both by the US Food and Drug Administration (FDA) and later by the European Medicines Agency (EMA) for the treatment of certain B-cell NHL types in adults, as well as relapsed/refractory (r/r) B-ALL in children and young adults. In addition to this, the potential of CAR-T-cell therapy is also being explored in other B-cell neoplasms, such as MM and B-CLL [68, 73].

Non-Hodgkin Lymphoma

B-cell NHL is the most frequent hematological malignancy, with diffuse large B-cell lymphoma (DLBCL) being the most common subtype. Despite therapeutic improvements, a substantial proportion of DLBCL patients develop chemorefractory disease. Currently, approximately two-thirds of patients with newly diagnosed DLBCL are cured with first-line cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP) therapy in combination with rituximab

[78]. The standard of care second-line treatment for fit patients with r/r DLBCL is salvage chemotherapy followed by autologous SCT (ASCT). Unfortunately, approximately half of the patients will remain refractory or experience a relapse after second-line treatment [78]. Relapsed/refractory DLBCL faces a grim prognosis; based on data from the SCHOLAR-1 study, a multicohort, retrospective study involving 636 patients with pooled data from two phase III studies (CORAL and LY.12) and two observational cohorts, the median overall survival (OS) for patients with r/r DLBCL is only 6.3 months (95% CI: 5.9–7.0 months) [11]. To overcome this chemorefractoriness in DLBCL, several novel therapeutic strategies have been explored, including CAR-T-cell therapy. Several early, single-center studies demonstrated significant anti-lymphoma activity of CD19-directed CAR-T-cell therapy in NHL patients and formed the basis for the design of three larger multicenter clinical trials [79, 80].

The phase II portion of the ZUMA-1 trial evaluated axi-cel in patients with refractory, high-grade B-cell lymphoma. In this study, no bridging therapy was allowed, and the LD regimen consisted of cyclophosphamide and fludarabine. Patients in the trial were divided in two cohorts: cohort 1—the largest cohort—included DLBCL patients, while cohort 2 consisted of patients with transformed follicular lymphoma (TFL) and primary mediastinal B-cell lymphoma (PMBCL) [81, 82]. The primary endpoint in ZUMA-1 was overall response rate (ORR) in patients with more than 6 months follow-up after axi-cel infusion, as compared with historical control (SCHOLAR-1 [11]). In total, 111 patients were enrolled of whom 101 received axi-cel. More than two-thirds of the patients were refractory to at least three lines of therapy and 21% relapsed within 12 months after an ASCT. In the most recent report of this trial, with a median follow-up of 27.1 months, an ORR of 83% was demonstrated with a complete remission (CR) rate of 58% [81]. This represents an eightfold higher CR rate in comparison with SCHOLAR-1 [11]. The median duration of response is still not reached for patients with a CR (95% CI: 12.9 months–not estimable), underscoring the durability of the responses to axi-cel [81]. A more detailed overview of the efficacy data in ZUMA-1 can be found in Table 1 [81, 82].

The JULIET trial was a phase II multicenter global study in patients with r/r B-cell NHL using the anti-CD19 CAR-T-cell product tisa-cel [83, 84]. Key eligibility criteria in JULIET included aggressive B-cell lymphoma (DLBCL, representing 80% of the treated patients, or TFL); about half of the patients had refractory disease with at least three prior lines of therapy (including ASCT in 49% of the patients). In contrast to ZUMA-1, cryopreserved apheresis products were utilized, and bridging chemotherapy was allowed for patients with rapidly progressive disease [84]. Overall, 92% of the patients received bridging chemotherapy. LD chemotherapy consisted of cyclophosphamide and fludarabine, or bendamustine. Similar to the ZUMA-1 trial, the primary endpoints of the trial were ORR and the rate of CR. A total of 165 patients were enrolled and 111 patients were infused with tisa-cel. In the 93 response-evaluable patients (at least 3 months of follow-up), the reported ORR and CR rates were 52% and 40%, respectively. More efficacy details are shown in Table 1 [83].

Table 1. Efficacy data of CD19-targeted CAR-T-cell therapies axi-cel, tisa-cel, and liso-cel in NHL.

Cell Product	**axi-cel**	**tisa-cel**	**liso-cel**
Trial [ref.]	**ZUMA-1 [81, 82]**	**JULIET [83, 84]**	**TRANSCEND [85-87]**
N enrolled (infused)	111 (101)	165 (111)	344 (269 + 25 [#])
N response-evaluable	101	93	256
Best ORR (CR)	83% (58%)	52% (40%)	73% (53%)
Median DoR	11.1 mo *(4.2 mo-n.e.)*	Not reached *(10.0 mo-n.e.)*	Not reached *(8.6 mo-n.e.)*
Median PFS	5.9 mo *(3.3–15.0 mo)*	NR	6.8 mo *(3.3–14.1 mo)*
PFS rate	24-mo PFS: 72% *(for pts in CR at 3 mo)*	12-mo PFS: 83% *(for pts in CR/PR at 3 mo)*	12-mo PFS: 65% *(for pts in CR)*
Median OS	Not reached *(12.8 mo-n.e.)*	12.0 mo [§] *(7.0 mo-n.e.)*	21.1 mo *(13.3 mo-n.e.)*
OS rate	Est. 24-mo OS: 50.5%	Est. 12-mo OS: 49% *(90% for pts in CR)*	Est. 12-mo OS: 58% *(86% for pts in CR)*

Axi-cel, axicabtagene ciloleucel; CR, complete response; DoR, duration of response; Est., estimated; liso-cel, lisocabtagene maraleucel; mo, months; N, number; n.e., not estimable; NHL, non-Hodgkin lymphoma; NR, not reported; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; PR, partial response; ref., reference; #, number of patients that received a nonconforming product (N = 25); §, median OS reported for the infused population (N = 111).

Based on the promising results of ZUMA-1 and JULIET, the US FDA approved axi-cel and tisa-cel for certain r/r B-cell NHL subtypes in October 2017 and May 2018, respectively. A couple of months later, both agents were also approved by the EMA. With the approval of axi-cel and tisa-cel, interest in reporting the efficacy of this therapy in real clinical practice grew. “Real-world” data on the use of axi-cel were reported by Nastoupil et al. [88], Jacobson et al. [89], and others [90]. Overall, 43% of the patients in the study by Nastoupil et al. did not meet the inclusion criteria of ZUMA-1. Moreover, 55% received bridging therapy whereas this was not allowed in ZUMA-1. Of the 294 leukapheresed patients, 274 were actually infused. Best ORR (81%) and CR (57%) rates were similar to those reported in ZUMA-1 (83% and 58%, respectively). This essentially confirms that the efficacy of axi-cel in r/r B-cell NHL (including DLBCL, TFL, and PMBCL) could be replicated outside the strict eligibility criteria of clinical trials [88-90].

The multicenter TRANSCEND NHL 001 study of lisocabtagene maraleucel (liso-cel) is the largest CD19 CAR-T cell study performed so far; 344 patients with a variety of r/r B-cell NHL types, including DLBCL, TFL, PMBCL, FL grade 3b, and other high-grade B-cell lymphomas, were leukapheresed [85-87]. Like in ZUMA-1 and JULIET, DLBCL was the most common histological subtype. Bridging therapy was allowed and required in approximately two-thirds of the patients. A cyclophosphamide and fludarabine combination was used for lymphodepletion. In total, 294 patients were infused in this trial, but 25 patients received a nonconforming

product. The best ORR and CR rates among the 256 response-evaluable patients were 73% and 53%, respectively [85]. The PFS and OS data are presented in Table 1 [85].

The most common acute toxicities observed after CAR-T-cell therapy are CRS and immune effector cell-associated neurotoxicity syndrome (ICANS, previously termed CAR-T-cell-related encephalopathy syndrome (CRES)), either of which can be lethal [91]. CRS is caused by cytokine elevations as a result of immune activation of large numbers of lymphocytes. The cardinal symptoms include fever, hypotension, and hypoxemia [91]. The median time to onset of CRS was 2–3 days with axi-cel in ZUMA-1 [81, 82] and tisa-cel in JULIET [83], and 5 days with liso-cel in TRANSCEND [85]. In recent years, guidelines for the uniform grading of CRS have been published, of which the guidelines by the American Society for Transplantation and Cellular Therapy (ASTCT) have become the most widely adopted [92]. CRS is graded with a score of 1 (mild) to 4 (life-threatening) [92]. In ZUMA-1 (axi-cel) [81, 82], JULIET (tisa-cel) [83], and TRANSCEND (liso-cel) [85], the incidence of any grade CRS was 92%, 58%, and 42%, respectively (Table 2). Grade ³3 CRS occurred in 11%, 22%, and 2%, respectively (Table 2). In the real-world study by Nastoupil et al., 7% of the patients developed severe CRS [88, 90].

Table 2. Toxicity data of CD19-targeted CAR-T-cell therapies axi-cel, tisa-cel, and liso-cel in NHL.

Cell Product	**axi-cel**	**tisa-cel**	**liso-cel**
Trial [ref.]	**ZUMA-1 [81, 82]**	**JULIET [83, 84]**	**TRANSCEND [85-87]**
CRS (gr. ≥ 3)	92% (11%)	58% (22%)	42% (2%)
NT (gr. ≥ 3)	67% (32%)	21% (12%)	30% (10%)
Tocilizumab use	43%	14%	19%
Corticosteroid use	27%	10%	21%

Axi-cel, axicabtagene ciloleucel; CRS, cytokine release syndrome; gr., grade; liso-cel, lisocabtagene maraleucel; NHL, non-Hodgkin lymphoma; NT, neurotoxicity; ref., reference; tisa-cel, tisagenlecleucel.

Interleukin-6 (IL-6) has been implicated as a central mediator of CRS [91]. This explains why tocilizumab, a therapeutic antibody blocking IL-6 receptors, has become the drug of choice for the management of moderate to severe CRS [92, 93]. It induces near-immediate reversal of CRS symptoms in most patients. Importantly, tocilizumab does not seem to affect the efficacy of CAR-T-cell therapy in terms of ORR, CR rate, or the durability of responses [93]. In ZUMA-1 (axi-cel) [81, 82], JULIET (tisa-cel) [83] and TRANSCEND (liso-cel) [85], tocilizumab was used in 43%, 14%, and 19% of the patients, respectively (Table 2). In the real world, tocilizumab is far more frequently used (in 63% of the cases in the study with axi-cel by Nastoupil et al.) [88-90]. Until recently, corticosteroids were used only in severe CRS cases due to concerns regarding their suppressive action on T-cell function [93]. However, it is becoming increasingly clear that corticosteroids can be used safely to treat CAR-T-cell-related toxicities without limiting efficacy. This statement is further strengthened by the real-world data on the use of axi-cel in

r/r B-cell NHL (i.e., similar efficacy in ZUMA-1 and real-world study by Nastoupil et al., despite the proportionally higher use of corticosteroids to treat CRS (55% vs. 27% in ZUMA-1)) [88, 90].

Neurotoxicity, termed ICANS or CRES, is the second most common serious adverse reaction after administration of CAR-T-cell therapy [92]. Affected patients develop toxic encephalopathy with confusion, aphasia, ataxia, delirium, seizures, and cerebral edema [92]. The causative pathophysiology of these neurological side effects is still not fully understood. IL-6 does not seem to play an important role in ICANS/CRES; in mouse models, it was elegantly shown that anti-IL-6 therapy with tocilizumab did not have a major impact on the development and evolution of ICANS/CRES [94]. Nevertheless, tocilizumab will often be used, especially if the neurotoxicity co-occurs with CRS. Otherwise, corticosteroids are the preferred treatment or, if available, the IL-1 blocker anakinra. The severity of ICANS can fluctuate rapidly, necessitating close patient monitoring. This is especially important for the very rare, but life-threatening cerebral edema, for which anti-IL-6 therapy is not effective [93]. Similar to CRS, management of ICANS is based on the severity of the neurological symptoms. The 10-point "Immune Effector Cell-Associated Encephalopathy (ICE)" scoring tool is now the gold standard for screening and grading of ICANS [92]. Neurotoxicity appears to be more common with axi-cel (67% with 32% grade ³3 in ZUMA-1 [81, 82]), as compared to tisa-cel (21% with 12% grade ³3 in JULIET [83]) and liso-cel (30% with 10% grade ³3 in TRANSCEND [85]) (Table 2).

B-Cell Acute Lymphoblastic Leukemia

The phase II ELIANA trial investigated the CD19-directed genetically modified autologous T-cell product tisa-cel as a single infusion for r/r pediatric and young adult B-cell ALL [95]. From the 107 patients who were screened, 92 were enrolled; 17 patients could not be infused for a variety of reasons: death (N = 7), serious adverse events (N = 3) or CAR-T-cell production failure (N = 7). Of the 75 tisa-cel-treated patients, 65 (87%) required bridging chemotherapy between enrolment and infusion, and 72 (96%) received LD chemotherapy (mostly fludarabine plus cyclophosphamide). Patients in the study received a median of three prior therapies, and 61% of patients previously underwent an alloSCT. The CR rate at 3 months was 81% and the median duration of the remission was not reached with a median follow-up of 1 year. All patients with a treatment response were negative for minimal residual disease (MRD). The event-free survival (EFS) and OS rates at 6 months were 73% and 90%, respectively, dropping to 50% and 76% at the 1-year landmark [95]. Long-term in vivo persistence was demonstrated. All patients with a response to treatment had B-cell aplasia, and most patients in the study received immunoglobulin replacement in accordance with local practice. Grade 3/4 adverse events (AEs) with a suspected relation to tisa-cel occurred in 73% of patients. CRS occurred in 77% of patients, of whom 48% received tocilizumab. Neurotoxicity was observed in 40% of patients; all these events took place within the first 2 months [95]. Tisa-cel has received regulatory approval for the treatment of pediatric and young adult patients up to 25 years of age with B-ALL that is refractory, in relapse after alloSCT or in second or later relapse.

Multiple Myeloma

Multiple myeloma is a B-cell neoplasm characterized by a malignant proliferation of plasma cells in the bone marrow. Over the last decade, we have witnessed enormous progress in the treatment of MM, but despite these advances, the disease remains incurable. Therefore, the development of new therapeutic drugs is needed, and CAR-T-cell therapy is considered promising. B-cell maturation antigen (BCMA) is the most widely used target antigen in CAR-T-cell studies for MM [96-98]. BCMA expression is largely restricted to (malignant) plasma cells and some mature B cells [99, 100]. BCMA appears to play an important role in the promotion of MM cell survival, proliferation, and was also found to be involved in the development of drug resistance [101]. Table 3 provides an overview of all BCMA CAR-T-cell clinical trials in MM that were published as full article on Web of Science/Pubmed (date of last search: 01 Jan 2020) [102-108]. Due to the early phase character of most trials, the number of infused patients was rather low. The ORR was in the range of 85–95% in most studies; only two studies, NCT02546167 [102] and NCT02215967 [103, 104], reported lower ORR and CR rates. Possible explanations are the suboptimal BCMA CAR-T cell doses that were used in these trials and the fact that most patients were heavily pretreated. The median PFS observed with BCMA CAR-T-cell therapy was in the range of 1 year [105-108]. As shown in Table 3, most patients developed CRS; grade 3 or higher CRS was observed in 5–41% of the patients. Neurotoxicity was an uncommon event, usually occurring in less than 10% of the patients. Only two studies reported neurotoxicity rates of 32% [102] and 42% [105].

Table 3. Efficacy and toxicity data of selected§ B-cell maturation antigen (BCMA)-targeted CAR-T-cell therapies in multiple myeloma (MM).

Trial Registry # [ref.]	N	ORR (CR)	Median PFS *(95% CI)*	CRS (gr. ≥ 3)	NT (gr. ≥ 3)
NCT02546167 [102]	25	48% (8%)	NR	88% (32%)	32% (12%)
NCT02215967 [103, 104]	26	58% (8%)	NR	69% (23%)	4% (4%)
NCT02658929 [105]	33	85% (45%)	11.8 mo *(6.2-17.8 mo)*	76% (6%)	42% (3%)
NCT03090659 [106]	17	88% (82%)	12.2 mo *(NR)*	100% (41%)	0% (0%)
NCT03090659 [107]	57	88% (74%)	15.0 mo *(11.0 mo-n.e.)*	89% (7%)	2% (0%)
ChiCTR-17011272 [108]	21	95% (57%)	8.0 mo ¶ *(NR)*	91% (5%)	10% (NR)

BCMA, B-cell maturation antigen; CI, confidence interval; CR, complete response; CRS, cytokine release syndrome; gr., grade; MM, multiple myeloma; mo, months; N, number; n.e.: not estimable; NR, not reported; NT, neurotoxicity; ORR, objective response rate; PFS, progression-free survival; ref., reference. Trial registry #, study registration number on ClinicalTrials.gov (NCT#) or in the Chinese Clinical Trial Registry (ChiCTR-#); §, only clinical studies published as full article in Web of Science/PubMed were selected (date of last search: 01 Jan 2020). ¶, PFS reported for patients with very good partial response or better.

Despite the relatively high ORR obtained with BCMA CAR-T-cell therapy, the observed therapeutic effect was often transient and relapses were frequently observed. Downregulation or loss of BCMA expression is likely an important mechanism underlying these relapses [109].

Therefore, targets other than BCMA, such as CD19 or CD138, have been investigated in CAR-T-cell studies, but yielded varying results [110, 111]. Dual antigen targeting, for example by combining BCMA and CD19 CAR-T cells, is also being pursued in an attempt to improve response durability [108]. CD19 is a rather unconventional target antigen in MM, because myeloma cells are mostly CD19-negative by flow cytometry. Nevertheless, more sensitive techniques have recently revealed that CD19 is expressed at ultra-low levels on MM cells, and that these levels are sufficient for recognition of MM cells by CD19 CAR T-cells [112]. Moreover, it appears that $CD19^+$ MM cells bear features of a cancer stem cell (i.e., self-renewal and drug resistance), making it an attractive target for immunotherapy [113]. Another strategy to avoid BCMA-negative relapses involves the combination of BCMA CAR-T cells with gamma-secretase inhibitors which prevent cleavage of BCMA from the MM cell surface [64]. In addition to this, other studies are looking into the potential of CAR T-cell therapies targeting other antigens, including CD38, SLAMF7, CD44v6, CD56, GPRC5D, amongst others [114]. There are currently no CAR-T-cell therapies for MM that have received regulatory approval yet, but the first approvals are expected later this year or in 2021.

Chronic Lymphocytic Leukemia

B-CLL was one of the first diseases in which CD19 CAR-T cells were tested. Since the first report of the efficacy of second-generation CAR-T cells against CLL in 2011 [115], results have been reported of CD19-targeted CAR-T-cell therapy in a total of 134 CLL patients [116]. Overall, the CLL patients who were treated with CAR-T-cell therapy had a particularly poor prognosis, with most of them being in relapse after a large number of treatment lines. In total, 74 of the 108 (68.5%) evaluated patients in these studies had p53 alterations, and 41 out of 70 (58.6%) had a complex karyotype [116]. A second observation from the different CAR-T-cell reports in CLL is that the efficacy is lower for CLL than for DLBCL or B-ALL: CR, according to the IWCLL criteria, was obtained in only a minority (20–30%) of patients with an estimated 18-month PFS of 25% [117-119]. Interestingly, responses appeared to be weaker in the lymph nodes than in the bone marrow and blood. In fact, in some series, a substantial proportion of patients treated with CAR-T cells obtained undetectable MRD in the bone marrow [118, 120, 121]. For example, in a study by Turtle et al. including 24 r/r CLL patients who previously received ibrutinib, an ORR of 71% (21% CR) was reported four weeks after the CAR-T-cell infusion, with bone marrow negativity in 58%. Among these MRD negative patients, the PFS and OS rate was almost 100% at a median follow-up of 6.6 months [118].

The lower efficacy of CAR-T cells in CLL may be partly due T-cell exhaustion in CLL patients resulting in decreased CAR-T-cell functionality [122]. To overcome this, several research groups are looking into ways to optimize the CAR constructs in CLL. In addition to this, studies are underway looking into the potential of combining CAR-T-cell therapy with other anti-CLL therapies. In this respect, data suggest that ibrutinib may improve the outcome in CLL patients receiving CAR-T cells [120, 121]. Based on these observations, a prospective

study will further evaluate the efficacy of ibrutinib maintenance at the time of injection of the CAR-T cells (NCT03331198).

Conclusions and Future Perspectives

CAR-T-cell therapy is becoming an important addition to the treatment of r/r B-cell malignancies [123]. CD19-targeted CAR-T-cell therapies have shown unprecedented clinical activity in certain aggressive B-cell NHL subtypes, including DLBCL. The three most advanced CD19 CAR-T-cell products for use in NHL are axi-cel, tisa-cel, and liso-cel; the first two have already received FDA and EMA approval and are now reimbursed in several countries [123]. In the absence of head-to-head clinical trial data, it is not possible to directly compare the effectiveness of these three agents. Nevertheless, the overall CR rate lies in the range of 50%, which is indeed exceptionally high in patients with chemorefractory DLBCL who have failed several prior lines of therapy [11]. Moreover, PFS curves for these three agents show a plateau at their tail, indicating that durable responses can be observed in approximately 1/3 NHL patients [81, 83]. This high efficacy, however, comes at a cost of substantial toxicity. Based on the toxicity data presented in this review (Table 2), it can be concluded that liso-cel has a favorable safety profile in terms of severe CRS and neurotoxicity [85] but whether this is product-dependent remains to be determined [124]. In B-ALL, tisa-cel is the only CD19 CAR-T-cell product that has received regulatory approval so far. It is marketed for the treatment of pediatric and young adult patients up to 25 years of age with r/r B-ALL. Toxicity is considerable, but generally accepted given the very few effective salvage treatment options available for these patients [95]. Patients with r/r MM can benefit from BCMA-targeted CAR-T-cell therapy. BCMA CAR-T cells are highly active in r/r MM, with ORRs of 85–95% (Table 3) and CR rates of up to 80% in selected studies [106]. The median PFS is around 12 months, which is also unprecedently high in heavily pretreated MM patients. Toxicity is common, with CRS reported in >75% of the patients. The occurrence of neurotoxicity appears to be product-specific (Table 3). Finally, in r/r B-CLL, CD19 CAR-T cells have been tested but response rates were rather disappointing [117-119]. In these patients, combination strategies with, for example, ibrutinib, may be required to unlock the full therapeutic potential of CD19 CAR-T-cell therapies [125].

Concerning efficacy, the focus must now be placed on improving response durability and thus also on developing strategies to tackle relapse. Two main mechanisms of relapse following CAR-T-cell therapy have been identified, including relapses due to loss or downregulation of the target antigen (antigen-negative relapses) and relapses due to poor persistence and exhaustion of the CAR-T cells (so-called antigen-positive relapses because the target antigen is still retained on the tumor cell surface) [126]. Antigen-negative relapses can be caused by selective pressure of the CAR-T cells on the tumor cells, resulting in outgrowth of antigen-negative clones or clones with reduced antigen expression [127]. Fry et al. were the first to

establish that r/r B-ALL patients experiencing an antigen-negative relapse after CD19-targeted CAR-T-cell therapy can be rescued by using CAR-T cells targeting an alternative antigen: CD22 [128]. This has fueled the development of dual antigen-targeted approaches to overcome antigen escape [129]. Several early-phase CAR-T-cell clinical trials investigating the combined targeting of CD19 and another antigen, such as CD22 and CD20, have now been initiated in patients with $CD19^+$ B-cell malignancies [130, 131]. Similarly, in MM, a dual antigen approach with BCMA and CD19 CAR-T cells has already been published [108], and novel MM antigens (e.g., GPRC5D) are being identified at rapid pace for rational combined targeting with BCMA [114, 132]. In MM, BCMA-negative relapses can also be prevented by the combined use of BCMA CAR-T cells and an inhibitor of γ-secretase, an enzyme responsible for active cleavage of BCMA from the MM cell surface [64].

Antigen-positive relapses can be overcome by improving persistence and by putting anti-exhaustion measures in place. One of the strategies is to use a low-affinity CD19 CAR (CAT) with a faster CD19 interaction time than the scFv from FMC63, which is the anti-CD19 antibody used in axi-cel, tisa-cel, and liso-cel (Figure 3). In a small cohort of pediatric r/r B-ALL patients (n = 14), prolonged CAR-T-cell persistence was observed with the use of this CAT CAR with durable responses [133]. Tonic signaling, i.e., constitutive CAR triggering in the absence of the target antigen, has been recognized as an important mechanism leading to CAR-T-cell exhaustion [74, 134]. Long et al. showed that the choice of co-stimulatory domain has an impact on this phenomenon, with CD28 augmenting and 4-1BB mitigating CAR-T-cell exhaustion following tonic CAR signaling [72]. A direct pairwise comparison of CD28 and 4-1BB co-stimulated CD19 CAR-T cells in NHL patients revealed that the 4-1BB variant results in improved persistence, indicating that 4-1BB co-stimulation favors more durable responses [135]. Nevertheless, the CD28 co-stimulatory domain may be required in the setting of low CD19 antigen density because CD28 co-stimulated CAR-T cells are far more efficient at targeting CD19-low tumor cells as compared to their 4-1BB counterparts [136]. A growing body of evidence indicates that CAR-T cells from non-responders or (early) relapsers are more prone to exhaustion and display increased expression of immune checkpoint molecules, such as PD-1 [119, 137]. Conceptually, immune checkpoint blockade could help to restore the function of these exhausted CAR-T cells and several studies combining CAR-T cells with checkpoint inhibitors are now underway [65, 138, 139]. To avoid the toxicities of systemically administered checkpoint inhibitors, CAR-T cells have also been genetically modified to locally release a PD-1 blocking antibody [140]. Alternatively, CAR-T cells can also be “armored” with c-Jun to prevent their exhaustion [134].

Concerning toxicity, several approaches are being explored to improve the overall safety profile of CAR-T-cell therapy. Tocilizumab and corticosteroids are now being used when early signs of CRS appear, leading to a decreased incidence of severe CRS [141]. Other strategies involve the incorporation of suicide genes in the CAR construct that can be activated in the event of uncontrolled toxicity [142]. Likewise, CAR-T cells can also be modified to co-express

a truncated (inactive) epidermal growth factor receptor (EGFR); use of the anti-EGFR mAb cetuximab will then allow selective depletion of the CAR-T cells in case of severe toxicity [142]. The drawback of these strategies is that they result in an irreversible elimination of the CAR-T cells. Recently, it was shown that the tyrosine kinase inhibitor dasatinib can be used as a pharmacologic “on/off” switch for CAR-T cells; it allows for an immediate and titratable inhibition of the CAR-T cells with a complete restoration of their function upon withdrawal of the drug [143]. Alternatively, when CAR-T cells are transiently modified using CAR-encoding mRNA, potential toxicities will be self-limiting [144], making this approach especially useful for evaluating safety of novel CAR constructs.

Despite these remaining challenges both on the level of efficacy, in particular with respect to improving the response durability, and on the level of toxicity, it is clear that CAR-T-cell therapy is here to stay as an important therapeutic modality for patients with r/r B-cell malignancies.

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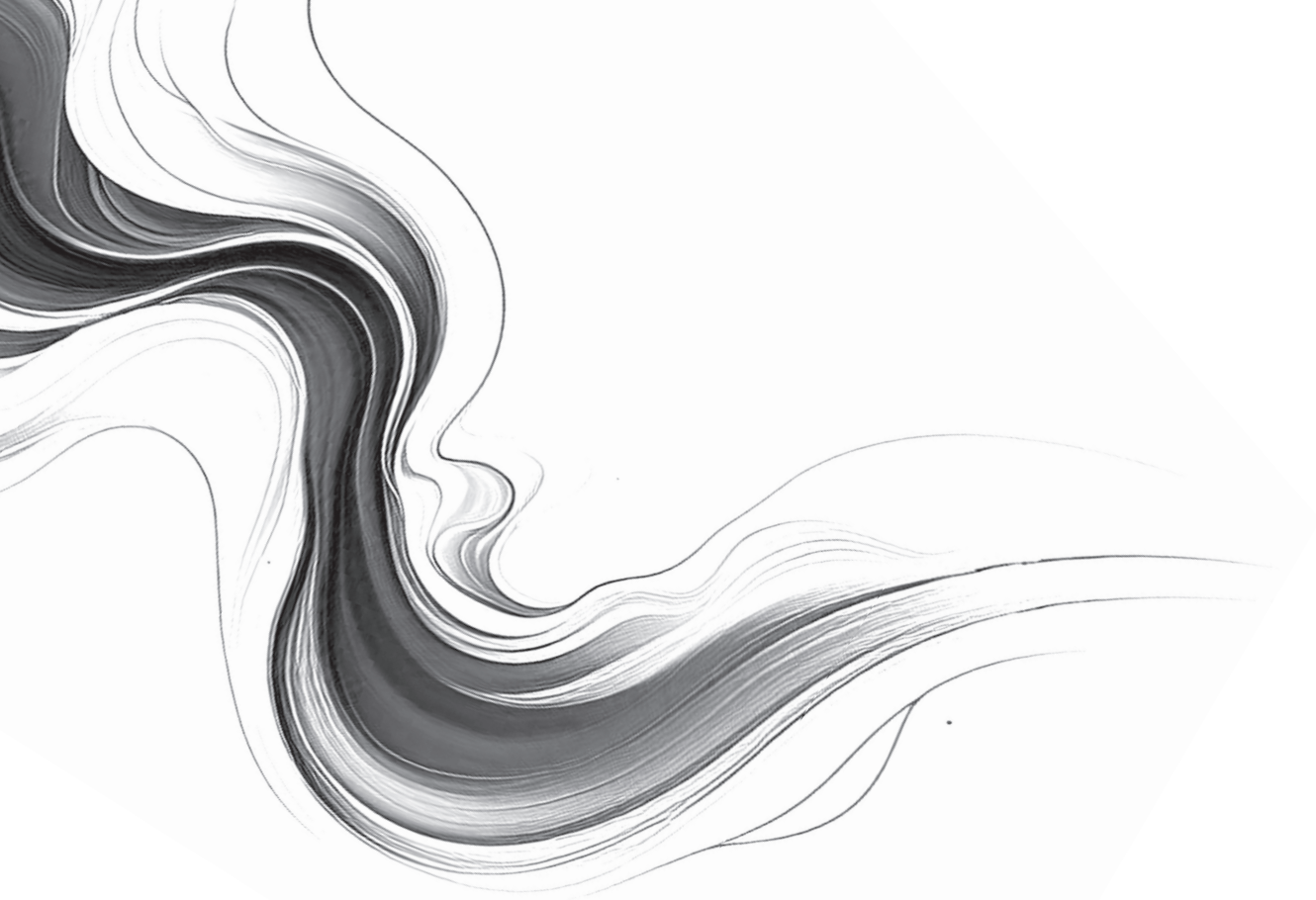

3

SAFETY AND CLINICAL EFFICACY OF BCMA CAR-T-CELL THERAPY IN MULTIPLE MYELOMA

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Abstract

Background: B-cell maturation antigen (BCMA)-targeted chimeric antigen receptor (CAR)-T-cell therapy is an emerging treatment option for multiple myeloma. The aim of this systematic review and meta-analysis was to determine its safety and clinical activity, and to identify factors influencing these outcomes.

Methods: We performed a database search using the terms "BCMA", "CAR", and "multiple myeloma" for clinical studies published between 01/01/2015-01/01/2020. The methodology is further detailed in PROSPERO (CRD42020125332).

Results: Twenty-three different CAR-T-cell products have been used so far in 640 patients. Cytokine release syndrome was observed in 80.3% (69.0–88.2); 10.5% (6.8–16.0) had neurotoxicity. A higher neurotoxicity rate was reported in studies that included more heavily pretreated patients: 19.1% (13.3–26.7; I^2=45%) versus 2.8% (1.3–6.1; I^2=0%) (p<0.0001). The pooled overall response rate was 80.5% (73.5–85.9); complete responses (CR) were observed in 44.8% (35.3–54.6). A pooled CR rate of 71.9% (62.8–79.6; I^2=0%) was noted in studies using alpaca/llama-based constructs, whereas it was only 18.0% (6.5–41.1; I^2=67%) in studies that used retroviral vectors for CAR transduction. Median progression-free survival (PFS) was 12.2 (11.4–17.4) months, which compared favorably to the expected PFS of 1.9 (1.5–3.7) months (HR 0.14; p<0.0001).

Conclusions: Although considerable toxicity was observed, BCMA-targeted CAR-T-cell therapy is highly efficacious even in advanced multiple myeloma. Subgroup analysis confirmed the anticipated inter-study heterogeneity and identified potential factors contributing to safety and efficacy. The results of this meta-analysis may assist the future design of CAR-T-cell studies and lead to optimized BCMA CAR-T-cell products.

Introduction

Multiple myeloma (MM) is defined by a malignant proliferation of plasma cells in the bone marrow (BM) [145, 146]. As the second most common haematological malignancy after lymphomas, it accounts for 1% of all cancers [147]. Recent epidemiological studies have indicated a steady increase in the incidence and prevalence of MM, mainly attributable to the aging population and therapeutic advances improving survival [148]. Indeed, over the past two decades, the landscape of myeloma treatment has dramatically changed with the advent of several novel therapies, including monoclonal antibodies (mAbs) [149].

Recently, chimeric antigen receptor (CAR)-T-cell immunotherapy has entered the clinical trial arena [59, 150]. CAR-T cells are autologous lymphocytes collected by leukapheresis and genetically modified (most often by lentiviral or retroviral transduction) to express a CAR. Following *ex vivo* expansion, the cells are then re-infused to the patient who is usually first conditioned with lymphodepleting chemotherapy (Figure 4) [151]. CARs are synthetic receptors that bear characteristics of a mAb and a T-cell receptor (TCR); they contain an antigen-recognition domain from a mAb (usually in single-chain variable fragment [scFv] format) and CD3ζ [152]. The mAb part is responsible for HLA-independent binding of the CAR-T cell to a target expressed on the tumor cell surface, whereas the CD3ζ chain triggers T-cell activation by mimicking TCR signaling. Most CAR constructs also contain one (2nd generation) or more (3rd generation) co-stimulatory domains, such as 4-1BB or CD28 (Figure 4) [151].

Although several antigens are undergoing clinical evaluation, B-cell maturation antigen (BCMA) has been the most popular myeloma target antigen so far [96, 114, 153]. BCMA is involved in cell survival and is expressed exclusively on the surface of B-cell lineage cells, including malignant plasma cells [96, 114]. The impressive clinical results of CD19-targeted CAR-T cells in CD19^{+} haematological malignancies [59, 154, 155] have created high expectations for CAR-T-cell therapy in other cancers [156]. However, it remains unclear whether these expectations are justified in the context of MM since doubts have recently been raised about the durability of therapeutic activity [157]. Moreover, CAR-T-cell therapy can produce potentially life-threatening toxicities, such as cytokine release syndrome (CRS) and neurotoxicity [158].

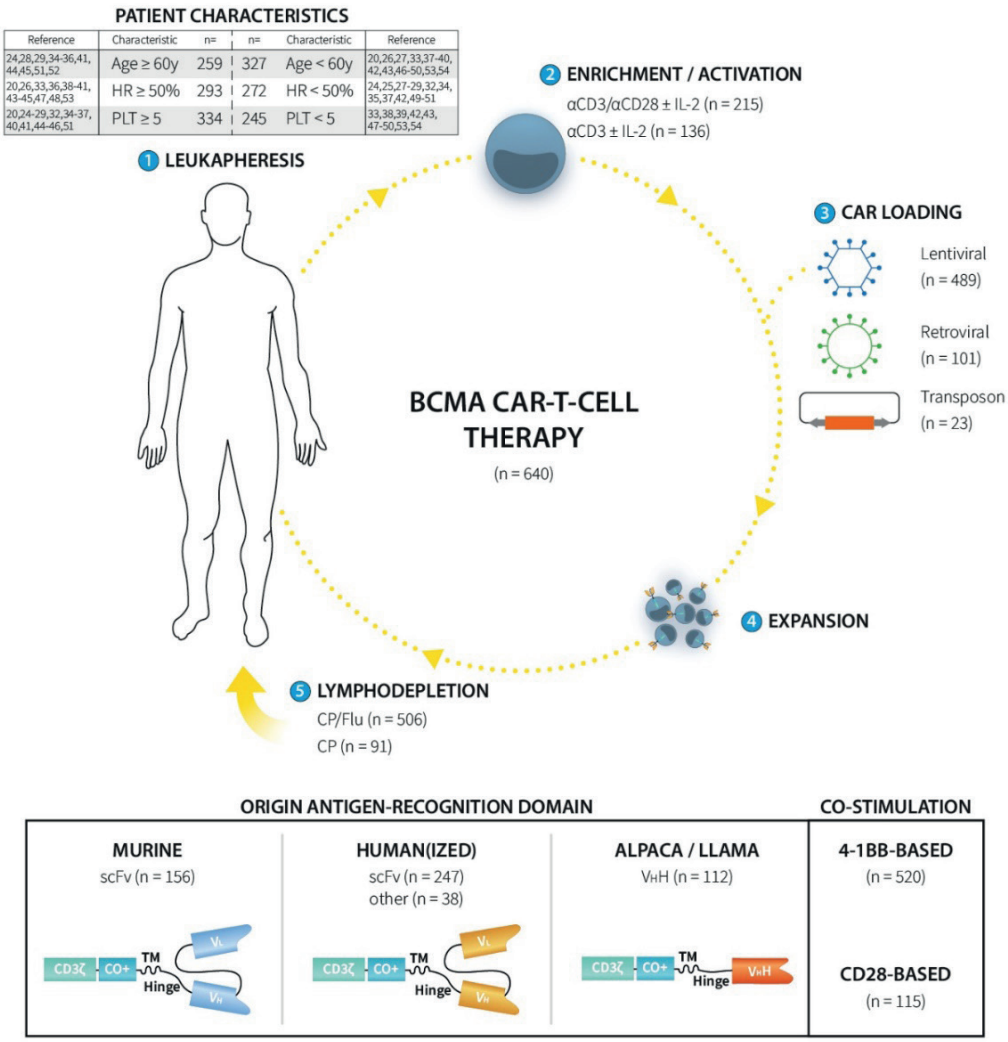

Figure 4. Overview of BCMA CAR-T-cell therapies used to date in multiple myeloma (MM) patients. Twenty-three different BCMA CAR-T-cell products involving 640 patients were identified. All products were derived from autologous T cells collected by apheresis (1), and enriched and activated ex vivo by anti-CD3/CD28 stimulation ± interleukin (IL)-2 or by single anti-CD3 stimulation ± IL-2 (2). The CAR gene was introduced in the T cells by lentiviral or retroviral transduction, or using a transposon (3). The resultant CAR-T cells were then further expanded (4) and administered to the patient by intravenous infusion, usually after lymphodepleting conditioning with cyclophosphamide (CP) ± fludarabine (Flu) (5). The BCMA CAR-T-cell products used to date can be divided in three groups based on the origin of the extracellular antigen-recognition domain: murine, human(ized) or alpaca/llama. The murine and human(ized) CAR constructs are usually based on the antigen-binding domain of a monoclonal antibody (mAb) in single-chain fragment variable (scFv) format with the variable regions of the heavy (V_H) and light chains (V_L) linked together in a single chain. Alpaca/llama BCMA CAR constructs are based on the structure of a camelid nanobody containing one or more V_HH domains. In addition, the intracellular co-stimulatory domain allows a further subdivision in 4-1BB-based and CD28-based BCMA CAR-T-cell products. Age=studies in whom the median patient age was ≥ or <60 years. CO+=co-stimulatory domain. HR=studies with a median of ≥ or <50% high-risk myeloma patients (based on cytogenetics and/or International Staging System [ISS] score). n=number of patients. PLT=studies in which the median number of prior lines of therapy was ≥ or <5. TM=transmembrane domain.

Current evidence on BCMA-targeted CAR-T-cell therapy in MM is restricted to relatively small, non-randomized early-phase clinical trials. Hence, at this stage, it is difficult to obtain a clear sight on the toxicity and efficacy that can be expected from this novel therapeutic approach in relapsed/refractory MM patients. To the best of our knowledge, there has been only one attempt so far to systematically aggregate the outcome data of BCMA CAR-T-cell clinical studies [159]. In that report, Gagelmann *et al.* included 15 studies comprising a total of 285 patients. Here, we were able to identify 27 studies involving 23 different BCMA CAR-T-cell products and a total of 640 patients, making it the most comprehensive systematic review and meta-analysis to date of the safety and clinical efficacy of BCMA-targeted CAR-T-cell therapy in MM. Moreover, this study is also the first to identify potential patient- and treatment-related factors influencing toxicity and efficacy, which helps us to understand the different outcomes between bb2121 (idecabtagene-vicleucel) and LCAR-B38M (ciltacabtagene autoleucel), the two most advanced, late-stage BCMA CAR-T-cell products which are likely to receive regulatory approval in the years to come. Furthermore, controlled trials are lacking, making it challenging to assess the true progression-free survival (PFS) benefit that is reported in individual clinical studies. In this meta-analysis, we incorporated a surrogate control arm, composed of patients treated with inactive doses of BCMA CAR-T cells. PFS data from this control population were used to determine the expected outcome in order to more accurately assess the therapeutic benefit of BCMA CAR-T-cell therapy in relapsed/refractory MM patients.

Methods

Search strategy and selection criteria

This study involves a systematic review and meta-analysis of the risks and benefits of BCMA CAR-T-cell therapy in MM patients. Relevant clinical studies were identified by a systematic search of Web of Science (Clarivate Analytics) and PubMed/MEDLINE using the following search terms: “B-cell maturation antigen” or “BCMA”, ”chimeric antigen receptor” or “CAR”, and “multiple myeloma”. Additional records were retrieved by screening published conference abstracts of American Society of Clinical Oncology (ASCO), American Society of Hematology (ASH), European Group for Blood and Marrow Transplantation (EBMT), and European Hematology Association (EHA). All clinical trial designs (i.e. controlled and uncontrolled studies) were considered. Since the first clinical report of CAR-T-cell therapy in MM was published in 2015 [160], the search was restricted to studies published between Jan 1, 2015, and Jan 1, 2020. Only clinical trials registered on Clinicaltrials.gov (NCT-number) or Chinese Clinical Trial Registry (ChiCTR-number) and published in English, either as full scientific article or as abstract during the annual scientific meetings of ASCO, ASH, EBMT or EHA, were taken into consideration. Patient data were solely extracted from these publications and no requests for additional original patient data were made to the authors of these studies. Reviews and non-scientific publications were not used for data collection to avoid duplicate data, but were used

to ensure accurate and appropriate data selection. Database searches and data collection were conducted independently by three authors (GR, MT, and SA). Data were omitted if no unanimous consensus over their inclusion was found. The PRISMA flow diagram in Figure 5 depicts the search strategy that was followed to identify the relevant publications (Figure 5).

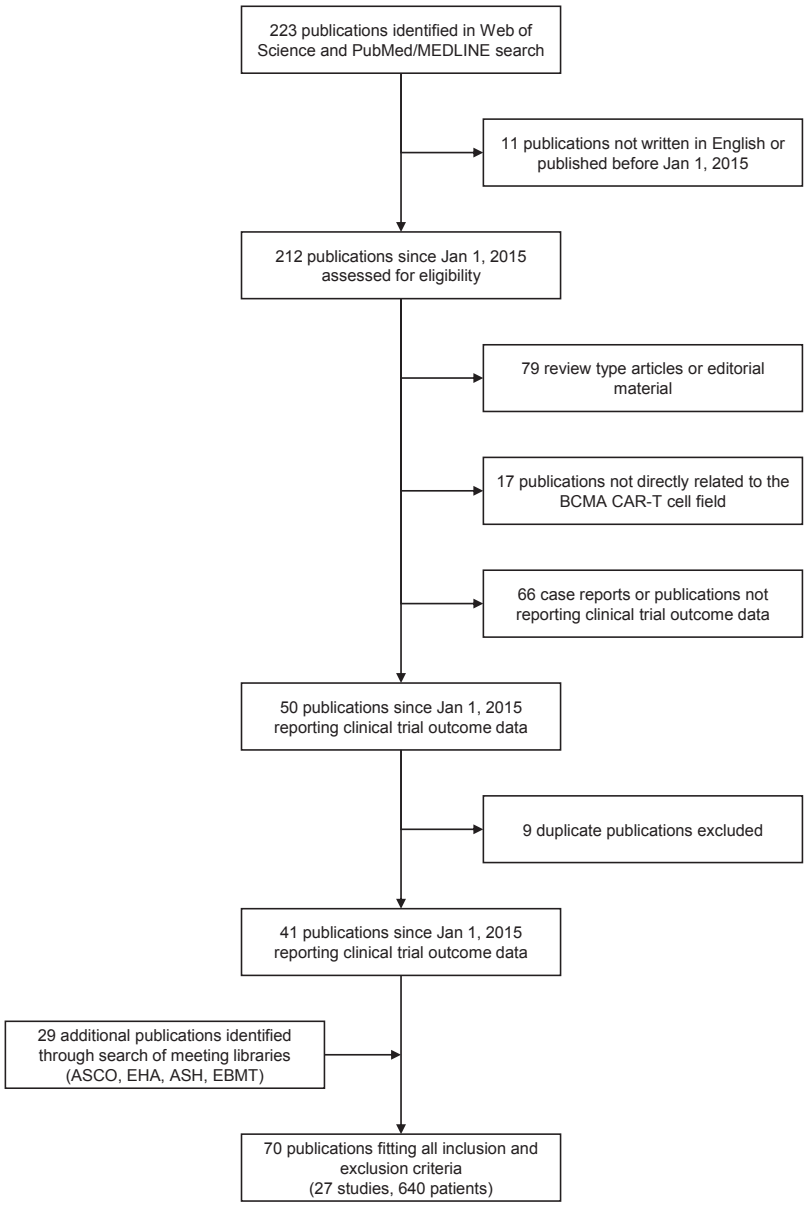

Figure 5. Search strategy and study selection. ASCO=American Society of Clinical Oncology. ASH=American Society of Hematology. BCMA=B-cell maturation antigen. CAR=chimeric antigen receptor. EBMT=European Group for Blood and Marrow Transplantation. EHA=European Hematology Association.

Data analysis

Table S1 provides an overview of the 61 publications that were retrieved following the PRISMA flow diagram depicted in Figure 5. Based on the clinical trial registration number (NCT-number or ChiCTR-number), the CAR-T-cell product name and the study group (lead author and affiliation), we were able to identify 27 different studies comprising 640 unique patients. For several studies, overlapping publications were identified; to avoid duplicate data, only the most recent and/or the largest (based on the number of included patients) records were considered (Table S1). As shown in Table S1, there were two exceptions to this rule. For study NCT02546167 (CART-BCMA UPenn), we decided to use the full publication [102] rather than the meeting abstract[161]. For study NCT03661554 (BCMA nanoantibody), the latest publication involving 16 CAR-T-infused patients was not considered because outcome data were incompletely reported (only for 7 patients) [162].

Primary outcome measures were CAR-T-cell-related toxicities (i.e. CRS and neurotoxicity) and objective response rate (ORR). ORR was defined as the sum of (stringent) complete responses ([s]CR) and (very good) partial responses ([VG]PR), according to IMWG criteria [163]. Progression-free survival (PFS) was used as secondary outcome measure. We collected data on the following patient- and disease-related variables: number of patients, median age, myeloma risk (based on cytogenetics and/or International Staging System [ISS] score), and prior lines of therapy. Information on the following treatment-related variables was extracted: origin and type of the CAR antigen-recognition domain, enrichment/activation method, loading strategy, type of co-stimulatory domain, cell dosage, and lymphodepletion regimen.

We conducted a meta-analysis for proportions to estimate the overall proportion of CRS/neurotoxicity and ORR/CR. Because of the diversity between the studies, a random-effects model was used. Heterogeneity was judged by forest plots and I^2. Results are reported as proportions with 95% confidence interval (CI). Subgroup analyses were performed to assess differences between groups of studies. P-values were calculated based on the between subgroups heterogeneity statistic.

Median PFS with 95% CI was calculated from individual patient data, which were retrieved using computerized analysis of published Swimmer plots and/or Kaplan-Meier survival curves. We verified the correctness of the retrieved data by back-checking that the calculated median PFS was identical to the published median PFS of each study. A comparative analysis was performed between CAR-T cells used at active doses with inactive doses, where an inactive dose was defined as a CAR-T cell dose that failed to produce both CRS and ORR rates of >50%. This corresponded to the patients included in the lowest dose cohorts of the following four early-phase BCMA CAR-T-cell studies with a dose-escalation design: NCT02658929 [105], NCT02546167 [102], NCT02215967 [164] and NCT03070327 [165]. In the absence of randomized controlled trials, the latter served as a surrogate control group to determine the

expected PFS. A marginal Cox regression model with clustering per study was used to assess differences in PFS between the subgroups. All statistical analyses were performed using R v3.4.4. (R Foundation for Statistical Computing, Vienna, Austria). This study was registered with PROSPERO (CRD42020125332).

Results

As shown in Table 4, Figure 4 and Figure 5, 27 studies involving 23 different BCMA CAR-T-cell products were identified. Data were available from 640 BCMA CAR-T-cell treated patients. For 11 CAR-T-cell products, the extracellular BCMA-recognition domain of the CAR consisted of a human(ized) mAb in scFv format (Table 4) [97]. In one study (NCT03288493), the antigen-recognition domain was composed of a centyrin, a human fibronectin type III-based antibody mimetic [166, 167], while another (NCT03602612) used a human heavy-chain-only binding domain [168]. All other studies used non-human antibodies, either murine scFv mAb or nanobodies derived from alpaca or llama [107, 169]. Bb2121 and LCAR-B38M, the two most advanced BCMA CAR-T-cell products, used a murine- and llama antibody-based CAR construct, respectively (Table 5). The method used for T-cell enrichment/activation was not reported in the majority of the studies; anti-CD3 and anti-CD28 antibodies (usually coupled to magnetic beads) or an anti-CD3 antibody alone, with or without interleukin (IL)-2, were mostly used [170]. Lentiviral (489/640 patients; 76.4%) and, to a lesser extent, gamma-retroviral transduction (101/640 patients; 15.8%) were the preferred transduction methods (Table 4). NCT03288493 (23/640 patients; 3.6%) was the only clinical trial so far in which a non-viral delivery method was applied (i.e. a transposon). In two trials (ChiCTR-1800018143 and ChiCTR-1900027678) the method of CAR loading was not defined (Table 4) [171, 172]. In 520/640 patients (81.3%), a 4-1BB-based second-generation CAR construct was used; the other patients received BCMA CAR-T cells with a CD28 co-stimulatory domain (either alone or in combination with OX40 or 4-1BB). One study (ChiCTR-1900027678) did not disclose the type of co-stimulatory domain [172]. CAR-T cell dosages varied considerably across the different studies, from 0.07×10^6/kg to $>1000 \times 10^6$ cells. This variation is also exemplified in Table 5, comparing bb2121 and LCAR-B38M, showing a 10-fold difference between both studies in CAR-T-cell dosage used (Table 5). Cyclophosphamide, usually in combination with fludarabine, was the most frequently used lymphodepleting chemotherapy regimen.

Among 639 patients evaluable for safety, 80.3% (69.0–88.2) experienced CRS (Table 4). CRS is graded on a scale from 1-4 [158]; severe CRS (i.e. grade ≥3) occurred in 14.1% of patients (9.6–20.4). As shown in Table 5, detailing the key differences between the two most advanced BCMA CAR-T products bb2121 and LCAR-B38M, the median time of CRS onset varied greatly between 1 and 9 days. The median duration was between 7 days and 9 days for bb2121 and LCAR-B38M, respectively; CRS could last to up to 2 months (Table 5). The pooled CRS rate

was 61.0% (35.3–81.8; I^2=84%), 83.8% (70.9–91.7; I^2=71%) and 91.0% (83.8–95.2; I^2=0%) in studies using CAR constructs with murine-based, human(ized) and alpaca/llama-derived antigen-binding domains, respectively (Table S2 and Figure S1). Despite the apparently lower CRS rate in studies using murine scFv-based CAR constructs, individual studies revealed a clear "dose-toxicity" relation. For example, with the bb2121 CAR-T product, which contains a murine anti-BCMA scFv, a CRS rate of 96.3% was noted at the recommended phase II dose of 450 × 10^6 cells (Table 5), whereas it was only 75.7% and 50.0% at the 300 × 10^6 and 150 × 10^6 dose levels, respectively [173].

The pooled neurotoxicity rate was 10.5% (6.8–16.0), with a considerable variation between the different studies. For example, in the bb2121 study, 20.4% of the patient experienced some sort of neurological symptoms, whereas only 1.8% of the LCAR-B38M-treated patients had neurotoxicity (Table 5). The origin of the antigen-recognition domain (murine, human(ized) or alpaca/llama) had no impact on neurotoxicity (Table S3). Lymphodepletion with cyclophosphamide and fludarabine, a known neurotoxic agent, did not lead to more neurological events as compared to cyclophosphamide alone or no lymphodepletion. A lower rate of neurotoxicity was observed in studies that used anti-CD3 mAbs alone instead of anti-CD3/CD28 mAbs for T-cell enrichment/activation (4.9% [2.1–10.9; I^2=0%] versus 15.9% [8.1–28.9; I^2=66%]; p=0.028). A similar observation was made for studies that used CD28 instead of 4-1BB as co-stimulatory backbone (3.4% [1.2–9.3; I^2=0%] versus 12.9% [8.2–19.6; I^2=59%]; p=0.018). A higher rate of neurotoxicity was observed in studies in which the median patient age was ≥60 years (20.5% [12.5–31.9; I^2=63%] versus 6.4% [3.3–12.0; I^2=38%]; p=0.0043), and in studies in which the median number of prior lines of therapy was ≥5 (19.1% [13.3–26.7; I^2=45%] versus 2.8% [1.3–6.1; I^2=0%]; p<0.0001; Figure S2).

Table 4. Multiple myeloma CAR-T-cell clinical trials targeting BCMA.

Trial # [ref.] (product name)	n=	Origin mAb	Expansion	Loading	Co-stimulation	T-cell dosage	Conditioning	Toxicity	Clinical response
ChiCTR-OIC17011272 [108] (CD19 & BCMA CAR-T)	21	Murine scFv	aCD3	Lentiviral	4-1BB	1×10^6/kg	CP/Flu	CRS gr. 1-2 (86%), gr. ≥3 (5%) Neurotoxicity (10%)	sCR/CR (57%) / VGPR (24%) PR (14%)
NCT02658929 [105, 174] (bb2121)	43/39	Murine scFv	aCD3+aCD28	Lentiviral	4-1BB	50-800×10^6	CP/Flu	CRS gr. 1-2 (58%), gr. ≥3 (5%) Neurotoxicity (33%)	sCR/CR (44%) / VGPR (23%) PR (10%)
NCT03274219 [175] (bb21217)	38	Murine scFv	aCD3+aCD28 + PI3k inhibitor	Lentiviral	4-1BB	150-450×10^6	CP/Flu	CRS gr. 1-2 (61%), gr. ≥3 (5%) Neurotoxicity (24%)	sCR/CR (13%) / VGPR (34%) PR (5%)
ChiCTR-OPC16009113 [176, 177] (BCMA-CAR T)	28	Murine scFv	aCD3	Lentiviral	CD28/4-1BB	5.4-25×10^6/kg	CP/Flu	CRS gr. ≥3 (14%)	sCR/CR (61%) / VGPR (4%) PR (21%)
NCT02215967 (1) [164, 178] (NCI BCMA CAR-T)	10	Murine scFv	aCD3 IL-2	Retroviral	CD28	0.3-3×10^6/kg	CP/Flu	CRS gr. 1-2 (30%)	VGPR (10%) PR (10%)
NCT02215967 (2) [164] (NCI BCMA CAR-T)	16	Murine scFv	aCD3 IL-2	Retroviral	CD28	9×10^6/kg	CP/Flu	CRS gr. 1-2 (56%), gr. ≥3 (38%) Neurotoxicity (6%)	sCR/CR (13%) / VGPR (50%) PR (19%)
ChiCTR-1800018143 [171] (BM38 CAR)	22	Humanized scFv	ND	ND	4-1BB	0.5-4×10^6/kg	CP/Flu	CRS gr. 1-2 (68%), gr. ≥3 (23%)	sCR/CR (55%) / VGPR (9%) PR (24%)
NCT02546167 [102] (CART-BCMA UPenn)	25	Human scFv	aCD3/CD28	Lentiviral	4-1BB	50-500×10^6	CP or none	CRS gr. 1-2 (56%), gr. ≥3 (32%) Neurotoxicity (32%)	sCR/CR (8%) / VGPR (20%) PR (20%)
NCT03302403, NCT03380039, NCT03716856 [179, 180] (CT053)	24	Human scFv	aCD3/CD28	Lentiviral	4-1BB	50-180×10^6	CP/Flu	CRS gr. 1-2 (63%) Neurotoxicity (8%)	sCR/CR (79%) / VGPR (4%) PR (4%)
NCT03430011 [181] (JCARH125)	44	Human scFv	ND	Lentiviral	4-1BB	50-450×10^6	CP/Flu	CRS gr. 1-2 (70%), gr. ≥3 (9%) Neurotoxicity (25%)	sCR/CR (27%) / VGPR (20%) PR (34%)

Trial # [ref.] (product name)	n=	Origin mAb	Expansion	Loading	Co-stimulation	T-cell dosage	Conditioning	Toxicity	Clinical response
NCT03815383 [182] (C-CAR088)	5	Human scFv	ND	Lentiviral	4-1BB	1-3×10^6/kg	CP/Flu	CRS gr. 1-2 (80%)	sCR/CR (20%) / VGPR (60%) PR (20%)
ChiCTR-1800018137 [183] (CT103A)	18	Human scFv	ND	Lentiviral	4-1BB	1-6×10^6/kg	CP/Flu	CRS gr. 1-2 (72%), gr. ≥3 (22%)	sCR/CR (67%) / VGPR (17%) PR (17%)
NCT03549442 [184] (CART-BCMA+CTL119)	16	Human scFv	ND	Lentiviral	4-1BB	500×10^6	CP/Flu	CRS gr. 1-2 (88%)	sCR/CR (19%) / VGPR (25%) PR (25%)
NCT03338972 [185] (FCARH143)	11	Human scFv	aCD3/CD28	Lentiviral	4-1BB + EGFRt	50-150×10^6	CP/Flu	CRS gr. 1-2 (91%) Neurotoxicity (9%)	sCR/CR (55%) / VGPR (36%) PR (9%)
NCT03502577 [186] (FCARH143+GSI)	10	Human scFv	ND	Lentiviral	4-1BB + EGFRt	50-300×10^6	CP/Flu	CRS gr. 1-2 (60%), gr. ≥3 (40%) Neurotoxicity (60%)	sCR/CR (30%) / VGPR (50%) PR (20%)
NCT03196414 [187] (SZ-MM-CART01)	29/28	Humanized scFv	aCD3	Lentiviral	CD28/OX40	20-82×10^6/kg	CP/Flu	CRS gr. 1-2 (66%), gr. ≥3 (34%) Neurotoxicity (3%)	sCR/CR (54%) / VGPR (4%) PR (29%)
NCT03455972 [188] (SZ-MM-CART02)	32	Humanized scFv	aCD3	Lentiviral	CD28/OX40	50×10^6/kg	BUCY or Mel + autoHSCT	CRS gr. 1-2 (97%), gr. ≥3 (3%)	sCR/CR (72%) / VGPR (ND) PR (ND)
NCT03070327 [165] (MCARH171)	10/11	Human scFv	ND	Retroviral	4-1BB + EGFRt	1×10^6/kg or 150-450×10^6	CP/Flu or CP	CRS gr. 1-2 (40%), gr. ≥3 (20%) Neurotoxicity (10%)	VGPR (45%) PR (18%)
NCT03602612 [168] (FHVH33)	15	Human VH	ND	Retroviral	4-1BB	ND	CP/Flu	CRS gr. 1-2 (87%), gr. ≥3 (7%) Neurotoxicity (27%)	sCR/CR (20%) / VGPR (7%) PR (53%)
NCT03288493 [166] (P-BCMA-101)	23/19	Human centyrin	None	Trans-poson	4-1BB + rimiducid SS	51-1143×10^6	CP/Flu	CRS gr. 1-2 (9%) Neurotoxicity (4%)	sCR/CR + VGPR (26%) PR (42%)

Trial # [ref.] (product name)	n=	Origin mAb	Expansion	Loading	Co-stimulation	T-cell dosage	Conditioning	Toxicity	Clinical response
NCT03661554 [169] (BCMA nanoantibody)	9	Alpaca V_HH	ND	Lentiviral	4-1BB	250-900×10^6	CP/Flu	CRS gr. 1-2 (67%), gr. ≥3 (22%) Neurotoxicity (11%)	sCR/CR (56%) / VGPR (33%) PR (11%)
NCT03090659 (1) [106, 189] (LCAR-B38M)	17	Llama V_HH	aCD3/CD28	Lentiviral	4-1BB	0.21-1.52×10^6/kg	CP/Flu or CP	CRS gr. 1-2 (59%), gr. ≥3 (41%)	sCR/CR (82%) / VGPR (6%)
NCT03090659 (2) [107, 190] (LCAR-B38M)	57	Llama V_HH	aCD3/CD28 IL-2	Lentiviral	4-1BB	0.07-2.1×10^6/kg	CP	CRS gr. 1-2 (82%), gr. ≥3 (7%) Neurotoxicity (2%)	sCR/CR (73%) / VGPR (4%) PR (11%)
NCT03548207 [191] (LCAR-B38M)	29	Llama V_HH	ND	Lentiviral	4-1BB	0.5-0.9×10^6/kg	CP/Flu	CRS gr. 1-2 (86%), gr. ≥3 (7%) Neurotoxicity (10%)	sCR/CR (69%) / VGPR (17%) PR (14%)
ChiCTR-1800017404 [192] (BCMA CAR-T)	33/32	ND	ND	Lentiviral	4-1BB	1-6×10^6/kg	CP/Flu	CRS gr. 1-2 (52%), gr. ≥3 (48%)	sCR/CR (66%) / VGPR (22%) PR (13%)
NCT03093168 [193] (HRAIN BCMA-CART)	49	ND	ND	Retroviral	4-1BB + EGFRt	9×10^6/kg	CP/Flu	CRS gr. 1-2 (12%), gr. ≥3 (6%)	sCR/CR (45%) / VGPR (18%) PR (14%)
ChiCTR-1900027678 [172] (GC012F)	5	ND	ND	ND	ND	1-2×10^6/kg	CP/Flu or none	CRS gr. 1-2 (80%)	sCR/CR (20%) / VGPR (80%)
Pooled studies	**639/630**							**CRS gr. 1-4 (80.3%) (95% CI 69.0-88.2; I^2=83%) Neurotoxicity (10.5%) (95% CI 6.8-16.0; I^2=58%)**	**ORR (80.5%) (95% CI 73.5-85.9; I^2=61%)**

aCD3+aCD28=anti-CD3 and anti-CD28 antibodies. aCD3/CD28 + IL-2=anti-CD3 and anti-CD28-coated beads plus interleukin-2. AutoHSCT=autologous hematopoietic stem cell transplant. BCMA=B-cell maturation antigen. BUCY=busulfan and cyclophosphamide. CAR=chimeric antigen receptor. CP=cyclophosphamide. CR=complete response. CRS=cytokine release syndrome. EGFRt=truncated epidermal growth factor receptor. Flu=fludarabine. Gr.=grade. GSI=gamma-secretase inhibitor. IL-2=interleukin-2. Mel=melphalan. n=number of patients evaluable for toxicity/clinical response. ND=not disclosed. PI3k=Phosphoinositide 3-kinase. PR=partial response. scFv=single-chain Fragment variable. SS=safety switch. sCR=stringent complete response. Trial #=study registration number in Clinicaltrials.gov (NCT#) or Chinese Clinical Trial Registry (ChiCTR-#). VGPR=very good partial response. V_HH=nanobody.

A total of 630 patients were evaluable for clinical response (Table 4). The pooled ORR was 80.5% (73.5–85.9) with (s)CR in 44.8% (35.3–54.6) of patients. Responses occurred rapidly, usually within the first month after CAR-T-cell infusion. Despite the higher likelihood to achieve a deep response in studies that included less pretreated patients (CR: 57.6% [45.2–69.0; I^2=63%]; p=0.011), a (s)CR rate of 32.9% (21.1–47.4; I^2=77%) was still achieved in studies with a median of ≥5 prior lines of therapy. Concerning the treatment-related variables, a superior CR rate of 71.9% (62.8–79.6; I^2=0) was noted in studies with an alpaca/llama-derived BCMA-recognition domain (p<0.0001 compared to their human and murine counterparts; Figure S3). Responses were usually deeper in studies that used an alpaca/llama-based anti-BCMA CAR construct, as exemplified by LCAR-B38M in Table 5. Finally, the CR rate was only 18.0% (6.5–41.1; I^2=67%) in studies that used a retroviral instead of a lentiviral vector (50.6% [39.8–61.4; I^2=77%]) for CAR-T-cell transduction (p=0.015; Table S4).

PFS data were available for 551 patients; the median PFS of patients treated with active BCMA CAR-T-cell doses was 12.2 months (11.4–17.4), comparing favorably to the 1.9-month PFS (1.5–3.7) observed in patients treated with inactive doses in the dose-escalation studies NCT02658929, NCT02546167, NCT02215967 and NCT03070327 (Hazard ratio [HR] 0.14; p<0.0001; Fig. 3A). In line with the superior clinical response rate, patients treated with lentivirally transduced CAR-T cells had a significantly longer PFS than those treated with retroviral constructs (12.8 months [11.4–19.9] versus 4.3 months [3.0–15.0]; HR 0.48; p=0.0065; Fig. 3B; Table S4). Although no difference was seen in terms of ORR, we observed a shorter PFS among patients treated with BCMA CAR-T cells containing a CD28-based co-stimulatory backbone (8.0 months [4.0–15.0] versus 12.2 months [10.8–17.4] with a 4-1BB-based co-stimulatory domain); however, this difference was not statistically significant (HR 0.63, p=0.061; Fig. 3C). The median PFS in the bb2121 study was 12.1 months (8.8–12.3); in the LCAR-B38M study, a median PFS of 19.9 months (9.6–31) was reported (Table 5). The longest PFS rates were observed in studies that used alpaca/llama constructs (p=0.0005; Fig. 3D; Table S2).

Table 5. Comparison of KarMMa (bb2121) and LEGEND-2 (LCAR-B38M) clinical studies.

	bb2121 / KarMMa [173]	**LCAR-B38M / LEGEND-2 (Xi'an site) [107, 190]**
Alternative product name	Ide-cel	Cilta-cel
Trial # (study phase)	NCT03361748 (phase II)	NCT03090659 (phase I)
n of patients	128	57
	(54 at RD of 450 × 10^6)	
Expansion method	aCD3+aCD28	aCD3/CD28 + IL-2
Loading method	Lentiviral	Lentiviral
CAR-T structure	Murine scFv	Llama 2xV_HH
		
Lymphodepletion	CP/Flu	CP
CAR-T cell dosage(s)	150-300-450 × 10^6	32.3 × 10^6 (3.3 to 126.2 × 10^6)
Patient characteristics		
Age (range), y	61 (33-78)	54 (27-72)
Median n PLT (range)	6 (3-16)	3 (1-9)
High-risk features[a]	51%	37%
CRS	96.3%[b]	89.5%
Gr. 1-2	90.7%	82.5%
Gr. ≥3	5.6%	7.0%
Median onset (range)	1d (1 – 10)	9d (1 – 19)
Median duration (range)	7d (1 – 63)	9d (3 – 57)
Tocilizumab use	67%	46%
Neurotoxicity	20.4%[§]	1.8%
ORR	82%[§]	88%
MRD^- CR	28%	68%
CR	11%	5%
VGPR	26%	4%
PR	17%	11%
Median PFS (95% CI)	12.1m (8.8 – 12.3)[§]	19.9m (9.6 – 31)

aCD3+aCD28=anti-CD3 and anti-CD28 antibodies. aCD3/CD28 + IL-2=anti-CD3 and anti-CD28-coated beads plus interleukin-2. cilta-cel=ciltacabtagene autoleucel. CP=cyclophosphamide. CP/Flu=cyclophosphamide plus fludarabine. CR=complete response. CRS=cytokine release syndrome. d=days. Gr.=grade. ide-cel=idecabtagene vicleucel. m=months. MRD=minimal residual disease. n=number. ORR=objective response rate. PFS=progression-free survival. PLT=prior lines of treatment. RD=recommended dose. scFv=single-chain variable fragment. (VG)PR=(very good) partial response. VHH=heavy-chain variable region. Trial #=study registration number in Clinicaltrials.gov (NCT#). y=years. [a]=high-risk defined as R-ISS stage 3 and/or high-risk genetics (del(17p), t(4;14), t(14;16)). [b]=data shown for the 450 × 10^6 dose cohort only.

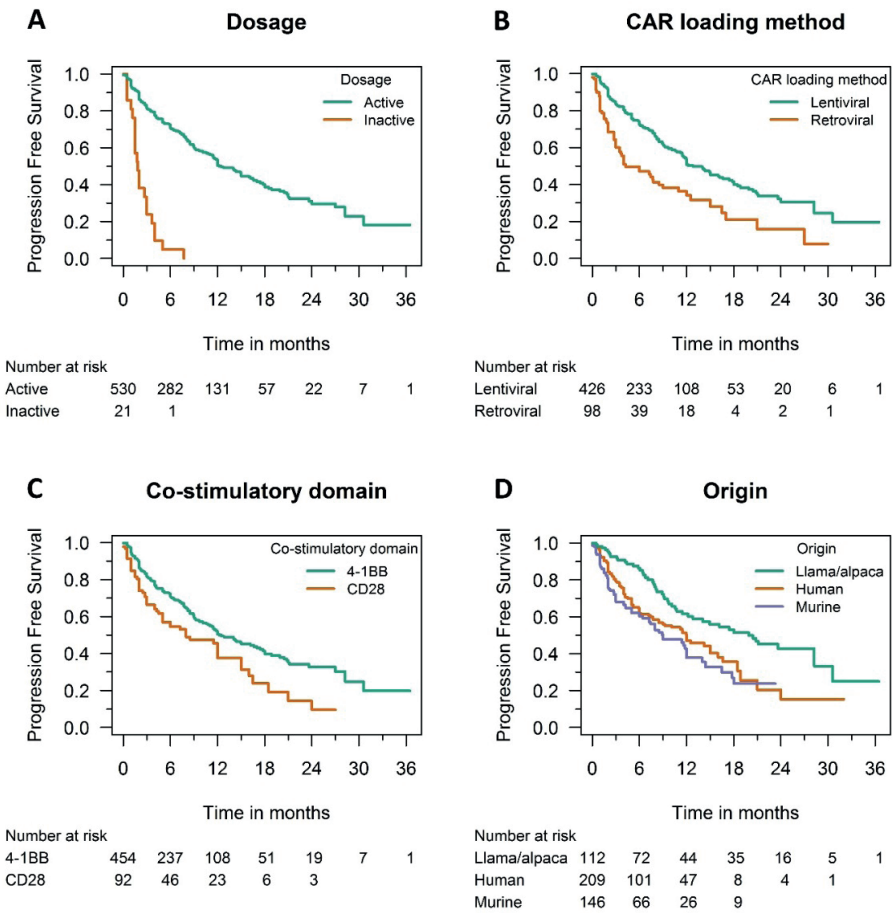

Figure 6. Kaplan-Meier progression-free survival curves.

Discussion

This meta-analysis provides insights into the risk and benefits of BCMA CAR-T-cell therapy in MM, into the diversity of the patient populations included and BCMA CAR-T-cell products used, and into the various factors that potentially contribute to toxicity and efficacy. As of Jan 1, 2020, 27 registered clinical studies have been published involving 23 different CAR-T-cell products and 640 patients. A high response rate was observed, with demonstrable responses in 8/10 patients (nearly half of whom had a CR). Toxicity was equally high, with CRS occurring in 8/10 patients and neurotoxicity in 1/10 patients. Despite the high initial response rate, responses were usually temporary and relapses were frequently observed, resulting in a median PFS of 12.2 months in patients receiving active doses of BCMA CAR-T cells. The two most advanced BCMA CAR-T products are bb2121 or idecabtagene-vicleucel (which contains a murine anti-

BCMA scFv as antigen-recognition domain) and LCAR-B38M or ciltacabtagene autoleucel (which contains two llama anti-BCMA heavy chain variable regions or V_HH) [105, 189, 190].

At the recommended phase II dose level of 450 × 10^6 cells, the murine BCMA CAR-T product bb2121 yielded comparable CRS rates as its human(ized) or alpaca/llama-based counterparts (Table 5) [173], indicating that not the species origin of the CAR antigen-recognition domain but the CAR-T cell dosage is a major determinant of CRS. Albeit mostly low grade, neurotoxicity occurred in up to 1 out of 5 patients treated with bb2121; in LCAR-B38M-treated patients, the neurotoxicity rate was 10-fold lower (Table 5). Since the origin of the antigen-recognition domain (murine, human or alpaca/llama) was not found to be a risk factor for neurotoxicity in this meta-analysis, other factors should have contributed to the observed difference in neurotoxicity rate between bb2121 and LCAR-B38M. In the LEGEND-2 study conducted at the Xi'An site in China (NCT03090659) [107, 190], the largest study with LCAR-B38M published to date, the lymphodepleting regimen consisted of cyclophosphamide alone whereas cyclophosphamide and fludarabine were used in the KarMMa pivotal phase II study with bb2121 (NCT03361748) (Table 5). The use of fludarabine for lymphodepletion, which by itself can cause neurotoxicity, was shown to increase the risk of neurologic adverse events in the CD19 CAR-T-cell field [60]. Our meta-analysis, however, failed to demonstrate a role for fludarabine in the higher rate of neurotoxicity in the KarMMa study of bb2121. Dual anti-CD3/CD28 stimulation during CAR-T-cell culture and 4-1BB as co-stimulatory domain were identified as potential risk factors for neurotoxicity in this study. As indicated in Table 5, both KarMMa (bb2121) and LEGEND-2 (LCAR-B38M) used anti-CD3/CD28-stimulated 4-1BB-based CAR-T cells. We, therefore, believe that these factors are not major drivers of neurotoxicity and, at least, do not explain the difference in neurotoxicity rates between both studies. Although this is in sharp contrast with what has been observed in studies with CD19 CAR-T cells [194, 195], this meta-analysis pointed to a higher risk of neurotoxicity in BCMA CAR-T-cell studies in which the median patient age was ≥60 years and/or in which the median number of prior lines of anti-myeloma treatments was ≥5. As shown in Table 5, LEGEND-2 (LCAR-B38M) tended to include younger and less pretreated patients, possibly explaining the lower frequency of neurological events as compared to KarMMa (bb2121).

Although a previous clinical trial of CD19 CAR-T-cell therapy in CLL failed to demonstrate such correlation [196], we observed a lower rate of deep responses ([s]CR) in studies that included more heavily pretreated patients (≥5 prior lines of treatment). This explains why in the LEGEND-2 study a higher proportion of LCAR-B38M-treated patients achieved an (MRD-negative) CR status as compared to the bb2121-treated patients in KarMMA. The rationale behind this is that apheresis products of less pretreated MM patients contain "fitter" T cells [197], resulting in better clinical responses. Autologous BCMA CAR-T-cell therapies are now being positioned earlier in the course of the disease (NCT03549442, NCT03455972) in an attempt to produce deeper and more durable clinical responses. The fact that KarMMa included more

high-risk MM patients as compared to LEGEND-2 (Table 5) likely played no role in the lower deep response rate. Indeed, in this meta-analysis, myeloma risk was not associated with reduced activity, indicating that BCMA CAR-T-cell therapy is also highly efficacious in the high need subgroup of high-risk MM patients. Another factor possibly contributing to the superior therapeutic activity of LCAR-B38M is related to the use of (two) llama V_HHs as antigen-binding domain in contrast to the murine scFv-based CAR construct of bb2121. It is known that CARs based on heavy-chain-only antibodies (such as alpaca or llama-derived V_HH) have superior BCMA-binding capability of V_HH compared to traditional scFv-based domains [198, 199]. This also reflected by the fact that 10-fold lower CAR-T cell dosages were required in LEGEND-2 (LCAR-B38M) as compared to KarMMa (bb2121). To summarize, although head-to-head trials between bb2121 and LCAR-B38M have not been conducted, the results of this meta-analysis indicate that the differences in terms of MRD-negativity, depth of response, and, consequently, PFS, between both products are in large part attributable to the different patient populations included and possibly also to the type of antigen-recognition domain used.

Although there was no statistically significant difference in terms of ORR, PFS was markedly longer in the 4-1BB subgroup. This corroborates recent research showing longer CAR-T-cell persistence and improved response durability with 4-1BB-based as compared to CD28-based CD19 CAR-T cells [135]. Although this should still be confirmed in a randomized controlled trial, our results also seem to favor the use of lentiviral over retroviral vectors for CAR-T-cell transduction given their superior clinical activity without increasing toxicity. Non-viral CAR loading methods, such as DNA transposons, are gaining popularity but how these compare to lentiviral or retroviral transduction in terms of toxicity and activity remains to be established.

We observed a 6-fold increase in median PFS in the treatment group compared to the control group, which received an inactive CAR-T-cell dose. The low PFS (~2 months) in the control group is congruent with previous literature [200], and illustrates the grim prognosis of the patients included so far in BCMA CAR-T-cell studies. In contrast to what is observed in the field of CD19-directed CAR-T-cell therapy for diffuse large B-cell lymphoma, the tail of the PFS curve did not reveal a plateau. This indicates that the majority of the patients will eventually relapse. Possible explanations are lack of CAR-T cell persistence, antigen escape, the hostile tumor microenvironment and exhaustion. Persistence can be improved by altering the CD4:CD8 composition of the infusion product [185, 201], or by enriching the product with stem cell memory T cells [166, 202]. BCMA downregulation or loss was observed in several trials [102, 164, 178, 185]; this can be mediated by shedding of BCMA from the cell surface [151] or by CAR-T cell-induced trogocytosis. The latter not only leads to reduced tumor cell recognition, but also to CAR-T-cell fratricide [203]. In order to prevent BCMA shedding, γ-secretase inhibitors are being combined with BCMA CAR-T cells (NCT03502577) [186]. Another approach to circumvent antigen escape is co-targeting of BCMA and another antigen, such as CD19 (NCT03196414, NCT03455972) [108, 188], or simultaneous targeting of two BCMA epitopes

as in LCAR-B38M [106, 107, 189-191]. Relapse can also occur despite CAR-T-cell persistence and maintained BCMA expression. The hypoxic niche in the BM, where MM cells reside, impairs cytokine secretion and granzyme B release from BCMA CAR-T cells [204]. In addition, upregulation of immune checkpoint molecules, such as programmed death-1 (PD-1), results in BCMA CAR-T-cell exhaustion which can be restored by PD-1 blockade [205]. Tonic signaling in the absence of antigen can induce CAR-T-cell exhaustion as well; proper selection of the antigen-recognition domain [166] and the co-stimulatory domain [203] can help to minimize CAR tonic signaling.

In conclusion, this meta-analysis provides robust evidence for the high clinical activity of BCMA CAR-T-cell therapies in MM and shows that several patient- and treatment-related factors might contribute to their toxicity and efficacy. These findings may inform the design of future CAR-T-cell studies in MM.

Acknowledgements

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Supplementary material

Table S1. Overview of the studies identified per clinical trial according to the search strategy and study selection. Publications are ordered based on their reported cut-off date or, if no cut-off date was stated, based on publication year. Reports used for data analysis are in bold. * indicates when abstract data was combined with data from the oral or poster presentation of this abstract.

Trial # (product name)	N (cut-off date)	Ref
ChiCTR-OIC17011272	**21 (20/01/2019)**	**[108]**
(CD19 & BCMA CAR-T)		
NCT02658929	36 (30/04/2018)	[105]
(bb2121)	**43 (29/03/2018)***	**[174]**
	21 (02/10/2017)	[206]
	10 (02/10/2017)	[207]
	21 (04/05/2017)	[208]
	11 (18/11/2016)	[209]
	09 (28/10/2016)	[210]
NCT03274219	**38 (04/09/2019)***	**[175]**
(bb21217)	8 (15/06/2018)	[202]
ChiCTR-OPC16009113	**28 (27/03/2018)**	**[177]**
(BCMA-CAR T)	2	[176]
NCT02215967 (1)	**10**	**[164]**
(NCI BCMA CAR-T)	10	[211]
	10	[212]
	6	[213]
NCT02215967 (2)	**16**	**[164]**
(NCI BCMA CAR-T)	13	[211]
ChiCTR-1800018143	**22 (31/10/2019)***	**[171]**
(BM38 CAR)	12 (31/01/2019)	[214]
NCT02546167	25 (07/09/2018)	[201]
(CART-BCMA UPenn)	**25 (09/07/2018)**	**[215]**
	21 (24/07/2017)	[216]
	6	[217]
NCT03302403, NCT03380039,	**24 (22/09/2018)**	**[179]**
NCT03716856	24 (28/02/2019)	[218]
(CT053)	16 (10/07/2018)	[180]
NCT03430011	**44**	**[181]**
(JCARH125)		
NCT03815383	**5 (30/11/2019)***	**[182]**
(C-CAR088)		
ChiCTR-1800018137	**18 (28/11/2019)***	**[183]**
(CT103A)	12 (18/06/2019)	[219]

Trial # (product name)	N (cut-off date)	Ref
	9 (04/02/2019)	[220]
NCT03549442	**16**	**[184]**
(CART-BCMA+CTL119)		
NCT03338972	**11***	**[185]**
(FCARH143)		
NCT03502577	**11***	**[186]**
(FCARH143+GSI)		
NCT03196414	**28 (31/01/2019)**	**[187]**
(SZ-MM-CART01)	8	[221]
NCT03455972	**32**	**[188]**
(SZ-MM-CART02)	10	[222]
	9	[223]
NCT03070327	**11 (16/07/2018)**	**[165]**
(MCARH171)	6	[224]
NCT03602612	**15***	**[168]**
(FHVH33)		
NCT03288493	**23 (29/11/2018)***	**[166]**
(P-BCMA-101)	2	[225]
NCT03661554	16 (31/12/2018)	[162]
(BCMA nanoantibody)	**9***	**[169]**
NCT03090659 (1)	**17 (20/07/2019)**	**[189]**
(LCAR-B38M)	17 (20/10/2018)	[106]
NCT03090659 (2)	**57 (31/12/2018)**	**[190]**
(LCAR-B38M)	57 (25/06/2018)	[226]
	57 (06/02/2018)	[107]
	19	[227]
	5 (31/07/2017)	[228]
	22 (20/02/2017)	[229]
NCT03549207	**29***	**[191]**
(LCAR-B38M)		
ChiCTR-1800017404	**33 (01/08/2019)**	**[192]**
(BCMA CAR-T)	19 (01/12/2018)	[230]
	17 (28/11/2018)	[231]
NCT03093168	**49***	**[193]**
(HRAIN BCMA-CART)	17 (06/07/2018)	[232]
	10 (31/12/2017)	[233]
ChiCTR-1900027678	**5 (31/07/2019)**	**[172]**
(GC012F)		

Table S2. Subgroup comparison for antigen-recognition domain origin.

	Human Proportion (95% CI)	Murine Proportion (95% CI)	Llama/alpaca Proportion (95% CI)	P-value
Number of studies	16	5	3	
Number of patients	285	156	112	
CRS grade 1-2	68.24% (56.59 – 77.98%)	53.30% (34.24 – 71.45%)	76.26% (61.88 – 86.41%)	0.15
CRS grade 3-4	15.58% (9.66 – 24.18%)	10.11% (4.08 - 22.93%)	15.74% (5.26 - 38.59%)	0.67
CRS all grades	83.83% (70.90 – 91.69%)	61.03% (35.32 – 81.79%)	91.00% (83.83 – 95.18%)	0.013
Neurotoxicity	12.63% (6.96 – 21.85%)	14.88% (7.00 – 28.87%)	6.48% (2.69 – 14.79%)	0.31
CR	39.88% (26.69 – 54.73%)	31.97% (16.02 – 53.66%)	71.91% (62.76 – 79.55%)	< 0.0001
CR/VGPR	56.40% (43.12 – 68.83%)	59.70% (44.92 – 72.90%)	81.53% (73.04 – 87.79%)	0.0013
ORR	78.72% (69.32 – 85.83%)	72.24% (50.95 – 86.70%)	89.52% (81.57 – 94.29)	0.060
Median PFS	12.0m (7.4 – 16.6)	9.0m (5.0 – 14.4)	19.9m (16.2 – 28.2)	0.0005

Table S3. Subgroup comparison for neurotoxicity.

Condition	Condition = FALSE		Condition = TRUE		
	N studies (patients)	Proportion neurotoxicity (95% CI)	N studies (patients)	Proportion neurotoxicity (95%CI)	P-value
Median age ≥ 60	16 (326)	6.41% (3.33% – 12.00%)	9 (259)	20.51% (12.47% – 31.86%)	0.0043
≥ 50% high risk	11 (321)	9.35% (4.74% – 17.61%)	13 (243)	13.58% (7.10% – 24.42%)	0.42
5 prior lines of treatment	10 (245)	2.78% (1.25% – 6.06%)	16 (333)	19.10% (13.30% – 26.66%)	< 0.0001
Recognition domain = Llama/alpaca	21 (440)	13.76% (8.84 – 20.80%)	4 (112)	6.48% (2.69 – 14.79%)	0.12
Enrichment/activation = aCD3/CD28	6 (136)	4.92% (2.14% – 10.90%)	7 (215)	15.88% (8.07% – 28.89%)	0.028
Loading method = lentiviral	5 (100)	8.73% (2.75% – 24.44%)	20 (489)	11.86% (7.22% – 18.90%)	0.62
Co-stimulation = 4-1BB	5 (115)	3.41% (1.20% – 9.31%)	22 (519)	12.85% (8.21% – 19.56%)	0.018
Lymphodepletion = CP/Flu	6 (138)	7.27% (1.89% – 24.25%)	23 (501)	11.28% (7.10% – 17.46%)	0.53

Table S4. Subgroup comparison for CAR loading method.

	Retroviral Proportion (95% CI)	Lentiviral Proportion (95% CI)	P-value
Number of studies	4	20	
Number of patients	101	489	
CRS grade 1-2	43.05% (17.82% – 72.49%)	69.63% (61.62% – 76.61%)	0.094
CRS grade 3-4	13.43% (4.92% – 31.75%)	14.32% (8.91% – 22.22%)	0.90
CRS all grades	62.23% (25.03% – 89.05%)	85.02% (75.61% – 91.22%)	0.16
Neurotoxicity	8.73% (2.75% – 24.44%)	11.86% (7.22% – 18.90%)	0.62
CR	18.01% (6.48% – 41.06%)	50.62% (39.77% – 61.42%)	0.015
CR/VGPR	44.92% (26.56% – 64.78%)	70.51% (60.87% – 78.61%)	0.022
ORR	68.33% (48.07% – 83.42%)	84.14% (75.96% – 89.91%)	0.076
Median PFS	4.3m (3.0 – 15.0)	12.8m (11.4 – 19.9)	0.0065

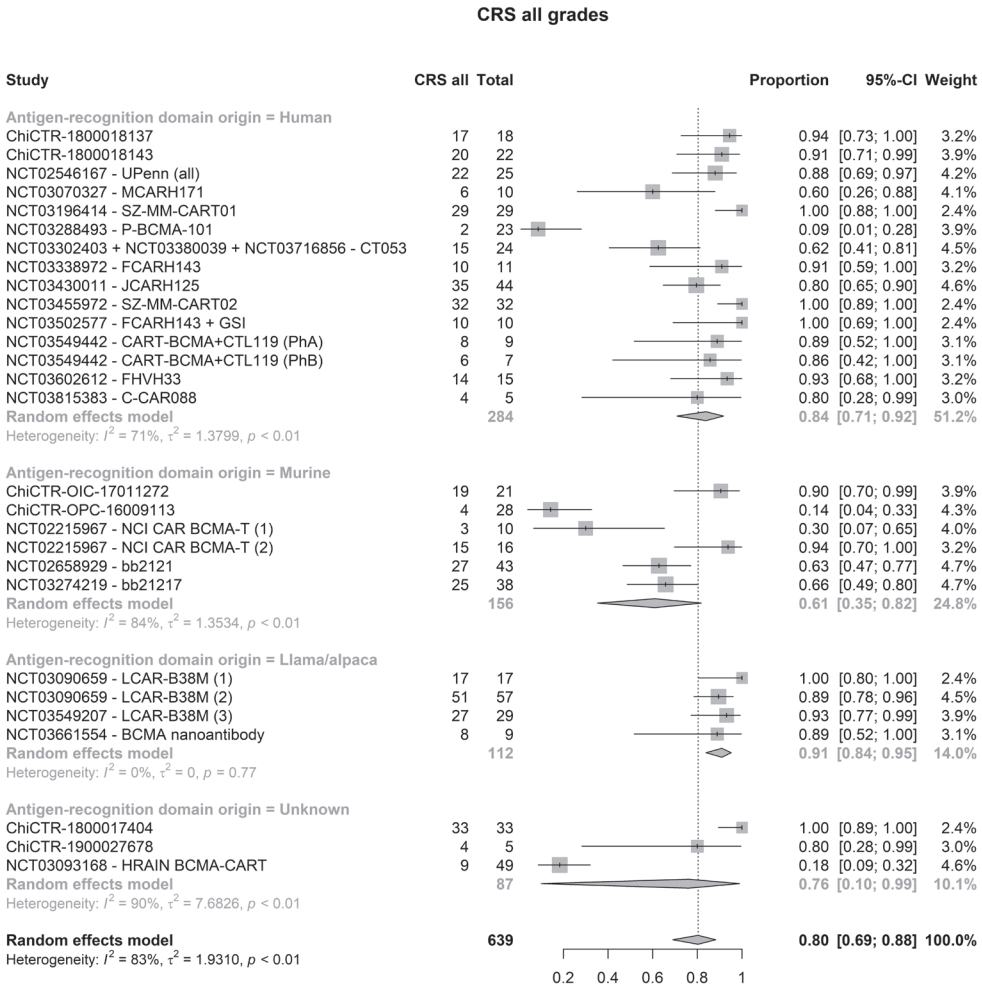


Figure S1. Forest plot for CRS, grouped by antigen-recognition domain origin.

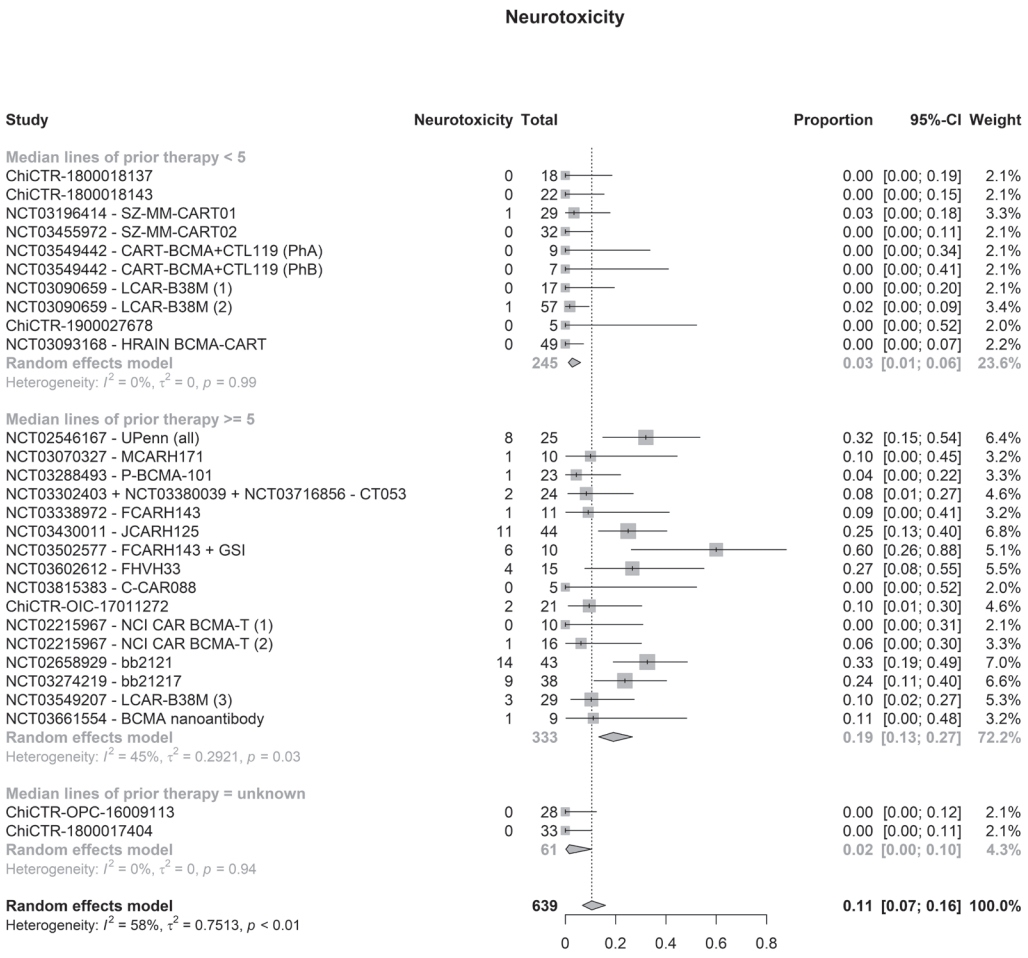


Figure S2. Forest plot for neurotoxicity, grouped by lines of prior therapy.

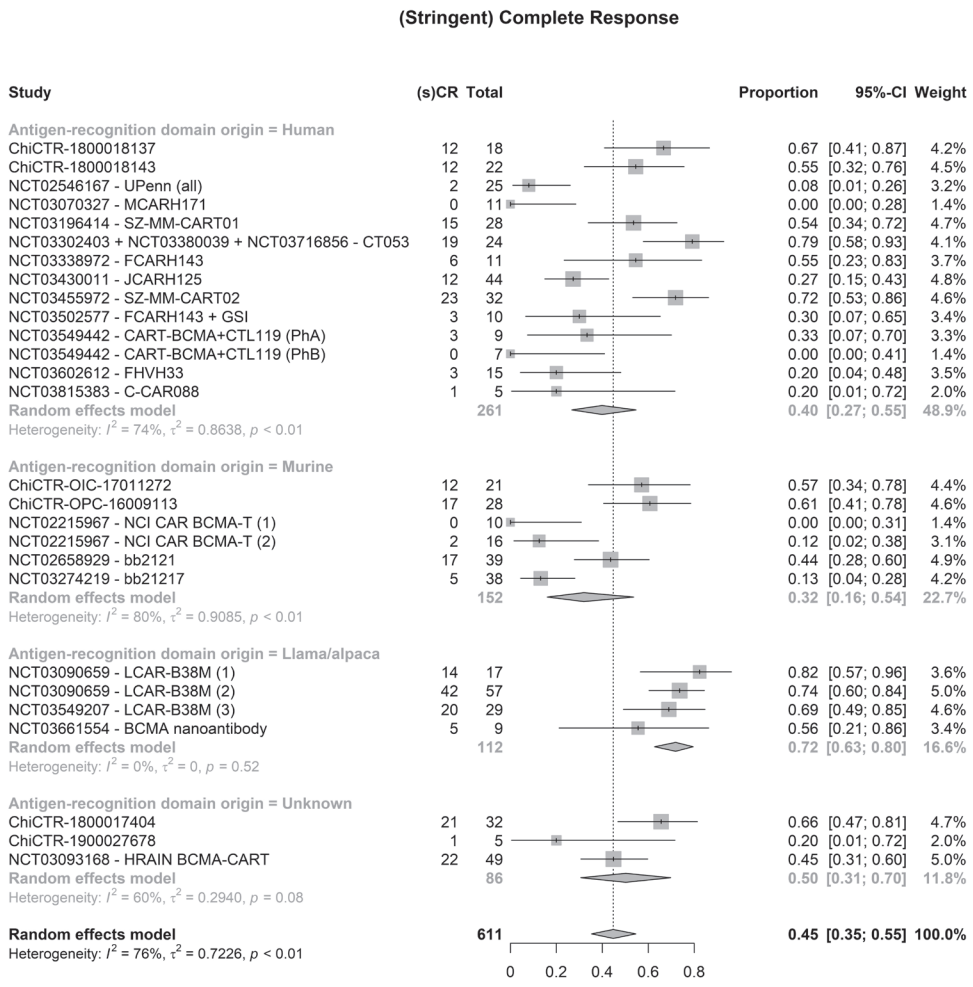
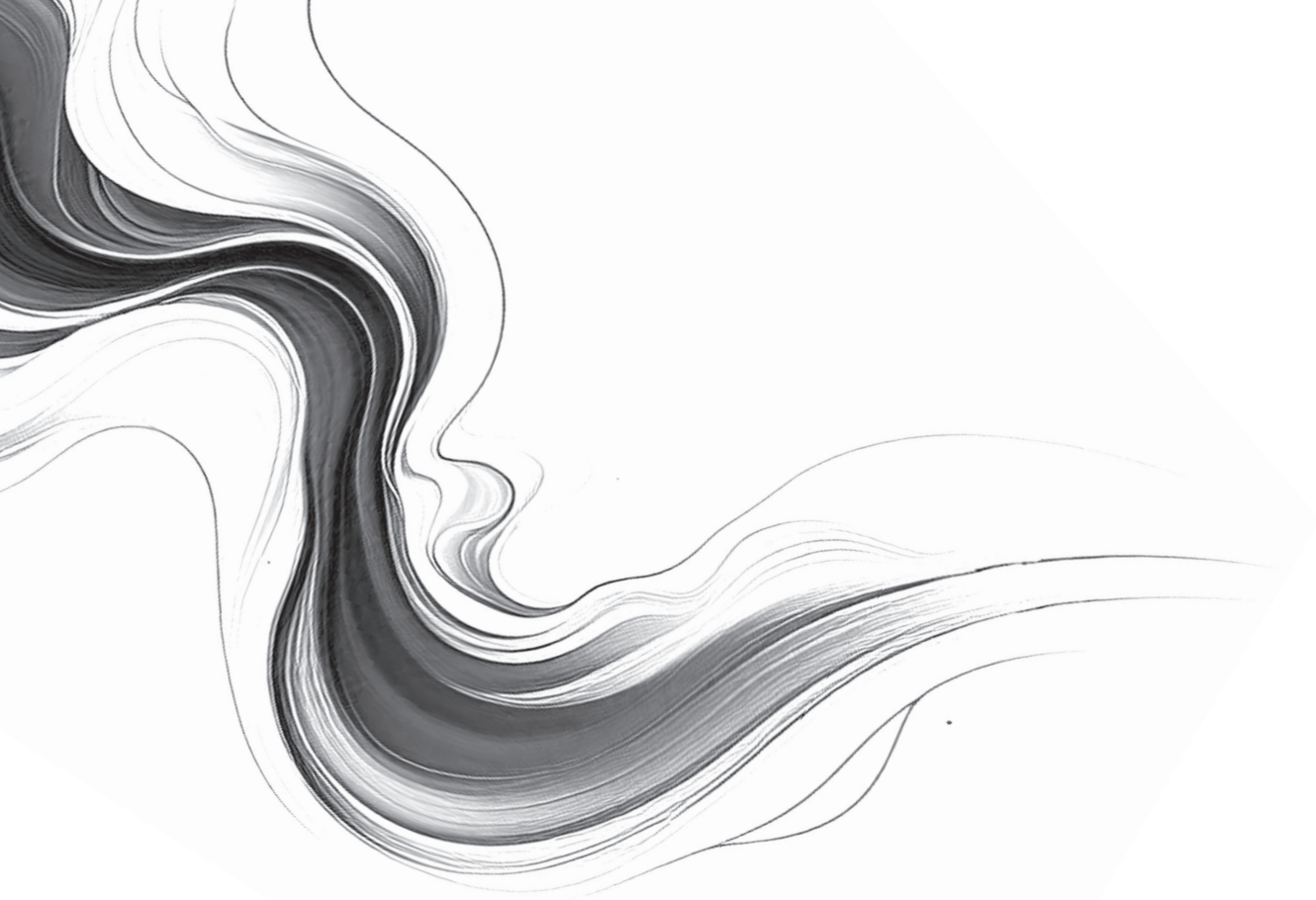


Figure S3. Forest plot for (s)CR, grouped by lines of prior therapy.



4

CHIMERIC ANTIGEN RECEPTOR-MODIFIED T CELL THERAPY IN MULTIPLE MYELOMA: BEYOND B CELL MATURATION ANTIGEN

Timmers M*, Roex G*, Wang Y, Campillo-Davo D, Van Tendeloo VFI, Chu Y, Berneman ZN, Luo F, Van Acker HH, Anguille S. * Shared first author.

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Abstract

Chimeric antigen receptor (CAR)-modified T cell therapy is a rapidly emerging immunotherapeutic approach that is revolutionizing cancer treatment. The impressive clinical results obtained with CAR-T cell therapy in patients with acute lymphoblastic leukemia and lymphoma have fueled the development of CAR-T cells targeting other malignancies, including multiple myeloma (MM). The field of CAR-T cell therapy for MM is still in its infancy, but remains promising. To date, most studies have been performed with B cell maturation antigen (BCMA)-targeted CARs, for which high response rates have been obtained in early-phase clinical trials. However, responses are usually temporary, and relapses have been observed due to loss of BCMA expression following CAR-T therapy. This has fostered a search for alternative target antigens that are expressed on the MM cell surface. In this review, we provide an overview of myeloma target antigens other than BCMA that are currently being evaluated in preclinical and clinical studies.

Introduction

Multiple myeloma (MM) is a malignant neoplasm of plasma cells that accumulates in the bone marrow, leading to bone destruction and marrow failure. With an incidence of 5 cases/100,000 individuals/year in Western countries, MM accounts for 1% of all cancers and for approximately 10% of all hematological malignancies [234]. MM arises from a premalignant asymptomatic proliferation of plasma cells (monoclonal gammopathy of unknown significance [MGUS] and smoldering MM). These can further evolve into symptomatic MM with end-organ damage, which is associated with significant patient morbidity [235]. Despite the availability of various therapeutic agents, including proteasome inhibitors (e.g., bortezomib), immunomodulatory drugs (e.g., lenalidomide), and monoclonal antibodies (e.g., daratumumab and elotuzumab), the disease remains incurable [236].

Cellular engineering has provided various opportunities to redirect the immune system against malignant cells. For example, adoptive transfer of chimeric antigen receptor (CAR)-engineered T cells is an emerging therapeutic strategy that has already shown unprecedented results in CD19-expressing hematological malignancies [79, 117, 237, 238]. Hence, these results have spurred new interest in the further development of this technology. The majority of CAR-T cell approaches have been applied to αβ T cells or occasionally natural killer (NK) cells [239], γδ T cells [240], or NK/T cells [241], as the effector cells of choice (Figure 7). The concept behind this therapy is that CAR-engineered immune cells and their effector functions are redirected against malignant cells bearing the antigen of interest, irrespective of the patients' human leukocyte antigen (HLA) genetics.

CARs comprise (i) an ectodomain binding directly a tumor-specific molecule on the cell surface, (ii) an extracellular hinge/spacer and a transmembrane domain spanning the membrane, and (iii) an endodomain providing T cell signaling (Figure 7). The ectodomain is generally derived from the antigen binding regions of a monoclonal antibody [242]. The endodomain usually includes the CD3ζ signaling chain, providing an activation signal termed signal 1. Second- and third-generation CARs have additional costimulatory molecule domains, e.g., CD28, OX40, or 4-1BB (signal 2). Fourth-generation CARs, also known as T cells redirected for universal cytokine-mediated killing, express additional molecules to enhance CAR-T cell efficacy, such as inducible interleukin (IL)-12 [243].

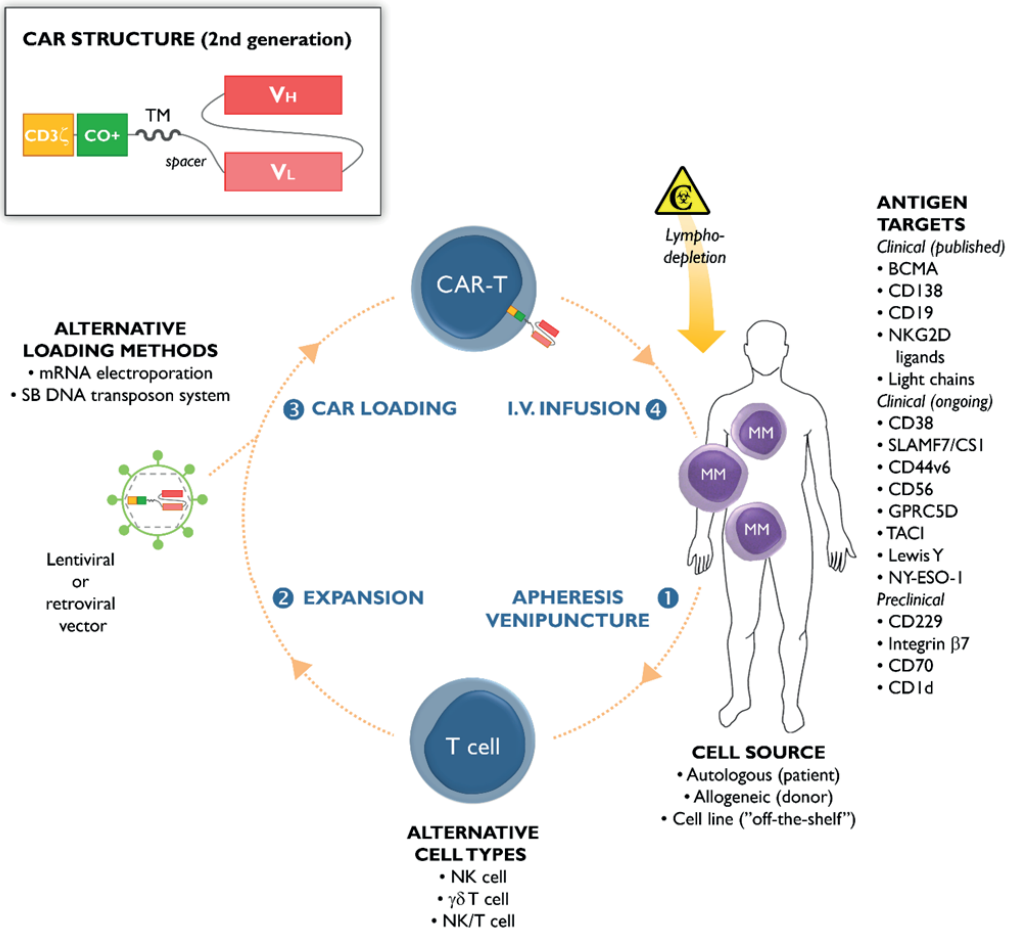

Figure 7. Chimeric antigen receptor (CAR)-T cells from multiple myeloma (MM) patients are usually manufactured from autologous T cells collected through leukapheresis or venipuncture (step 1). Apart from autologous cells, allogeneic cells or cell lines have been used as starting material [244]. Natural killer (NK) cells, γδ T cells and NK/T cells have been used as alternative lymphocyte subsets for CAR-T manufacturing. In a next step, the cells are expanded *ex vivo* (step 2) and loaded (step 3) with a lentiviral or retroviral vector carrying the CAR molecule. CAR loading can also be accomplished by non-viral methods, including messenger RNA (mRNA) electroporation or using the Sleeping-Beauty DNA transposon system. The CAR-loaded T cells are administered by intravenous infusion (step 4) to the patient, who has usually received prior lymphodepleting chemotherapy (such as cyclophosphamide or fludarabine). The different MM antigens that can serve as targets for CAR-T cell-based immunotherapy are schematically depicted, including their stage of clinical development (published clinical trials, ongoing clinical trials, preclinical studies). The insert shows the common structure of a second-generation CAR construct. The extracellular part of a CAR is composed of the antigen-recognition domain from a monoclonal antibody (usually with the V_H and V_L chains in single-chain variable fragment [scFv] format), and an extracellular spacer. The transmembrane (TM) and intracellular domains are the other CAR constituting parts. The latter contains a costimulatory (CO+) domain (e.g., 4-1BB or CD28), and the CD3ζ chain of the T-cell receptor.

To date, two CD19-specific CAR-T cell products (Kymriah and Yescarta) have been approved by the US Food and Drug Administration and the European Medicines Agency. Although the use of CAR-T cells in the treatment of MM is still confined to a handful of antigens and early-phase clinical trials, CAR-T cell therapy holds the potential to fulfill the unmet medical needs of patients with relapsed/refractory MM.

In multiple myeloma, B-cell maturation antigen (BCMA) is a commonly used target antigen in CAR-T cell clinical trials [96-98]. BCMA, also known as tumor necrosis factor receptor superfamily member 17, is highly expressed on malignant plasma cells [99, 100]. No expression of BCMA has been observed in normal cells/tissues, except for healthy, differentiated B cells where it is usually expressed at low level. BCMA appears to be a vital in promoting MM cell survival, proliferation, and drug resistance [101, 245] and can be used to monitor the disease course and predict patient outcomes [246].

Table 1 summarizes the clinical outcome of all hitherto published clinical trials of BCMA-targeting CAR-T cell therapies in MM [102-107]. BCMA CAR-T cell therapy produces objective response rates of up to 88% (Table 6). Nevertheless, the therapeutic effect is often temporary and relapses are commonly being reported. As shown in Table 6, the median progression-free survival of BCMA CAR-T cell therapy is in the range of 12 months. Downregulation or loss of BCMA expression is likely an important mechanism underlying these relapses [109, 203]. Hence, alternatives for BCMA are now under intensive investigation in the field of CAR-T cell therapy for MM [98, 247]. The goal of this review is to outline the different target antigens other than BCMA that are currently being evaluated. In the first part, summarized in Table 7, an overview is given of non-BCMA CAR-T cell trials for which (preliminary) results have already been published in Web of Science-listed papers. In the second part, we will focus on alternative target antigens that have entered into CAR-T cell clinical trials. In the third and final part, we will briefly touch upon new antigens that are undergoing pre-clinical evaluation for use in CAR-T cell therapy for MM (schematically depicted in Figure 7).

Published clinical trials

CD138

CD138 or syndecan 1, a member of the syndecan family of type I transmembrane proteoglycans, is highly expressed on the MM cell surface and is directly involved in disease progression [248]. The latter works through binding to a proliferation-inducing ligand (APRIL), a survival factor [249], and cell proliferation-inducing growth factors [250]. Interestingly, the expression of CD138 on MM cells of patients in relapse or with progressive disease is more pronounced than that on MM cells of newly diagnosed patients [248]. Previous preclinical studies with NK

cells expressing an anti-CD138 CAR showed potent antimyeloma activity both *in vitro* and *in vivo* [251]. Therefore, CD138 is a very attractive target for anti-MM therapy.

Table 6. Clinical outcome of BCMA-targeted CAR-T cell therapy in multiple myeloma.

CAR-T cell product [ref.]	*n* =	ORR *(n =)*	median PFS (95% CI)
bb2121 [105]	33	85% (28)	11.8 months (6.2-n.e.)[$]
CART-BCMA Upenn [102]	25	48% (12)	2.0 months (ND)
NCI CAR BCMA-T [103]	10	20% (2)	1.5 months (ND)
NCI CAR BCMA-T [104]	16	81% (13)	7.25 months (ND)
LCAR-B38M [106]	17	88% (15)	12.2 months (ND)
LCAR-B38M [107]	57	88% (50)	15.0 months (11.0-n.e.)

Only fully published clinical studies were included (last search: May 1, 2019). (ref.), bibliography reference; n =, number of patients; ORR, objective response rate, defined as the sum of complete responses and (very good) partial responses; PFS, progression-free survival; 95% CI, 95% confidence interval; n.e., not estimable, ND, no data; [$]PFS calculated for 30 patients treated with active doses of bb2121 only (i.e., ≥150 × 10^6 CAR-T cells); [#]lower dose cohorts (i.e., 0.3-1-3 × 10^6 CAR-T cells/kg), [*]highest dose cohort (i.e., 9 × 10^6 CAR-T cells/kg).

As shown in Table 7, one report recorded the use of anti-CD138 CAR-T cells in a patient with refractory MM with extramedullary involvement. Here, the administration of 1.5 × 10^8 CAR-T cells led to partial response (PR) [252]. A pilot clinical trial (ClinicalTrials.gov identifier, NCT01886976) reported the results of five patients with refractory and relapsed MM, pretreated with chemotherapy and stem cell transplantation, who received an average dose of 0.756 × 10^7 cells/kg of autologous CD138 CAR-T cells (Table 7) [110]. All patients underwent a bone marrow examination, demonstrating CD138 expression in aspirates and by biopsy. The *CAR* gene was continuously observed in the patients' blood for at least 4 weeks, and high levels of CAR-T cells were detected in the bone marrow at the first two months. Stable disease (SD) was achieved in four patients, ranging from three to seven months, whereas the fifth patient progressed, even though CAR-T cells could be detected in the bone marrow for 90 days.

Although promising, CD138-targeted CARs should still be used with caution owing to the broad expression of CD138 in human tissues, including epithelial cells. For example, treatment with BT062, an antibody-drug conjugate directed against CD138, resulted in skin and/or mucosal toxicity [253]. Nevertheless, pre-clinical work by Sun et al. has shown that CD138 CAR-T cells are safe and lack activity against normal epithelial cells [254]. Like BCMA, CD138 can be shed from MM cells, a possible escape route disrupting the effector functions of CD138-targeted immune cells [255]. This underlines the importance of combining CD138 CAR-T cells with other CAR target antigens. Based on a search of the ClinicalTrials.gov registry using the search terms "multiple myeloma" and "chimeric antigen receptor" or "CAR" (date of search May 1, 2019), numerous studies of CD138-targeted CAR-T cell therapy in combination with other CARs are ongoing or planned (NCT03196414, NCT03473496, NCT03271632). Apart from the above-

mentioned NCT01886976 trial, only one other study could be identified which CD138 CAR-T cells were used as stand-alone therapy (NCT03672318).

CD19

Most myeloma cells resemble fully differentiated plasma cells and are CD19-negative. There is, however, a small subset of CD19-positive myeloma cells that are more premature and have drug-resistant and disease-promoting qualities [256, 257]. Targeting these MM stem cell-like cells could be of interest. In general, low expression of CD19 appears to be more common on MM cells than previously thought, correlating with poor survival [160, 258].

Garfall et al. conducted a pilot clinical trial (NCT02135406) of CD19 CAR-T cell therapy (CTL019) involving 10 MM patients with a progression-free survival of <1 year after their first stem cell transplantation (Table 7) [111]. Patients were treated with a combination of high-dose melphalan, a second autologous stem cell transplantation (ASCT), and 1–5 × 10^7 CTL019 cells (administered ~2 weeks post-ASCT). A case report was first published for one patient with a minimal residual disease-negative complete response (CR), persisting up to 12 months after CTL019 infusion [160]. When looking at the complete dataset, 6 out of 10 patients experienced a very good partial response (VGPR) at day 100 post-transplant, and an additional 2 patients had a PR [111]. The same group is also conducting a phase II clinical trial (NCT02794246) in which high-risk MM patients will receive CD19 CAR-T cells in the maintenance setting ~60 days after first-line ASCT.

Combining CD19 and BCMA

CD19-specific CAR-T cells have also been used in combination with BCMA-targeted CAR-T cells, both in the relapsed/refractory setting [259] and in a newly diagnosed setting [223]. Yan et al. reported on eight patients with relapsed/refractory MM; all patients experienced CAR-T cell-related cytokine release syndrome (CRS) but no neurological toxicity (Table 7) [259]. Among the five patients with sufficiently long follow-up to evaluate for clinical response, one went into CR, one into VGPR and two into PR (Table 7).

Table 7. Web of Science published results of multiple myeloma CAR-T cell clinical trials targeting antigens other than BCMA.

	Antigen	Signaling domains	Cell source / type	Transfer method	Conditioning	T-cell dosage	Therapy-related side effects	Clinical effects
n=1 [252]	CD138	ND	Autologous T cells	ND	CP/Flu	1.5 $\times 10^8$	CRS gr. 2 (1)	PR (1)
n=5 [110]	CD138	4-1BB / CD3ζ	Autologous T cells	Lentiviral	PCD, CP or VAD	0.756 $\times 10^7$/kg	• Infusion-related fever (4) • Nausea and vomiting (3) • ↑ Liver function tests (1) • Possible TLS (1)	• SD >3m (4) • ↓ circulating PCL cells (1)
n=10 [111]	CD19	4-1BB / CD3ζ	Autologous T cells	Lentiviral	HDM + ASCT	1-5 $\times 10^7$	• Hypogammaglobulinemia (1) • Autologous GvHD (1) • Mucositis (1)	• CR (1) • VGPR (6/10) at d100 post-ASCT • PR (2/10) at d100 post-ASCT
n=5/8 [259]	CD19 + BCMA	OX40 / CD28	Autologous or allogeneic T cells	Lentiviral	CP/Flu	1 $\times 10^7$/kg	• CRS gr. 1-2 (7), gr.≥3 (1) • Prolonged cytopenias (5/5) • Coagulopathy (5) • ↑ Liver function tests (4) • Pulmonary edema (3) • Pleural effusion and ascites (1)	• sCR (1/5) • VGPR (1/5) • PR (2/5) • SD (1/5)
n=10 [223]	CD19 + BCMA	OX40 / CD28	Autologous T cells	Lentiviral	Bu-CP + ASCT	1 $\times 10^7$/kg	• CRS gr. 1-2 (10) • Coagulopathy (7) • ↑ Troponin levels (4) • Atrial flutter (1)	• CR (7/10) • VGPR (3/10)
n=5 [260]	NKG2D ligands	CD3ζ	Autologous T cells	Retroviral	None	1-3 $\times 10^{6-7}$	• None	• None
n=7 [261]	κLC	CD28 / CD3ζ	Autologous T cells	Retroviral	CP (4) or none (3)	0.92-1.9$\times 10^8$/m²	• Lymphopenia gr. 3 (1)	• SD 6wk-24m (4)

n=, number of patients; (number), reference. ***Abbreviations used***: ASCT, autologous stem cell transplantation; BCMA, B cell maturation antigen; Bu, busulphan; CP, cyclophosphamide; CRS, cytokine release syndrome; d, days; Flu, fludarabine; GvHD, graft-versus-host disease; HDM, high-dose melphalan; κLC, kappa light chain; m, months; ND, no data; NKG2D, natural killer group 2, member D; PCD, pomalidomide-cyclophosphamide-dexamethasone; PCL, plasma cell leukemia; PR, partial response; (s)CR, (stringent) complete response; SD, stable disease; TLS, tumor lysis syndrome; VAD, vincristine-doxorubicin-dexamethasone; VGPR, very good partial response; wk, weeks.

The same group also evaluated the safety and efficacy of combined CD19/BCMA CAR-T cell infusion in 10 patients with newly diagnosed MM after standard induction chemotherapy and autologous hematopoietic stem cell transplantation [223]. The study, registered with ClinicalTrials.gov under number NCT03455972, showed that CAR-T cells can be used as post-remission therapy to deepen the clinical response; of the four patients who were only in PR after transplantation, three went into VGPR following CAR-T cell administration and one obtained a CR. Toxicities, which included CRS in all patients, were mild and manageable.

Several other groups are also currently conducting clinical studies of the combination of CD19 and BCMA-targeted CAR-T cells (NCT03549442, NCT03706547, NCT03767725). The study by Garfall at University of Pennsylvania (NCT03549442) involves a randomization between BCMA CAR-T cells alone vs. the combination of BCMA CAR-T cells and CD19 CAR-T cells. The randomization phase of this trial aims to assess the value of BCMA ± CD19 CAR-T cell infusions as consolidation therapy in high-risk MM patients responding to frontline treatment. The combination of CD19 CAR-modified cells together with CD138 CAR-engineered immune effector cells is being evaluated in a preclinical context [262]. Clinical trials of the latter combination are being awaited.

Natural killer group 2, member D (NKG2D) ligands

The activating cell surface receptor NKG2D is commonly found on effector lymphocytes, including NK cells, $CD8^+$ T cells, NK/T cells, and γδ T cells. Its ligands include major histocompatibility complex class I polypeptide-related sequence A/B and UL16 binding protein 1–6 [263]. Under physiological conditions, tissues do not express NKG2D ligands on their surface. In contrast, neoplastic transformation will induce the upregulation of NKG2D ligands, including that in MM [264, 265].

Baumeister et al. constructed a CAR that targets multiple NKG2D ligands and performed a first-in human phase 1 clinical trial [260]. They included five patients with relapsed/refractory progressive MM and seven patients with acute myeloid leukemia/myelodysplastic syndrome. None of the patients experienced CRS or neurotoxicity. NKG2D CAR-T cell persistence was limited and no objective clinical responses were observed (Table 7).

It is unclear whether or not this treatment failure was due to the fact that no lymphodepleting chemotherapy was used prior to CAR-T cell infusion (Table 7). Indeed, lymphodepletion appears to be important for CAR-T cell engraftment, and for clinical efficacy [161, 266]. The fact that a first-generation CAR construct (i.e., without intracellular costimulatory domain) was used, may also have contributed to the lack of response. Another possible explanation for the absence of clinical activity may be the cell type that was used for CAR-T cell therapy. Pre-clinical work by the group of Leivas et al. revealed that only NKG2D CAR-transduced NK cells, but not T cells, are capable of killing MM cells and halting MM growth [267].

Immunoglobulin light chains

Because impaired humoral immunity (i.e., B-cell depletion and profound hypogammaglobulinemia) is a well-known consequence of CD19-directed CAR-T cell therapy and CAR persistence, a more selective construct, sparing some B cells and hence preserving partial humoral immunity function, may have improved applications. Mature B cells express either κ or λ light chains, but not both; thus, one of the two subsets can be targeted, leaving the other subset alone. Hence, this concept could be used to kill monoclonal MM cells expressing a certain type of light chain but not normal B cells expressing the reciprocal type of light chain. However, it should be noted that plasma cells generally do not express immunoglobulins on their surface, but secrete them into the bloodstream. Cases of MM-propagating cells expressing surface immunoglobulins have nevertheless been reported [268]. Another possible drawback is that most patients who are candidates for CAR-T cell therapy already have B cell depletion at baseline due to previous therapies, making the evaluation of selective light chain therapy difficult.

Ramos et al. created the κ.CAR, a CAR construct specific for the κ light chain [261]. In a phase 1 trial (NCT00881920), seven patients with MM and nine patients with non-Hodgkin lymphoma were included. These patients had heterogeneous prior therapy histories and salvage chemotherapies. In the seven patients with MM, no objective responses were observed (Table 7). One patient maintained stable minimal residual disease for 17 months; another patient maintained SD for two years, and two other patients exhibited transient SD (Table 7). CAR-T cell infusion was repeated in one patient after 1.5 years (after conventional therapy), which again led to transient SD. In the other three patients, no response to CAR-T cell therapy was documented. No severe CRS was observed, and no other complications were described [261].

Ongoing clinical trials

CD38

CD38 has been shown to be a promising target for the treatment of MM, considering the established clinical efficacy of anti-CD38 monoclonal antibodies, i.e., daratumumab [269]. Unfortunately, CD38 is not only highly expressed on myeloma cells but also expressed at an intermediate level on hematopoietic cells, creating a real risk for on-target, off-tumor toxicity [270]. For example, daratumumab has been shown to deplete NK cells, known to express CD38, in MM patients [269].

This potential of on-target, off-tumor toxicity can be a stumbling block to clinical implementation of CD38-directed CAR-T cell therapies. One of the strategies to circumvent this problem, involves the use of CARs with single chain variable fragments (scFvs) of lower affinity, generated through ‘light-chain exchange technology’. These low-affinity CD38 CAR-T cells are able to kill

$CD38^{high}$ MM cells, while having no effect on the viability of $CD38^{low}$ healthy cells, as validated both *in vitro* and *in vivo* [271]. A similar effect has been observed with CARs based on a CD38 nanobody instead of a scFv derived from a CD38 monoclonal antibody. Such CD38 nanobody-based CAR-T cells displayed potent cytotoxicity towards MM cells but only limited toxicity towards CD38-expressing normal hematopoietic cells [272].

Alternatively, researchers are also looking at selectively increasing the intensity of CD38 expression on the targeted tumor cells in order to maximize tumor-specific cytotoxicity and minimize on-target, off-tumor toxicity. CD38 can be upregulated on tumor cells by all-trans retinoic acid [273], or by the histone deacetylase inhibitor panobinostat [274]. The combination of all-trans retinoic acid and CD38 CAR-T cell therapy has already been shown to be effective in a model of acute myeloid leukemia [273, 275].

Another strategy to control off-tumor effects is building CARs with a safety mechanism. Drent et al. produced a CD38 CAR based on the tetracycline-controlled Tet-on/off technology. CAR gene expression can be activated by the administration of low doses of the tetracycline doxycycline [276]. The off-tumor effects produced by these CAR-T cells can be stopped within 24 h after doxycycline withdrawal. CAR expression can re-emerge upon rechallenge with doxycycline.

CD38 serves as the target antigen in several CAR-T cell clinical trials [277]. In one study, CD38 CAR-T cells are used as monotherapy in patients with relapsed/refractory MM (NCT03464916). All other clinical trials are exploring potential combinations of CD38 CAR-T cells with other target antigens; with CD19 (NCT03125577), with BCMA (NCT03767751), with BCMA, CD138 or CD56 (NCT03473496, NCT03271632), and with BCMA and NY-ESO-1 (NCT03638206). Our group is currently investigating the possibility to simultaneously load lymphocytes with three different CARs, including CD38, CD19 and BCMA, by means of mRNA electroporation.

Signaling lymphocytic activation (SLAM) family member 7 (SLAMF7)/CS1

SLAMF7, also known as CS1, is a member of the SLAM family of transmembrane receptors. First identified as a NK cell receptor, SLAMF7 also controls different functions of other immune cells, including subsets of CD4 and CD8 T cells, as well as B cells [278, 279]. Moreover, SLAMF7 has been shown to be vital for phagocytosis of hematopoietic malignant cells by macrophages [280]. No indications, however, have been found for SLAMF7 expression on other healthy cells and tissues. Because SLAMF7 is a robust marker of malignant plasma cells, it could be an interesting target for CAR-T cell therapy. Indeed, SLAMF7 expression has been observed on plasma cells of premalignant MM stages (i.e., MGUS and smoldering myeloma) and in newly diagnosed MM. SLAMF7 expression is further retained, even after several lines of therapy [281, 282].

A CAR construct was derived from the anti-SLAMF7 antibody elotuzumab and transduced into T cells from healthy donors and patients with MM [283]. The generated CAR-T cells could efficiently kill MM tumor cell lines and primary MM cells. Like CD38, SLAMF7 is expressed on normal lymphocytes, including activated T cells, entailing a risk of CAR-T cell fratricide [283, 284]. Indeed, it was confirmed that SLAMF7 CAR-T cells also killed healthy lymphocytes, but only those with high expression of SLAMF7. Lymphocytes with low expression of SLAMF7 were spared. This is very interesting considering that SLAMF7 CAR-T cells adopted a SLAMF7^{low} phenotype while in culture, ruling out problems due to CAR-T cell fratricide. Another strategy to decrease the risk of CAR-T cell fratricide involves the use of the transcription activator-like effector nuclease (TALEN) gene-editing technology during CAR-T cell manufacturing [284]. Such TALEN-edited CAR-T cells no longer express endogenous SLAMF7 and are thus resistant to SLAMF7-driven CAR-T cell fratricide.

Currently, ongoing research focuses on identifying the optimal costimulatory moiety for the SLAMF7 CAR construct (i.e., 4-1BB or CD28) [283], the optimal lymphocyte source (i.e., autologous or allogeneic) [285], and the optimal cell type (i.e., T cells or NK cells) [239, 286]. Preclinical work is also investigating whether dual SLAMF7/BCMA CAR-engineered T cells are superior to CAR-T cells expressing a single CAR molecule [287]. In addition, SLAMF7 CAR-T cells are being tested in combination with other myeloma drugs, such as lenalidomide [288] and daratumumab [289].

To the best of our knowledge, three SLAMF7/CS1-based CAR-T cell products have entered the clinical trial pipeline. One study will use autologous, memory-enriched T cells lentivirally transduced to express a SLAMF7/CS1 CAR construct (NCT03710421). This CAR construct contains a truncated EGFR (EGFRt) molecule, permitting depletion of the CAR-T cells in case of severe side effects by administration of the anti-EGFR monoclonal antibody cetuximab [290]. The European Union (EU), through the Horizon2020 program, is supporting a phase I/II clinical trial of SLAMF7 CAR-T cell therapy in MM, known as the CARAMBA project (for more details, see https://www.caramba-cart.eu/). These CAR-T cells are also equipped with the EGFRt safety switch, but the particularity about this product is the non-viral, Sleeping Beauty transposon-based method to transfer the *CAR* gene into the T cells. Finally, an “off-the-shelf” SLAMF7/CS1-directed CAR-T cell product for MM has recently been approved for clinical trial use. The product, also called UCARTCS1, contains healthy, allogeneic T cells loaded with a SLAMF7/CS1 CAR TALEN technology is used prior to *CAR* gene transfer to disrupt the endogenous TCR and SLAMF7 expression in order to prevent alloreactivity and fratricide, respectively [285].

CD44v6

CD44, the major hyaluronan receptor, is expressed on hematological cancer cells and is thought to play a role in cancer initiation [291]. Unfortunately, CD44 is also expressed on the surface of healthy cells. However, the expression of the CD44v6 isoform is more restricted and

can frequently be detected on advanced, high-risk MM cells [292]. Casucci and colleagues created an anti-CD44v6 CAR for the treatment of acute myeloid leukemia and MM [293]. T cells activated with CD3/CD28 beads, IL-7, and IL-15 were transduced with the CD44v6 CAR, and displayed potent cytotoxic effects against MM. No effects were observed against normal hematopoietic stem cells and CD44v6^{low} keratinocytes; however, CD44v6 CAR-T cells did cause a reversible decrease in monocyte count. This side effect can be beneficial, since monocytes are the main cause of CRS [94]. Nevertheless, to minimize the risk of toxicity, safety switches under the form of suicide genes (i.e., thymidine kinase gene, or inducible caspase 9 gene) were incorporated [293]. The same group also incorporated an extracellular spacer from the nerve-growth-factor receptor (NGFR) into the CD44v6 CAR construct. Using anti-NGFR immunomagnetic beads, the CAR-T cell product could be highly enriched for CD44v6 CAR-expressing T cells. This method opens up the possibility to purify T cells expressing the CAR and to omit the non-transduced cells [294]. The EURE-CART project, supported by the EU Horizon 2020 program, involves a phase I/IIa clinical trial to determine the safety and efficacy of CD44v6 CAR-T cell therapy in patients with acute myeloid leukemia and MM (for more details, see https://www.eure-cart.eu/) [295].

CD56

CD56 expression is found on a broad range of cells, including on NK cells and other immune effector cells [296-298]. Although it is not expressed on healthy plasma cells, CD56 is frequently expressed on MM cells [299]. Lorvotuzumab mertansine, an antibody-drug conjugate against CD56, has recently been tested in a dose-escalation phase 1 clinical trial of 37 patients with relapsed MM [300]. Treatment was well tolerated, and some early signs of anti-MM activity were observed, strengthening further investigations of CD56 as a target in MM. One report described a CD56-directed CAR-T cell therapy with potent antimyeloma activity [301]. However, no further results were published. The clinical benefits and potential toxicities of targeting CD56 are not known, but caution should be exercised owing to the broad expression of CD56. Indeed, depletion of CD56-positive immune effectors cells by treatment with lorvotuzumab entails a risk of infection; infection-related deaths were observed with this antibody-drug conjugate in a clinical trial of patients with small cell lung cancer [298, 302]. As discussed above, CD56 – in combination with other target antigens – has been adopted in two CAR-T cell clinical trial protocols for MM (NCT03473496, NCT03271632).

G protein-coupled receptor class C group 5 member D (GPRC5D)

GPRC5D, a myeloma cell surface antigen whose precise function remains to be defined, has recently been proposed as an attractive candidate for anti-myeloma CAR-T cell therapy [303]. The antigen is expressed on CD138-positive MM cells; it also expressed in the hair follicle, a potentially immune-privileged site therapy limiting the risk for on-target, off-tumor toxicity. Most interestingly, the expression of GPRC5D is independent of BCMA. Hence, GPRC5D-targeted CAR-T cells could potentially rescue patients experiencing an antigen-loss relapse

under BCMA-directed CAR-T cell therapy [303]. This hypothesis has been confirmed in a murine BCMA antigen escape model [132] and has paved the way for the MCARH109 trial, a phase I clinical trial to evaluate GPRC5D CAR-T cell therapy in relapsed/refractory MM patients including those who have received prior BCMA-directed therapies [303].

Transmembrane activator and CAML interactor (TACI)

Like BCMA, TACI is a member of the tumor necrosis factor receptor superfamily that is expressed on malignant plasma cells, albeit usually at lower levels [304]. APRIL is a naturally occurring ligand for both BCMA and TACI; as discussed above, CD138 is required as a co-receptor for binding of APRIL to TACI [305]. APRIL-based CAR-T cells have been developed for dual targeting of BCMA and TACI on myeloma cells [304, 306], and clinical studies have been initiated (ClinicalTrials.gov identifier NCT03287804). Interestingly, preclinical work by Lee et al. has shown that APRIL-based CAR-T cells can kill $BCMA^{+}TACI^{+}$ as well as $BCMA^{-}TACI^{+}$ myeloma cells. This indicates that APRIL CAR-T cell therapy can maintain tumor control in case of BCMA downregulation, which is a well-described tumor escape mechanism in BCMA-directed CAR-T cell studies [304]. Furthermore, it was recently shown that TACI is also expressed on regulatory T (T_{reg}) cells in patients with MM. As such, APRIL-based CAR-T cells have the potential not only of targeting MM cells directly, but also indirectly by suppressing Treg cells [307].

Lewis Y

The Lewis Y (LeY) antigen is a carbohydrate antigen that is overexpressed on a variety of tumor cells, including MM cells. LeY expression is found in approximately 50% of MM cases [308]. The antigen is related to the Lewis blood group antigen system, but not expressed on the red blood cell membrane. Overall, LeY has limited expression in normal cells and tissues, and no evidence of on-target, off-tumor toxicity was found with anti-LeY CAR-T cells in preclinical studies [309]. A phase I clinical trial of anti-LeY CAR-T cell therapy for hematological malignancies (including MM) was registered with ClinicalTrials.gov already in 2012 (NCT01716364), but the status of this study is unknown and – to the best of our knowledge – no results have been published yet.

New York esophageal squamous cell carcinoma 1 (NY-ESO-1)

One of the main limitations of CAR-T cell therapy is that it is only applicable to cell surface antigens, but not to intracellular oncoproteins. Such antigens are usually expressed in the context of HLA molecules and recognized by the T-cell receptor (TCR). NY-ESO-1 is an example of an intracellular oncoprotein that serves as target for TCR-engineered T cell immunotherapy in MM [310]. TCR-mimetic CARs recognizing the NY-ESO-1/HLA complex, have been developed [311, 312]. In a mouse model of NY-ESO-1/$HLA\text{-}A2^{+}$ MM, NY-ESO-1-directed CAR-T cells were capable of delaying MM growth [312]. The anti-myeloma activity could by further improved by co-infusion of T cells that were genetically engineered to express the NY-ESO-1 antigen and membrane-bound IL-15. These NY-ESO-1/$IL\text{-}15^{+}$ T cells served as antigen-presenting cells and

were found to improve the persistence of NY-ESO-1 CAR-T cells with a memory phenotype [312]. One NY-ESO-1-directed CAR-T cell clinical trial (combined with other target antigens) for MM has been registered with ClinicalTrials.gov (NCT03638206).

Preclinical studies

CD229

SLAMF3, also known as Ly9 or CD229, is another receptor of the SLAM family. It has a homogeneous expression on MM cells, which is stable regardless of the disease stage and exposure to different treatments, and it plays an essential role in the survival of MM cells [313-316]. Moreover, $CD19^{-}CD138^{-}$ MM cells, which represent a quiescent, drug-resistant myeloma-propagating cell population [317], are highly-positive for CD229 [314]. This implies that CD229 CAR-T cells would be able to eradicate both the bulk of MM cells, and chemotherapy-resistant minimal residual disease. The first CD229 CAR-T cell construct was generated by the group of Atanackovic and colleagues [318]. CD229 CAR-T cells demonstrated a strong cytotoxic activity against CD229-positive myeloma cell lines, with only minor activity against B cells and resting T cells. The most interesting finding came from a mouse model engrafted with luciferase-expressing U266 MM cells. Whereas mice treated with CD19 CAR-T cells or phosphate-buffered saline still showed a clearly detectable bioluminescence signal after 18 days, the CD229 CAR-T cells had completely eradicated the MM cells [318]. To the best of our knowledge, a clinical trial with CD229 CAR-T cells in MM has not yet been registered.

Integrin β7

Because finding a myeloma-specific target antigen is quite difficult, some recent research has focused on non-cancer-specific epitopes that become specific after post-translational events, such as glycosylation or conformational changes. As such, integrin β7 has been identified as a potential target for MM by screening more than 10,000 hybridomas against MM tumor cells [319]. MMG49, a monoclonal antibody identified from that screening assay as having the highest potential, is able to specifically recognize cancer-specific conformation of integrin β7 and a small fraction of CD19-positive B cells. Further studies showed that MMG49 was directed at a configuration-sensitive epitope of integrin β7, targeting the activated state that is highly expressed by MM cells. *In vitro*, MMG49 CAR-T cells were able to proliferate, secrete the immunostimulatory cytokines interferon-γ and IL-2, and efficiently eradicate MM cells. There was no indication of myeloma cells escaping therapy. Healthy hematopoietic cells were left untouched, even when integrin β7 was activated. After humanizing the mouse-derived scFv, MMG49 CAR-T cells will be tested in clinical trials [319].

CD70

One of the first CARs developed for MM was directed against the tumor necrosis factor family member CD70 (CD27L), which plays a role in plasma cell differentiation [320]. Shaffer et al. constructed a CD70 CAR with an antigen-binding domain derived from CD27 and fused to the intracellular domain of the CD3ζ chain [321]. In this way, the CAR-T cells were able the kill CD70-positive MM cells and, at the same time, take advantage of CD27/CD70 co-stimulation, leading to enhanced T cell survival. In a murine xenograft model, CD70-specific CAR-T cells led to sustained regression of established lymphoma. The low and variable expression of CD70 on myeloma cells limits the use of CD70-directed CAR-T cells in MM [322].

CD1d

The MHC class I-like molecule CD1d is highly expressed on premalignant and early MM cells, followed by a gradual decline in expression level with disease progression [323]. The immune cells known to respond to glycolipids presented in the context of CD1d, are NK/T cells. Taking advantage of the intrinsic characteristics of NK/T cells, CD19 CAR-NK/T cells are able to target both CD19 and CD1d on MM cells, resulting in a reinforced anti-tumor effect as compared to CD19 CAR-T cells [241]. Strengthening this therapeutic avenue is the low cytotoxicity of the CD19 CAR-NK/T cells against monocytes, the highest CD1d-expressing blood cells [323]. Hence, NK/T cells are interesting effector cells for CAR-based cellular immunotherapy against CD1d-expressing malignant cells, including (early-stage) MM. Certain drugs, such as EZH2 inhibitors and ATRA, are known to increase CD1d expression on the MM cell surface [241], opening up the perspective for combination therapy.

Conclusions and future perspectives

While the experience with BCMA-targeted CAR-T cells has provided robust evidence for the high therapeutic potential of CAR-T cell therapy in MM, we must not lose sight of the fact that responses are often temporary and that half of the patients will have relapsed or progressed after 1 year (Table 6). One of the main reasons for these relapses is downregulation or loss of BCMA expression on the MM surface. The exact mechanism for this downregulation is still unclear. Shedding of BCMA into the bloodstream is one possibility. Moreover, it was recently elucidated that the downregulation of BCMA can be the result of CAR-T cell-induced trogocytosis, a process in which the BCMA molecule is transferred from the tumor cell to the CAR-T cell surface. The CAR-T cells then become BCMA-positive and will start recognizing each other, leading to CAR-T cell fratricide [203]. Whether or not CAR-T cell therapy will revolutionize the treatment of MM will largely depend on how we will be able to deal with this problem of antigen escape. The answer to this question probably lies in the identification of additional antigens that can be targeted in combination with BCMA.

One potential strategy involves the combined infusion of two (or more) CAR-T cell products, such as BCMA CAR-T cells and CD19 CAR-T cells. The goal here is to eradicate not only the bulk myeloma cells (BCMA-positive) but also the small reservoir of myeloma "stem cells" (CD19-positive), thereby increasing the likelihood of achieving a durable clinical response. As discussed above, the combination of BCMA and CD19 CAR-T cells has already proven to be highly clinically efficacious [223, 259]. Nevertheless, the results of an ongoing randomized study comparing BCMA/CD19 CAR-T cells with BCMA CAR-T cells alone (NCT03549442) need to be awaited in order to draw conclusions about the potential superiority of this combinatorial approach.

Instead of co-administering two separate CAR-T cell products, compound CAR-T cells are gaining increasing attention [324]. Compound CAR-T cells are T cells expressing two (or more) different CARs [287]. The idea is to target multiple antigens at the same time in order the overcome the limitation of loss of one particular antigen [324]. Chen et al. have developed a compound CAR-T cell co-expressing a BCMA and SLAMF7/CS1 CAR. The authors found that BCMA CAR-T cells alone left a small population of (SLAMF7/CS1$^+$) myeloma cells whereas the compound CAR-T cells effectively depleted both the BCMA$^+$ and SLAMF7/CS1$^+$ cells [287].

The strategy proposed by Smith et al. deserves further consideration [132]. In a murine BCMA CAR-T cell model, the authors have elegantly shown that BCMA loss-mediated relapses can be avoided by subsequent targeting of a different myeloma surface antigen (i.e., GPRC5D) [132]. The drawback of this approach is the need to manufacture different batches of CAR-T cells and further increasing costs. Moreover, in a recently published mouse model of CD19/CD22 CAR-T cell therapy, it was shown that concomitant targeting was more effective than the sequential approach at preventing antigen escape [203].

The same study also indicated that the choice of costimulatory domain might be critical for therapeutic success in combinatorial CAR-T cell approaches [203]. For example, incorporation of the CD28 costimulatory domain in the CD19 CAR construct and 4-1BB in the CD22 CAR construct proved to be best combination of costimulatory domains in terms of synergistic activity. This combination was also the most effective in case of diminished expression of CD19 by the target cells. Although it remains to be examined whether these results are extrapolatable to the myeloma CAR-T cell field, the study clearly highlights the importance of rational CAR design especially in combination CAR-T cell therapy.

In conclusion, our knowledge of the mechanisms responsible for relapses following BCMA-CAR-T cell therapy is rapidly expanding. Besides tumor antigen downregulation or loss, other contributors of relapse, such as the development of anti-CAR-T antibodies, insufficient CAR-T cell persistence, or perhaps even more importantly, T cell exhaustion, are important topics of research [161]. This increasing knowledge of the mechanisms of relapse, along with the

identification of novel CAR target antigens, increases the likelihood that the full therapeutic potential of CAR-T cell therapy for MM will be unleashed in the near future.

Acknowledgements

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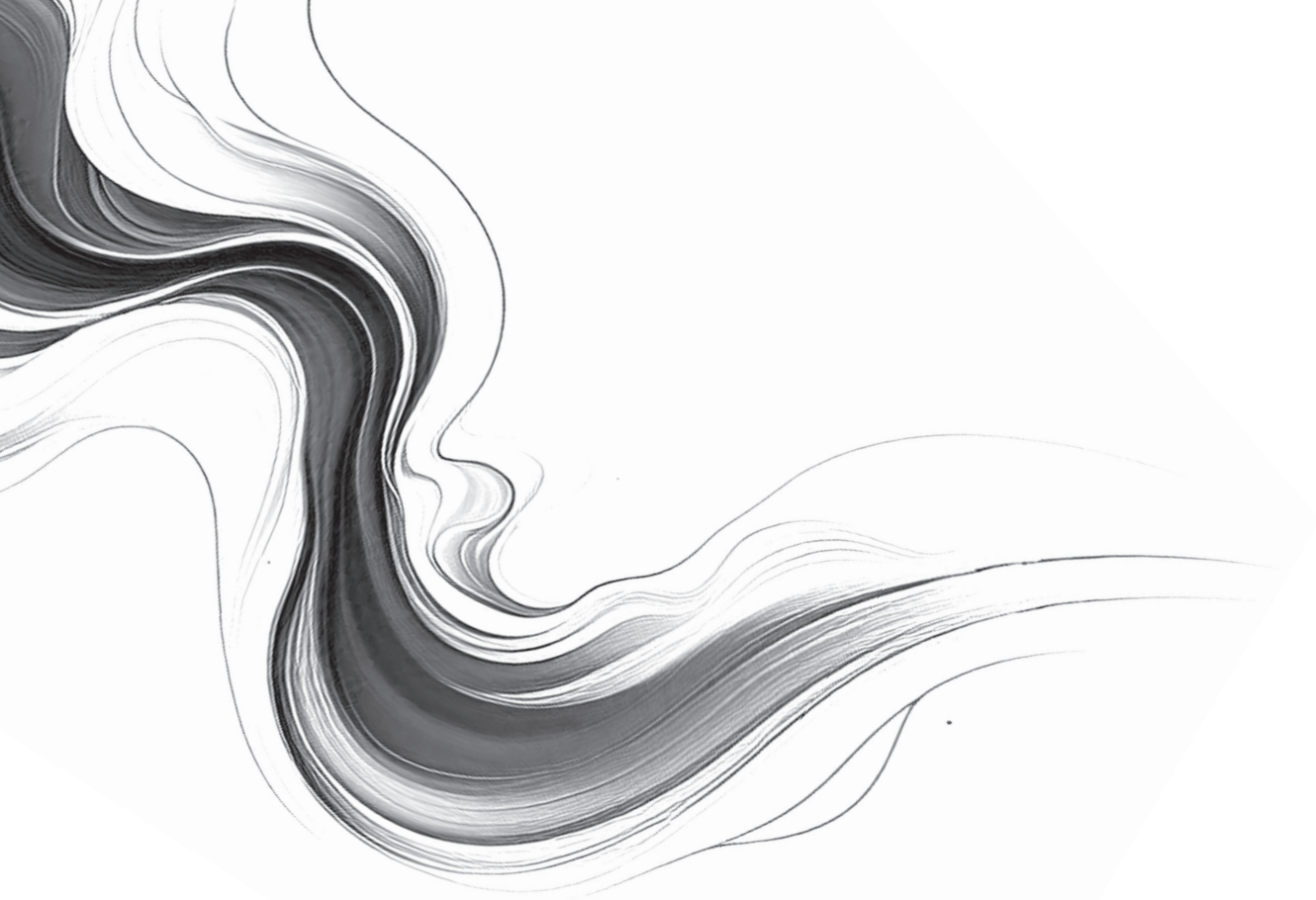

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TWO FOR ONE: TARGETING BCMA AND CD19 IN B-CELL MALIGNANCIES WITH OFF-THE-SHELF DUAL-CAR NK-92 CELLS

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Abstract

Background: Chimeric antigen receptor (CAR) T-cell therapy has proven to be a valuable new treatment option for patients with B-cell malignancies. However, by applying selective pressure, outgrowth of antigen-negative tumor cells can occur, eventually resulting in relapse. Subsequent rescue by administration of CAR-T cells with different antigen-specificity indicates that those tumor cells are still sensitive to CAR-T treatment and points towards a multi-target strategy. Due to their natural tumor sensitivity and highly cytotoxic nature, natural killer (NK) cells are a compelling alternative to T cells, especially considering the availability of an off-the-shelf unlimited supply in the form of the clinically validated NK-92 cell line.

Methods: Given our goal to develop a flexible system whereby the CAR expression repertoire of the effector cells can be rapidly adapted to the changing antigen expression profile of the target cells, electrotransfection with CD19-/BCMA-CAR mRNA was chosen as CAR loading method in this study. We evaluated the functionality of mRNA-engineered dual-CAR NK-92 against tumor B-cell lines and primary patient samples. In order to test the clinical applicability of the proposed cell therapy product, the effect of irradiation on the proliferative rate and functionality of dual-CAR NK-92 cells was investigated.

Results: Co-electroporation of CD19 and BMCA CAR mRNA was highly efficient, resulting in 88.1% dual-CAR NK-92 cells. In terms of CD107a degranulation, and secretion of interferon (IFN)-γ and granzyme B, dual-CAR NK-92 significantly outperformed single-CAR NK-92. More importantly, the killing capacity of dual-CAR NK-92 exceeded 60% of single and dual antigen-expressing cell lines, as well as primary tumor cells, in a 4-hour co-culture assay at low effector to target ratios, matching that of single-CAR counterparts. Furthermore, our results confirm that dual-CAR NK-92 irradiated with 10 Gy cease to proliferate and are gradually cleared while maintaining their killing capacity.

Conclusions: Here, using the clinically validated NK-92 cell line as a therapeutic cell source, we established a readily accessible and flexible platform for the generation of highly functional dual-targeted CAR-NK cells.

Introduction

Genetic engineering of T cells with a chimeric antigen receptor (CAR) has demonstrated high clinical activity in several B-cell malignancies [59]. This has led to the recent regulatory approval of several CAR-T-cell products, either targeting CD19 or B-cell maturation antigen (BCMA) [81, 83, 325-328]. Due to its ubiquitous expression on malignant B-cells, CD19 was an evident choice as a target antigen for B-cell leukemia and lymphoma. Depending on the cell product, overall response rates and complete responses range from 52-85% and 40-59%, respectively [59, 328]. These impressive results sparked the development of CARs for a myriad of other malignancies and led to the recent wave of BCMA-targeted CARs under clinical investigation for multiple myeloma (MM). BCMA is highly expressed on myeloma cells, whereas its presence on healthy cells is restricted to lower levels on mature B-cells and plasma cells [108]. Its expression profile, combined with its vital role in the proliferation, survival and drug resistance of MM cells makes BCMA an excellent target for MM.

Despite remarkable initial response rates mediated by CD19- and BCMA CAR-T cells in different hematological malignancies, a majority of treated patients ultimately relapsed [54, 59]. A considerable fraction of these relapse cases can be attributed to the selection and outgrowth of a small tumor cell population with downregulated or absent target antigen expression [67, 329-331]. Subsequent administration of CAR-T cells targeting other cell surface antigens, such as CD20 and CD22 in B-cell leukemia, can reinstate remission [128, 332, 333].

Due to the developmental relationship between B-cells and plasma cells, it comes as no surprise that some overlap exists between CD19 and BCMA expression. In this regard, several groups confirmed the presence of BCMA antigen on a subset of $CD19^+$ cells from B-cell leukemia and lymphoma patient samples, as recently reviewed by Dogan et al. [334]. Similarly, a small, less differentiated $BCMA^+CD19^+$ MM subpopulation was recently described, which was shown to be chemoresistant and to have cancer stem cell-like properties [257, 258]. Therefore, combinatorial approaches employing chemotherapeutics together with CD19- and BCMA-targeted CAR therapies are already under investigation in the context of MM [108, 111, 335].

Combinatorial multi-target therapies in the clinic are currently relying on simultaneous or sequential administration of the different CAR-T-cell products [108, 336-339]. However, co-administration of two single-CAR-T-cell products could lead to one product outcompeting the other, indicating that a single effector cell carrying multiple CARs would be more desirable [336]. There are, however, several drawbacks related to the production of single-antigen targeted autologous CAR-T cells [340]. First, the quality of autologous, patient-derived T cells is generally poor due to the detrimental effects of prior treatments [340, 341]. Second, CAR-T-cell production is already associated with a substantial price tag [342], further reducing financial accessibility when adding a second production run. Third, current manufacturing processes

are lengthy, making them unsuitable for patients with aggressive disease. The use of allogeneic T cells derived from healthy donors could represent a solution but this field is still in its infancy ([343-346] and ClinicalTrials.gov identifier: NCT04142619) and their applicability is beset by the need for additional genetic modifications in order to reduce the risk of graft-versus-host disease (GvHD) [347]. Another population of effector cells that is gaining attention as an alternative for T cells is the natural killer (NK) cell. NK cells are of particular interest due to their innate anti-tumor capacity mediated through their activating receptors, their favorable cytokine profile and the lack of GvHD [347]. However, primary NK cells generally face the same issues for clinical application as T cells, namely their limited ex vivo expansion capacity and population heterogeneity, in addition to their considerable resistance to genetic modification [348]. In contrast, the allogeneic NK-92 cell line provides a continuously expanding, homogeneous and easily engineerable off-the-shelf source of NK cells that is increasingly used in the clinic. This is exemplified by the fact that the NK-92 cell line has obtained FDA investigational new drug application status and by the growing number of clinical trials using (CAR-modified) NK-92 cells [347].

Here, we present a dual-targeting strategy with NK-92 co-expressing two complete CD19- and BCMA-specific CARs. We demonstrate that simultaneous transfection of multiple CAR-encoding mRNAs is feasible and results in high dual-CAR expression. Dual-CAR NK-92 cells efficiently recognize and eliminate single- and double-positive target cells, including primary tumor cells, even at low effector to target ratios. Furthermore, we confirm that dual-CAR NK-92 cells maintain their functionality after gamma-irradiation, which supports their off-the-shelf clinical applicability.

Methods

Primary cells, cell lines and culture conditions

Human Burkitt's lymphoma cell lines Daudi and Namalwa were purchased from the American Type Culture Collection. The NK-92 cell line was purchased from the German Collection of Microorganisms and Cell Cultures. U266 is a multiple myeloma cell line kindly gifted by Dr. Wilfred T.V. Germeraad (GROW School for Oncology & Developmental Biology, Maastricht University, Maastricht, The Netherlands). The enhanced green fluorescent protein (eGFP)-transduced erythroleukemia cell line K562 was generated in-house [349] (parental K562 was a kind gift from Dr. Cedrik Britten [R&D Oncology, GlaxoSmithKline, Stevenage, UK]). CD19.eGFP- and BCMA.eGFP-modified K562 (referred to as CD19-K562 and BCMA-K562, respectively) were kind gifts from Dr. Michael Hudecek (Hudecek Lab, University of Würzburg, Würzburg, Germany). Daudi, Namalwa, U266, K562, CD19-K562 and BCMA-K562 were maintained in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Gibco). NK-92 cells were maintained in GlutaMAX alpha

Minimum Essential Medium (α-MEM; Gibco) supplemented with 12.5% FBS and 12.5% horse serum (Gibco) (NK-92 medium) and 100 U/mL recombinant human (rh) interleukin (IL)-2 (ImmunoTools). All cell lines were maintained in logarithmic growth phase at 37 °C in a humidified atmosphere supplemented with 5% CO_2. For potential future clinical applications, NK-92 cells need to be irradiated prior to administration to avoid further cell proliferation in vivo. Therefore, where specified, NK-92 were irradiated with 10 Gy in the X-RAD320 (Accela) 4 h after *CAR* mRNA electroporation, and were incubated another 20 h before use in subsequent in vitro assays. Primary B-cell acute lymphoblastic leukemia (B-ALL) blasts were isolated from the peripheral blood of two patients using $CD19^+$ magnetic selection (Stemcell Technologies) and cryopreserved for further use. In contrast, assays against MM were performed on freshly isolated bulk bone marrow mononuclear cells (BMMNC) obtained from bone marrow samples from MM patients.

Generation of CAR-expressing NK-92

Two second generation CAR constructs against the target antigens CD19 and BCMA were designed using the same backbone: a CD8α leader peptide, an antibody-derived single-chain variable fragment (scFv), a CD8α hinge and transmembrane domain (referred to as "CD8"), a 4-1BB (CD137; referred to as "BB") co-stimulatory region and CD3ζ signaling domain. The sequence of the fully human scFv against BCMA was obtained from patent WO2016090320A1 (Seq No. 85), whereas the fully human scFv targeting CD19 was found in patent US20100104509A1 (47G-4). The synthetic genes CD8-CD19-CD8BBz and CD8-BCMA-CD8BBz were assembled from synthetic oligonucleotides and/or PCR products. The fragments were inserted into pST1-Rhamm (GeneArt, Thermo Fisher Scientific). Subsequent production of CAR-encoding mRNA through in vitro transcription (IVT) was previously described [350, 351]. Prior to electroporation, 200 µL of $25x10^6$ NK-92 cells/mL in Opti-MEM (Life Technologies) was mixed with nuclease-free water (IDT, Leuven, Belgium) (mock NK-92), 50 µg/mL *BCMA CAR* mRNA (BCMA-CAR NK-92), 50 µg/mL *CD19-CAR* mRNA (CD19-CAR NK-92) or both (dual-CAR NK-92) in a 4 mm cuvette (ImmunoSource). Cells were pulsed using a Gene Pulser Xcell (Bio-Rad) with a time constant protocol (300 V, 12 ms) and recovered in NK-92 medium without IL-2 for use in downstream applications. CAR surface expression was evaluated 24 h later by staining $2x10^5$ cells with 300 ng rhBCMA-FITC or 1 µg rhCD19-PE (AcroBiosystems) for 1 h at 4°C prior to acquisition on a CytoFLEX flow cytometer (Beckman Coulter).

NK-92 degranulation

CD107a was used as a marker of NK-92 degranulation upon target recognition. Cell membranes of target cells were labeled with CellTrace Violet (Molecular Probes, Invitrogen) according to manufacturer's instructions. Of the stained cells, $2x10^5$ were subsequently co-cultured with transfected NK-92 cells at an effector to target ratio of 1:2 in U-bottom 96-well plates for 5 h. At the start of the incubation period, 10 µL anti-CD107a-PE (BD Biosciences) was added to each well. As a protein transport blocker, 1X monensin (Biolegend) was added 1 h into the

co-culture. Samples were acquired on the FACSAria II (BD Biosciences) and gates were set based on appropriate fluorescence-minus-one controls.

Flow cytometric cytotoxicity assays

In case of tumor cell lines and primary B-ALL cells, target cells were membrane labeled with PKH26 (Sigma Aldrich) or CellTrace Violet directly prior to co-culture. Twenty-four hours after electroporation, CAR-transfected NK-92 and membrane labeled target cells were distributed in a U-bottom 96-well plate at different E:T ratios, briefly spun down (120 *g*, 2 min) to optimize cell contact and incubated for 4 h. Cell pools were subsequently stained with 7-AAD (BD Biosciences) and annexin V-FITC (Invitrogen) or -APC (BD Biosciences) and measured on the a CytoFLEX or FACSAria II flow cytometer, respectively. The proportion of cytotoxicity was calculated based on the fraction of live cells (double negative for 7-AAD and annexin V) using the formula: % cytotoxicity = 100 – (live target cells with effector cells/live target cells without effector cells)*100. Specific lysis was further calculated by subtraction of cytotoxicity induced by the mock NK-92 control.

Due to the limited quantity of MM cells in patient bone marrow aspirates, we conducted a flow cytometric killing assay based on counting beads using complete BMMNC. CellTrace Violet labeled CAR NK-92 were co-cultured for 4 h with $5x10^4$ BMMNC at different E:T ratios. Co-cultures were subsequently harvested and stained with LIVE/DEAD Fixable Near-IR (Life Technologies), anti-CD38-FITC (clone HIT2), anti-CD45-BV650 (clone HI30), anti-CD56-BV785 (clone 5.1H11), anti-CD19-APC (clone HIB19; all Biolegend), anti-CD3-PE-Cy7 (clone UCHT1) and anti-CD138-PE-CF594 (clone MI15; BD Biosciences). Precision counting beads (Biolegend) were added immediately prior to acquisition on a NovoCyte Quanteon (Agilent) to determine absolute counts of viable $CD138^+CD38^+$ MM cells. Cytotoxicity against primary MM cells was calculated using the formula: % cytotoxicity = 100 – (absolute number of viable MM cells in treated wells/mean absolute number of viable MM cells in untreated wells)*100 [352].

Quantification of granzyme B and IFN-γ secretion

Transfected NK-92 and target cells were resuspended at a concentration of $1x10^6$/mL and 100 µL of each was added to a U-bottom 96-well plate in triplicate. After 4 or 16 h of incubation, supernatant was harvested for the quantification of granzyme B or IFN-γ using enzyme-linked immunosorbent assay (ELISA; R&D Systems and Peprotech, respectively) according to manufacturer's instructions. After development of the plates, absorbance was measured on a VICTOR^3 multilabel plate reader (PerkinElmer).

Statistical analysis

Flow cytometric data was analyzed using FlowJo v10.7.1 software (TreeStar Inc). GraphPad Prism 9 (GraphPad Software) was used for graphical presentation and statistical analysis of the data. For normally distributed endpoints, a two-tailed unpaired t test was performed for

comparison between two groups. Alternatively, data consisting of three or more groups were analyzed using one-way analysis of variance (ANOVA), performing Dunnett's or Tukey's *post hoc* tests for multiple comparisons where appropriate. Results were considered statistically significant with a p value < 0.05. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$.

Results

Generation and specificity of BCMA- and CD19-CAR NK-92

For this study, we designed two IVT plasmid vectors encoding either a CD19- or BCMA-specific CAR. The extracellular antigen-recognition domains of our CAR constructs consisted of a fully human scFv. The remainder of the CAR building blocks, i.e. the CD8α hinge and transmembrane domain, and intracellular 4-1BB costimulatory and CD3ζ signaling domains, were identical to those used in the first-in-class CD19 and BCMA CAR-T cell therapies, tisagenlecleucel [353] and idecabtagene vicleucel [170], respectively (Figure 8A). NK-92 cells were electroporated with either BCMA- or CD19-specific *CAR* mRNA in order to generate BCMA-CAR NK-92 or CD19-CAR NK-92, respectively. We were able to efficiently transfect NK-92 cells, obtaining 76.0 ± 2.7% CD19-CAR$^+$ and 58.1 ± 3.3% BCMA-CAR$^+$ NK-92 cells 24 h after electroporation (Figure 8B). CAR expression peaked at 24 h and gradually decreased towards baseline over the course of four days (Figure 8C). Antigen specificity is an important requirement for CAR products to avoid off-target toxicity. We evaluated specificity of BCMA-CAR NK-92 and CD19-CAR NK-92 through a flow cytometric cytotoxicity assay against parental K562 (control), and K562 overexpressing CD19 (CD19-K562) or BCMA (BCMA-K562). BCMA-CAR NK-92 and CD19-CAR NK-92 showed lysis of K562 cells expressing their cognate antigens (47.8 ± 10.3% and 51.7 ± 2.3%, respectively), but left parental K562 cells and those transduced with irrelevant antigen unharmed, confirming antigen specificity (Figure 8D). By depriving the transfected NK-92 cells of IL-2 for 24 h, the background toxicity towards the parental NK-sensitive K562 cells was reduced to negligible levels (Figure 8D), confirming that the observed cytotoxic effects are largely attributable to CAR-driven activation of NK-92 cells.

Following confirmation of the specificity of each CAR, we investigated dual-CAR-expressing NK-92 (dual-CAR NK-92) in the context of antigen escape. NK-92 cells were simultaneously loaded with equal amounts of *BCMA-CAR* and *CD19-CAR* mRNA. Dual-CAR NK-92 cells displayed high CAR expression with 88.1 ± 0.8% of the cells expressing both CARs (Figure 8E), confirming that electroporation of two CAR constructs does not lead to a competitive reduction in CAR expression (Figure S4). Importantly, dual-CAR NK-92 are capable of recognizing and eliminating CD19-K562 and BCMA-K562, displaying only one target antigen, with equal efficiency to their single-CAR-expressing counterparts (Figure 8F), suggesting they are capable of overcoming antigen escape. Moreover, cytotoxicity of dual-CAR NK-92 cells

towards the Daudi lymphoma cell line, expressing both CD19 and BCMA, was high (80.1 ± 3.4%) and compared favorably to that of their single antigen-expressing counterparts (CD19-CAR NK-92 and BCMA-CAR NK-92: 75.2 ± 4.7% and 56.5 ± 0.4%, respectively), confirming that dual-CAR loading is not detrimental to the cytotoxic function (Figure 8F).

Antigen-specific degranulation and activation of dual-CAR NK-92

In order to quantitatively assess the activation status of CAR-loaded NK-92 cells, we first evaluated the degree of CD107a surface expression upon NK cell degranulation, a marker for identification of NK activity (Figure 9A). The intrinsic anti-tumor activity of NK-92 cells observed in the background levels of CD107a surface expression in mock NK-92 cells, which was slightly more pronounced against the NK-sensitive U266 cells compared to the NK-resistant Daudi cells (25.8 ± 3.3% and 18.4 ± 4% $CD107a^+$, respectively). Mock NK-92 (25.7 ± 3.3% $CD107a^+$) and CD19-CAR NK-92 (24.9 ± 3.9% $CD107a^+$) displayed equal levels of CD107a expression in response to $CD19^-$ $BCMA^+$ U266, indicating that CAR transfection does not impact their natural tumor reactivity. CD107a expression was significantly upregulated in dual-CAR NK-92 cells compared to BCMA-CAR NK-92 cells co-cultured with U266 cells (78.0 ± 0.5% and 54.4 ± 2.9% $CD107a^+$, respectively). A similar observation was made between CD19-CAR NK-92 cells and dual-CAR NK-92 cells against Daudi cells (64.2 ± 4.0% and 71.0 ± 1.1% $CD107a^+$, respectively).

These findings were further corroborated by the detection of secreted granzyme B in co-culture supernatant in the presence of the relevant antigen, but not in the absence thereof (Figure 9B). Generally, dual-CAR NK-92 cells significantly secreted more granzyme B against Daudi (7626 ± 1433 pg/mL), Namalwa (6801 ± 2346 pg/mL) and U266 (12008 ± 1710 pg/mL) compared to CD19-CAR NK-92 cells (3972 ± 873 pg/mL, 2909 ± 926 pg/mL and 1425 ± 478 pg/mL, respectively) and BCMA-CAR NK-92 cells (2894 ± 831 pg/mL, 1482 ± 615 pg/mL and 5842 ± 1251 pg/mL, respectively). Furthermore, IFN-γ secretion by dual-CAR NK-92 cells (Figure 9C) was also considerably elevated in comparison to CD19-CAR NK-92 cells against Daudi cells (660 ± 334 pg/mL vs 382 ± 209 pg/mL, respectively) and Namalwa (539 ± 126 vs 344 ± 57 pg/mL, respectively), and in comparison to BCMA-CAR NK-92 cells against U266 (1946 ± 77 pg/mL vs 866 ± 107 pg/mL, respectively). Taken together, these results verify that both CARs are capable of robustly activating NK-92 cells, separately as well as combined.

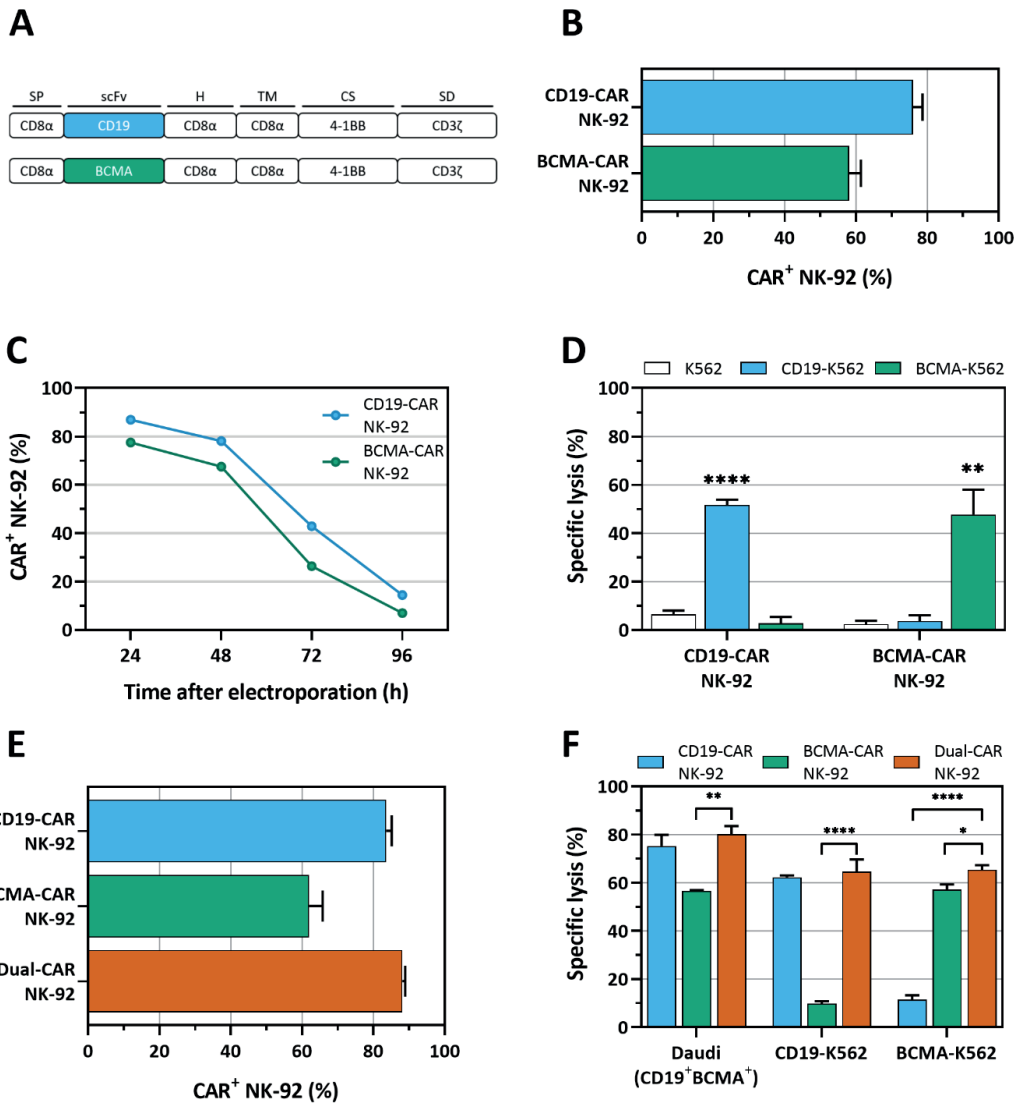

Figure 8. Generation and functional validation of dual-CAR NK-92. (A) Structural composition of the BCMA- and CD19-specific CARs used for the dual-CAR approach. **(B)** High CAR expression in transfected NK-92 cells, 24 h after electroporation with 50 µg/mL CAR-encoding mRNA (N=26-28). **(C)** CD19 and BCMA-CAR surface expression kinetics in NK-92 over four days (N=1). **(D)** Only target cells expressing cognate antigen are lysed, confirming CAR specificity. Statistical analysis was performed using ANOVA with Tukey's correction for multiple comparisons (N=3). **(E)** CAR expression of NK-92 transfected with mRNA encoding one (CD19-CAR NK-92 and BCMA-CAR NK-92) or both CARs (dual-CAR NK-92). Expression for dual-CAR NK-92 represents cells positive for both CARs (N=19). **(F)** Dual-CAR NK-92 lyse BCMA$^+$CD19$^+$ (Daudi), as well as single BCMA$^+$ (BCMA-K562) or CD19$^+$ (CD19-K562) tumor cells (N=3). Statistical analysis was performed using ANOVA with Dunnett's correction for multiple comparisons relative to the dual-CAR NK-92 condition. BCMA, B-cell maturation antigen; CAR, chimeric antigen receptor; CS, co-stimulatory domain; H, hinge domain; scFv, single-chain variable fragment; SD, signaling domain; SP, signal peptide; TM, transmembrane domain.

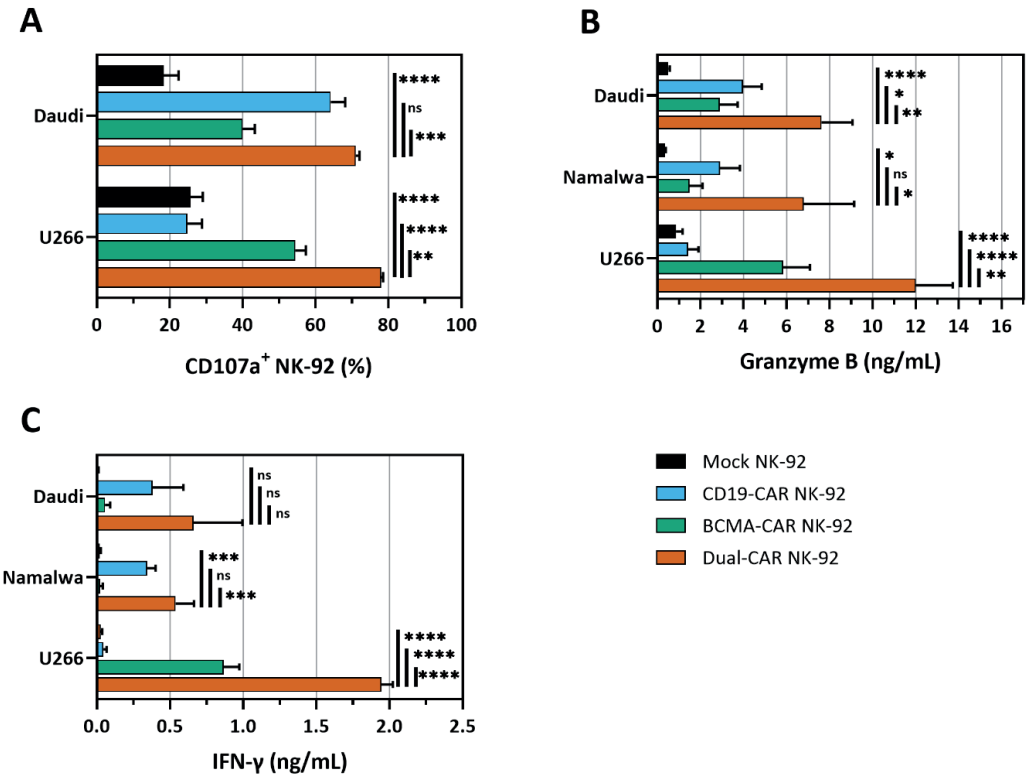

Figure 9. Degranulation and activation of dual-CAR NK-92. (A) CD107a expression demonstrated antigen-specific degranulation of CAR-engineered NK-92 during 5 h of co-culture at a 1:2 E:T ratio (N=3). **(B)** High granzyme B secretion by dual-CAR NK-92 in the supernatant of 4 h co-cultures (1:1 E:T ratio; N=3-6). **(C)** Dual-CAR NK-92 also significantly secrete IFN-γ upon activation (16 h co-culture at 1:1 E:T ratio; N=3). Statistical analysis was performed using ANOVA with Dunnett's correction for multiple comparisons with dual-CAR NK-92 as a reference. BCMA, B-cell maturation antigen; CAR, chimeric antigen receptor; ns, not significant.

Irradiation effectively halts NK-92 proliferation, but does not affect cytotoxicity

The NK-92 cell line requires inactivation prior to administration in order to prevent in vivo proliferation. This is usually achieved by gamma-irradiation, with 10 Gy being established as the recommended dose [354, 355]. Therefore, we irradiated CAR-transfected NK-92 cells 4 h after electroporation with 10 Gy and tracked viability and cell count up to one week. As shown in Figure 10A, non-irradiated parental NK-92, mock NK-92 and dual-CAR NK-92 cells showed similar growth rates, whereas their irradiated counterparts did not proliferate. Additionally, we saw a steady decrease in viability of the irradiated cells over the course of a week, leaving less than 5% of viable cells at the end of day 7, relative to the start of the culture (Figure 10A). To exclude any potential inhibitory effects on the functional properties of NK-92 cells caused by irradiation[341, 354, 356], we next investigated CAR expression levels and cytotoxic effector

function of irradiated single- and dual-CAR NK-92 cells (Figure 10B and Figure 10C). As shown in Figure 10B, 24 h after CAR mRNA electroporation, irradiated NK-92 cells maintained high expression of CD19 and BCMA single-CARs and dual-CAR (80.0 ± 4.3%, 59.1 ± 2.9% and 85.8 ± 0.9%, respectively), similar to non-irradiated CD19-CAR, BCMA-CAR and dual-CAR NK-92 cells (78.5 ± 1.1%, 66.2 ± 4.4% and 89.2 ± 0.4%, respectively). As observed in non-irradiated CAR NK-92, surface expression of the CAR is maximal at 24 h after mRNA electroporation and gradually decreases in the following days due to the transient nature of mRNA (Figure S5).

Figure 10C depicts the specific lysis of irradiated and non-irradiated CAR NK-92 after a 4 h co-culture with Daudi and Namalwa lymphoma cell lines (both $CD19^+$ and $BCMA^+$), and with the U266 myeloma cell line ($CD19^-$ and $BCMA^+$) at an E:T ratio of 1:1. In terms of anti-tumor killing activity of double-positive target cells, no significant differences between non-irradiated and irradiated CAR NK-92 cells were observed (Figure 10C). Irradiation had no impact on the lytic activity of CD19-CAR NK-92, BCMA-CAR NK-92 and dual-CAR NK-92 against Daudi (72.9 ± 3.4%, 33.9 ± 6.1% and 85.0 ± 1.7%, respectively) or Namalwa cells (67.3 ± 4.4%, 23.0 ± 5.5% and 79.8 ± 2.4%, respectively). Likewise, irradiated dual-CAR NK-92 cells were still capable of efficiently lysing U266 cells (41.4 ± 1.9%) compared to non-irradiated dual-CAR NK-92 cells (49.8 ± 2.3%). Moreover, irradiated dual-CAR NK-92-mediated killing capacity against Daudi (85.0 ± 1.7%) and Namalwa (79.8 ± 2.4%) matched or exceeded that of CD19-CAR NK-92 cells (72.9 ± 3.4% and 67.3 ± 4.4%, respectively). Regarding U266 cells, irradiated dual-CAR NK-92 considerably outperformed the BCMA-CAR NK-92 condition (41.4 ± 1.9% vs. 27.5 ± 5.6%, respectively). Altogether, irradiation effectively inhibits proliferation and persistence of CAR NK-92 while CAR expression and performance are comparable to non-irradiated controls.

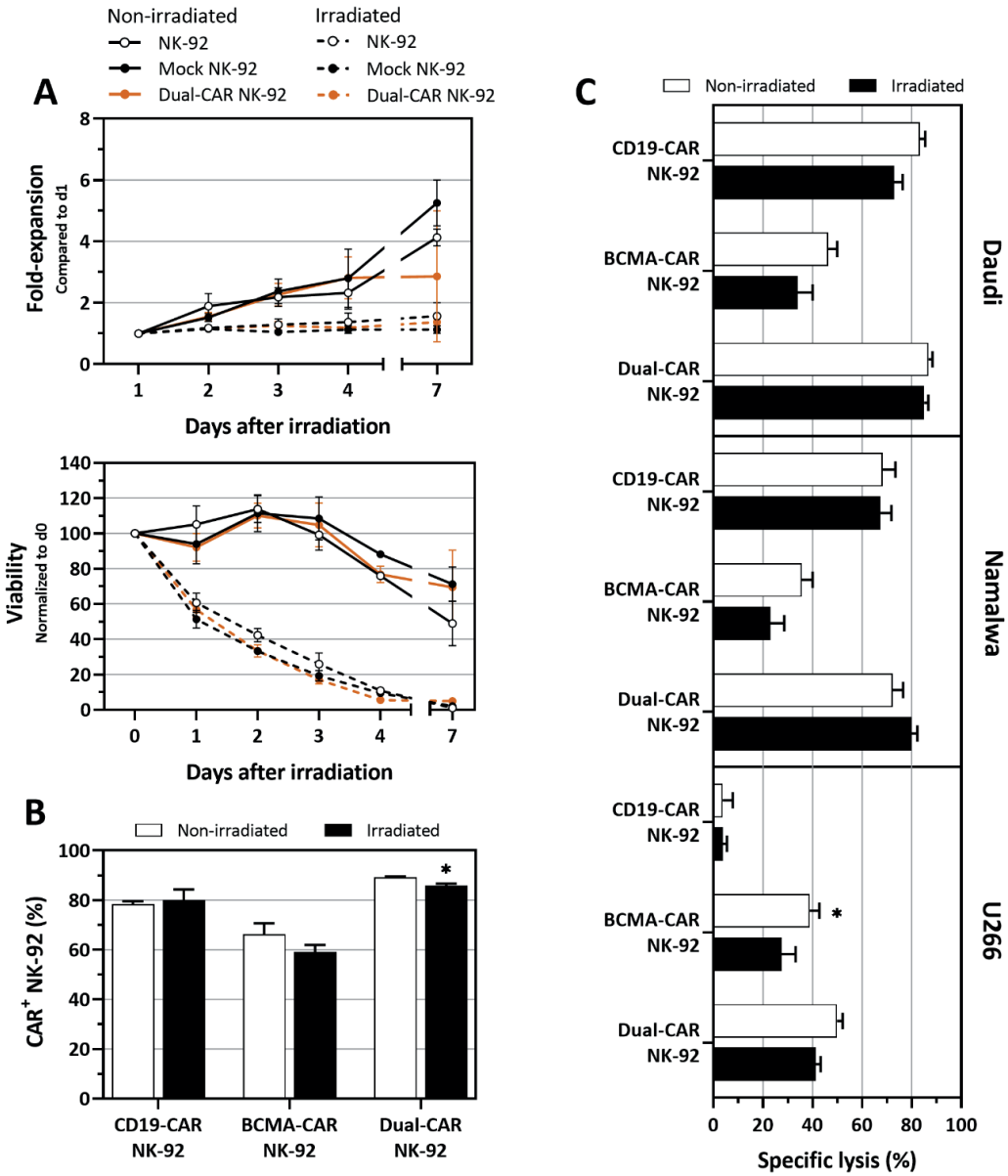

Figure 10. The effect of irradiation on proliferation, viability, CAR expression and functionality of dual-CAR NK-92. **(A)** Proliferation of NK-92 cells (top) was successfully inhibited after irradiation and viability (bottom) gradually declined over the course of a week (N=3). Follow-up on day 5 and 6 was not performed and, therefore, not shown in the graph. **(B)** Peak CAR expression (24 h post electroporation) is maintained following irradiation (N=3). **(C)** Despite irradiation, cytotoxic activity of NK-92 towards Daudi, Namalwa and U266 cells was largely preserved (4 h co-culture at 1:1 E:T ratio). Statistical analysis in B and C between non-irradiated and irradiated conditions was performed using an unpaired, two-tailed student t test. BCMA, B-cell maturation antigen; CAR, chimeric antigen receptor.

Cytotoxicity of dual-CAR NK-92 towards primary tumor cells

Finally, we investigated whether our dual-CAR NK-92 cells could also eradicate primary tumor samples (Figure 11). Comparable to our CD19$^+$ tumor cell line models, primary B-ALL blasts were effectively killed by CD19-CAR NK-92 and dual-CAR NK-92 at the different E:T ratios examined (Figure 11; e.g., ALL1 at a 1:1 E:T ratio, CD19-CAR: 72.9 ± 11.3% and dual-CAR: 78.0 ± 7.6%). Of interest, we could not observe any lysis mediated by BCMA-CAR NK-92 cells, indicating that the activity was CD19-CAR-mediated and not the result of any natural cytotoxic activity of NK-92 cells towards primary ALL cells. Similarly, both BCMA-CAR NK-92 and dual-CAR NK-92 killed primary MM cells at all tested E:T ratios while CD19-CAR NK-92 left them unharmed. Collectively, these results support the finding that simultaneous expression of two CARs does not impede the killing capacity of the NK-92 and that they possess cytotoxic activity not only towards cell lines but also towards primary tumor cells.

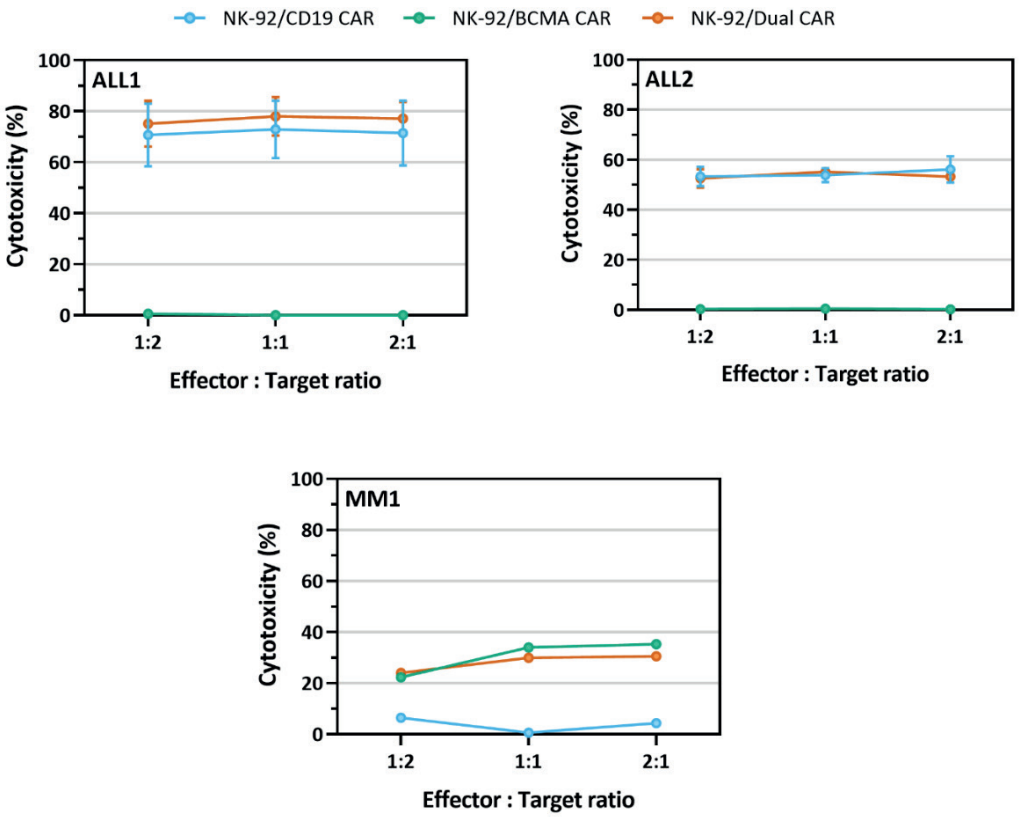

Figure 11. Lysis of primary tumor samples by CAR NK-92. High cytotoxicity of CAR-engineered NK-92 towards two primary B-ALL (N=2) and one primary MM (N=1) tumor samples after a 4 h co-culture at different E:T ratios. ALL#, B-ALL sample number. MM#, MM sample number.

Discussion

CAR-T-cell therapy has demonstrated immense therapeutic potential in hematological malignancies, including B-ALL, B-cell lymphoma and MM, even in patients in whom all standard treatment options have been exhausted. Yet, cancer cells exploit several escape mechanisms to thwart this potent immunotherapy, eventually leading to relapse in a large fraction of patients. Two main types of relapses after CAR-T-cell therapy can be discerned, one being the result of downregulation or complete loss of the target antigen on the tumor cell surface resulting in an inability of the CAR-T cells to recognize the tumor cells (antigen-negative relapses) and the other being the result of the loss of (functional) CAR-T cells; in this case, antigen expression is retained, hence the term antigen-positive relapses. There is now an intensive search for strategies to overcome the problem of antigen-negative as well as antigen-positive relapses. One way to tackle the problem of relapse due to antigen loss is to target more than one antigen [128, 332, 333, 357, 358], whereas the second problem could be addressed by producing and re-administering a second batch of CAR-T cells. However, the latter solution is cumbersome given the complex logistics and lengthy duration of autologous CAR-T-cell manufacturing, which is sometimes not possible due to the low numbers or low quality of patient-derived T cells. This problem could in turn be addressed by using an unlimited, off-the-shelf source of functionally active effector cells, such as the NK-92 cell line.

Here, we tackled the aforementioned root causes of relapse following CAR-T-cell therapy by developing a dual-CAR NK-92 cellular therapeutic targeted towards CD19 and BCMA. The reasons to select these two particular targets are obvious; CD19 and BCMA are highly relevant in the context of CAR-T-cell therapy for B-cell hematological malignancies, and, up until now, only CD19- and BCMA-targeted CAR-T-cell products have received regulatory approval. Furthermore, CD19 and BCMA are expressed across different stages in the development of B-cells to mature plasma cells. Hence, dual targeting of CD19 and BCMA offers the prospect of a broadly applicable therapeutic product that can be used in a spectrum of B-cell hematological malignancies ranging from B-cell leukemia and lymphoma to multiple myeloma. Some antigens are co-expressed on cells at the same developmental stage. This is, for example, the case for CD19 and CD20 on B cells; dual-CD19/CD20 CAR-T cells are now under active investigation in B-cell leukemia and lymphoma [359-362]. Similarly, for MM, combinatorial approaches with BCMA and other plasma cell surface antigens such as CD38 are under development [287, 304, 358, 363-365]. In vivo models investigating these dual CAR strategies consistently showed superior tumor clearance and prevention of antigen escape, offering the prospect for deeper and more durable clinical responses [357, 361, 364, 366].

Very recently, Luanpitpong et al. described a dual-CAR NK-92 approach similar to ours, but using a different combination of antigens (CD19 and CD138) and using lentiviral transduction as CAR loading strategy [367]. Here, the two fully human CAR constructs were introduced in

the NK-92 by means of mRNA electroporation. In contrast to lentiviral transduction, mRNA electroporation is a rapid, simple, relatively low-cost and highly efficient method for gene transfer in human cells. Boissel et al. previously applied the mRNA electroporation technology for introduction of a single CD19 CAR in NK-92 cells, reaching transfection efficiencies of approximately 50% [368, 369]. CD19 *CAR* mRNA electrotransfection of primary human NK cells yielded comparable results [370, 371]. In this study, we confirmed that mRNA electroporation is a suitable method for CAR loading of NK-92 cells, with either the CD19-CAR and BCMA-CAR being expressed at high levels. In addition, for the first time, we demonstrated that this technology can be used in the NK-92 therapeutic cell source to simultaneously introduce two different CAR constructs without hampering the expression of either CAR molecule. Despite its obvious advantages, such as potentially reducing the duration of severe side effects in treated patients, the temporary CAR expression following mRNA electroporation could imply a need for repeated administration of the therapeutic cell product [372]. However, in the NK-92 model, permanent gene expression is not an inherent requirement, since NK-92 cells have a relatively short lifespan after administration [341]. In this regard, it is of critical importance to carefully consider the origin of the extracellular antigen-recognition domains. As repeated administration of CAR products containing murine-derived components can cause immunization and anaphylaxis, severely limiting safety and therapeutic efficacy [373], we have opted for the use of two fully-human CARs.

For clinical application, proliferation of NK-92 cells needs to be halted prior to infusion to avoid NK-92 cell engraftment in vivo, which could lead to the development of NK cell lymphoma. Although alternative methods are being examined[374], this “inactivation” step is most commonly performed by gamma-irradiation. We confirmed that 10 Gy gamma-irradiation effectively blocks NK-92 cell proliferation and leads to a gradual decrease in cell viability down to zero over the course of one week, paralleling the CAR expression kinetics after mRNA electroporation. Importantly, gamma-irradiation did not affect the functionality of the (dual) *CAR* mRNA-electroporated NK-92 cells, underlining the potential clinical applicability of the proposed therapeutic cell-based product.

Another potential advantage of using NK cells over the gold-standard CAR-T cells is the natural anti-tumoral activity of NK cells. However, this intrinsic cytotoxic capacity is largely dependent on exogenous activation stimuli, such as IL-2 [375]. IL-2 is an essential cytokine for NK cell growth and is, therefore, indispensable during NK-92 cell culture. IL-2 administration in humans can cause severe toxicity and can lead to regulatory T-cell activation, which is an undesired effect in the context of cancer immunotherapy due to their counterproductive inhibitory effect on cytotoxic lymphocytes [375]. Therefore, to pave the way towards clinical application, we omitted the supplementation of IL-2 during the last 24 h of NK-92 culture. As expected, this led to an almost complete abrogation of the natural cytotoxicity of NK-92 towards the NK-sensitive tumor cell line K562 [375-377]. In addition, as exemplified here both in the Daudi lymphoma cell

line model as well as in the primary patient samples, some B-cell hematological malignancies are largely resistant to NK cell lysis [378]. Here, we show that CAR engineering of NK-92 cells overcomes the IL-2 dependence and restores their anti-tumor cytolytic activity. Corroborating the results of other dual-targeted CAR products [367, 379, 380], dual-CAR NK-92 were at least equally effective as their single-CAR counterparts in eliminating single and dual antigen expressing target cells, effectively reducing the probability of antigen escape. Interestingly, dual-CAR NK-92 display higher cytotoxicity towards $CD19^{-}BCMA^{+}$ cells compared to BCMA-CAR NK-92. The reason for this discrepancy, remains to be elucidated but the increased frequency of BCMA CAR^{+} cells in the dual-CAR NK-92 population compared to the BCMA-CAR NK-92 provides a likely explanation for the heightened lytic activity against $BCMA^{+}$ target cells in the dual-CAR NK-92 conditions. Moreover, it was recently reported that dual CD19- and BCMA-CAR-T cells were able to completely ablate regulatory B-cells from the bone marrow of MM patients, contributing to a favorable environment for clearance of myeloma cells in the bone marrow [339]. Hence, in addition to their direct anti-tumor activity, our dual-CAR NK-92 cells could also play an important role in reshaping the tumor microenvironment.

The use of dual-CAR NK-92 cells as presented in this study contains some limitations. First, supplementing the NK-92 culture medium with animal serum instead of human serum limits the clinical translational potential. To the best of our knowledge, there have been no comparative studies between the two, leaving uncertainty on whether the serum source affects CAR NK-92 performance. Of interest, one study on serum-free NK-92 culture reports no significant difference in viability, proliferation, receptor expression levels, or perforin and granzyme levels, but a significantly decreased degranulation and cytotoxic potential in vitro which could be partly recovered after the addition of serum [381]. Second, our follow-up period of NK-92 cell viability and proliferation after irradiation was limited to 7 days. However, others have reported on the complete abrogation of NK-92 expansion for more than 30 days using the same irradiation protocol as described here [355, 382]. In our view, this provides sufficient proof for the safe clinical application of dual-CAR NK-92. Finally, we did not directly investigate potential “off-tumor” effects in our work. As discussed above, so far, NK-92 clinical studies have not revealed any toxicity towards normal cells or tissues. Moreover, given that “on-target/off-tumor” toxicities of current CD19- and BCMA-CAR-T products, such as hypogammaglobulinemia following B-cell depletion, are well described and manageable [54, 59], it seems unlikely that combinatorial targeting of these antigens will result in any unanticipated off-tumor side effects. Moreover, in the event such toxicities occur with our dual-CAR NK-92 approach, repeated administration of the cells can be terminated, benefiting from their limited persistence after irradiation and the transient nature of the CAR-encoding mRNA.

Conclusions

In conclusion, we demonstrate the efficient generation of dual CD19- and BCMA-CAR expressing NK-92 cells. These dual-CAR NK-92 cells have potent and specific cytotoxic activity towards $CD19^+$ and $BCMA^+$ tumor cell lines, as well as primary B-ALL and MM cells. Gamma-irradiation was confirmed to be adequate to avoid proliferation and persistence of the NK-92 cells while maintaining functionality. Future work should be conducted to validate our findings in vivo before proceeding to early phase clinical testing to evaluate safety and feasibility. However, in the long term, we envision that our CD19/BCMA-targeted dual-CAR NK-92 can be used as an off-the-shelf therapeutic for B-cell leukemia, lymphoma and myeloma patients to address the current problems of antigen-negative and antigen-positive relapses after autologous single CAR-T administration.

Acknowledgements

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Supplementary material

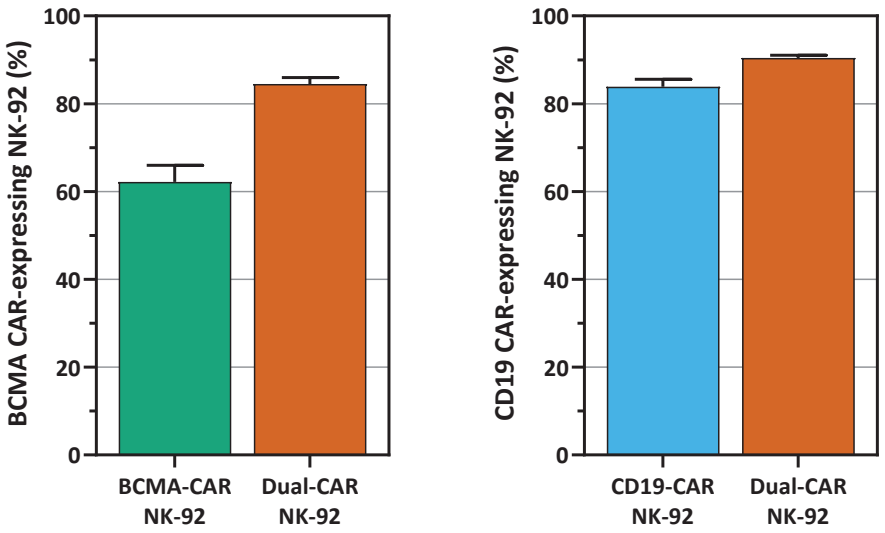


Figure S4. Decomposition of CAR expression of single- and dual-CAR NK-92 cells. BCMA-CAR (left) and CD19-CAR (right) expression in each of the relevant NK-92 cells (N=19).

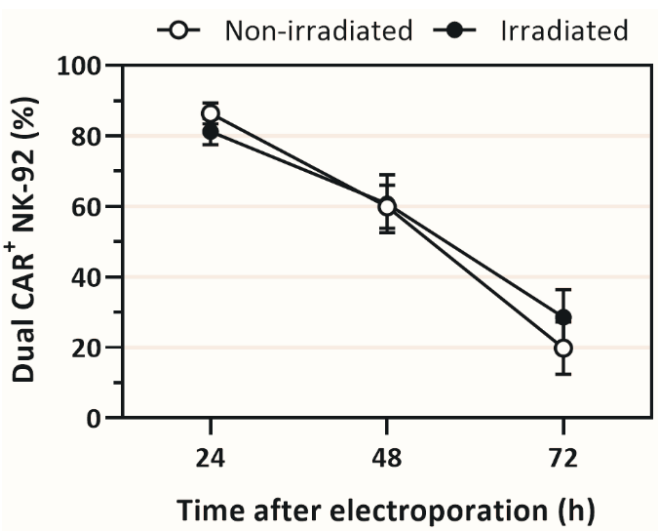
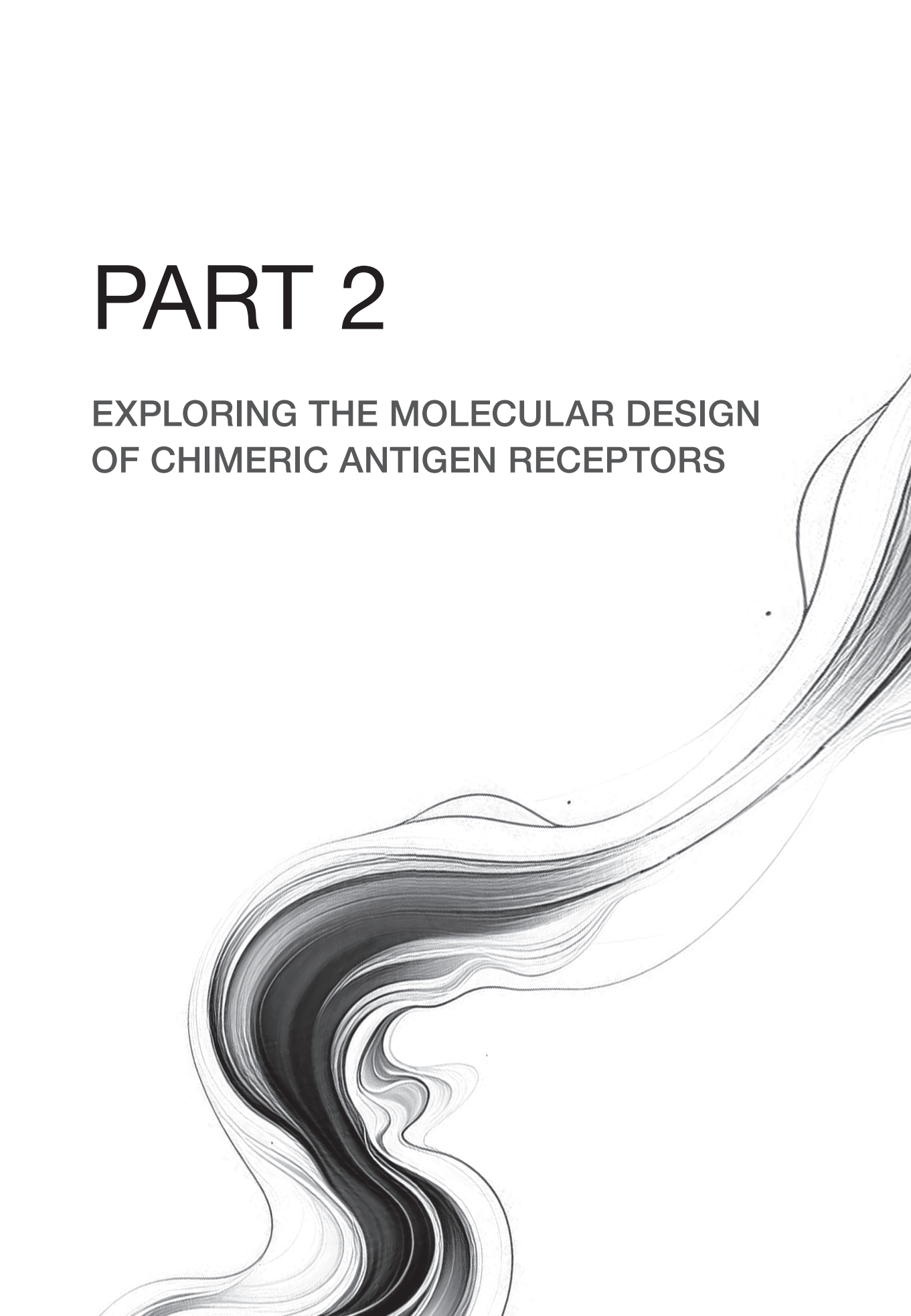
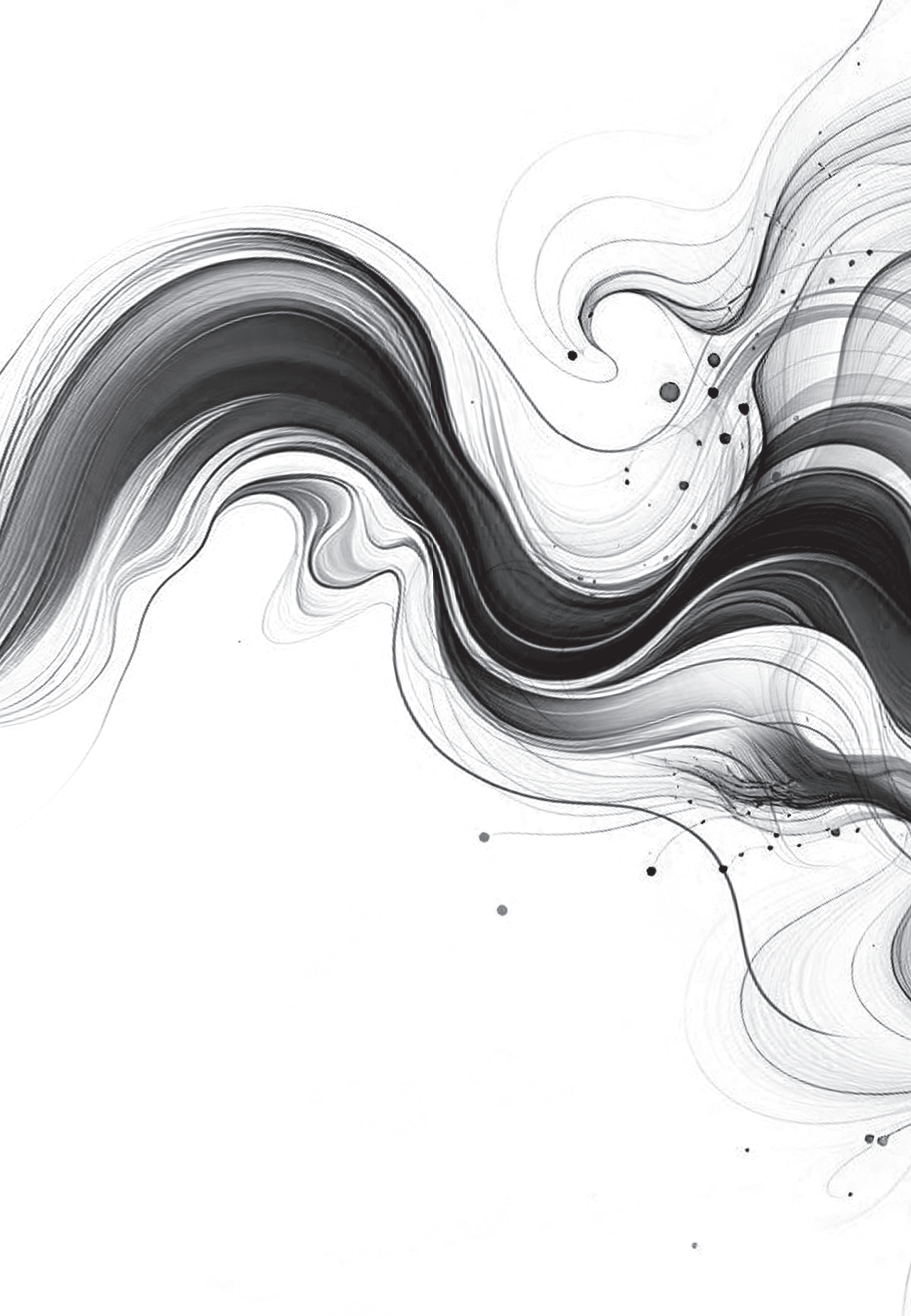


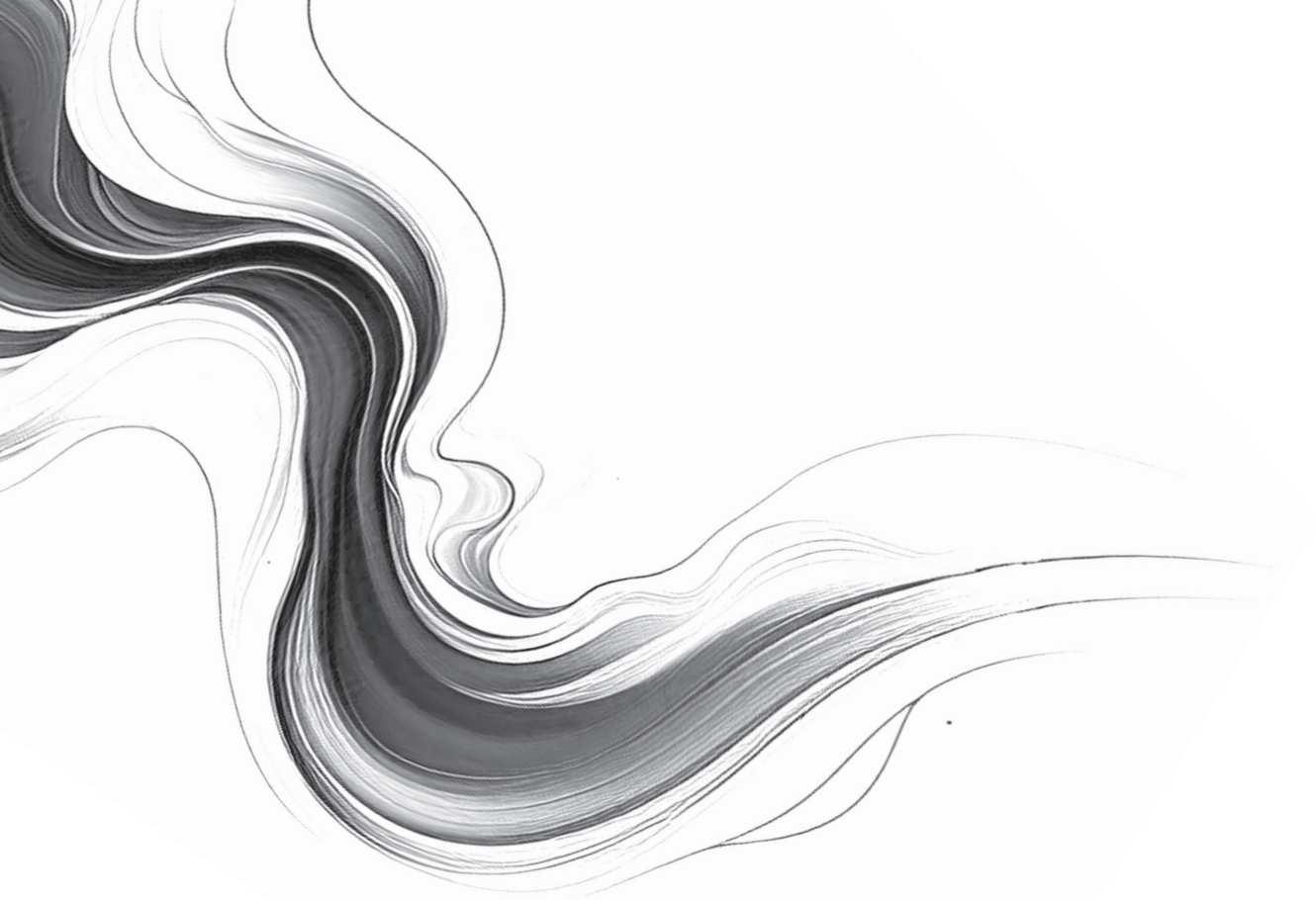
Figure S5. Dual-CAR expression kinetics after irradiation of dual-CAR NK-92. Follow-up of dual-CAR expression over the course of three days (N=3).

PART 2

EXPLORING THE MOLECULAR DESIGN OF CHIMERIC ANTIGEN RECEPTORS







6

EXPANDING THE CAR TOOLBOX WITH HIGH THROUGHPUT SCREENING STRATEGIES FOR CAR DOMAIN EXPLORATION: A COMPREHENSIVE REVIEW

Roex G, Gordon KS, Lion E, Birnbaum ME, Anguille S.

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Abstract

Chimeric antigen receptor (CAR)-T-cell therapy has been highly successful in the treatment of B-cell hematological malignancies. CARs are modular synthetic molecules that can redirect immune cells towards target cells with antibody-like specificity. Despite their modularity, CARs used in the clinic are currently composed of a limited set of domains, mostly derived from IgG, CD8α, 4-1BB, CD28 and CD3ζ. The current low throughput CAR screening workflows are labor intensive and time-consuming, and lie at the basis of the limited toolbox of CAR building blocks available. High throughput screening methods facilitate simultaneous investigation of hundreds of thousands of CAR domain combinations, allowing discovery of novel domains and increasing our understanding on how they behave in the context of a CAR. Here we review the growing body of reports that employ these high throughput screening and computational methods to advance CAR design. We summarize and highlight the important differences between the different studies and discuss their limitations and future considerations for further improvements. In conclusion, while still in its infancy, high throughput screening of CARs has the capacity to vastly expand the CAR domain toolbox and improve our understanding of CAR design. This knowledge could be foundational for translating CAR therapy beyond hematological malignancies and push the frontiers in personalized medicine.

Introduction

Treatment of B-cell leukemia and lymphoma has seen a tremendous improvement in clinical outcomes with the advent of chimeric antigen receptor (CAR-)T-cell therapies. This wave of novel therapeutics was led by the CD19-targeted CAR-T-cell products Kymriah[95] and Yescarta[81] in the context of relapsed/refractory diffuse large B-cell lymphoma and acute B-lymphocytic leukemia, which were approved in late 2017. Since then, we have seen the regulatory approval of two additional CD19-specific CAR-T-cell products for B-cell malignancies[325, 328], as well as two products targeted against B-cell maturation antigen for the treatment of myeloma[326, 327]. Although revolutionary, current CAR therapies are not flawless. In addition to concerns of on-target off-tumor toxicities [383, 384], simultaneous activation of massive numbers of cancer-directed immune cells cause side effects, such as cytokine release syndrome and neurotoxicity, often requiring additional medical interventions[385, 386]. Perhaps more importantly, a considerable portion of CAR-treated patients eventually relapse because of either antigen escape or lack of persistence [54, 387]. Furthermore, tumor-specific target selection, migration towards and infiltration into the tumor site, and expansion and function in a hostile tumor microenvironment provide additional challenges for CAR-T-cell therapy in solid tumors[388]. These drawbacks led researchers to scrutinize the design of CARs currently used in the clinic.

CARs are synthetic molecules that generally consist of five major sequential components (Figure 12A): (1) an antigen-binding domain (ABD), often derived from a conventional monoclonal antibody in single-chain variable fragment (scFv) format, (2) a flexible hinge domain (HD) or spacer, (3) a transmembrane domain (TMD), (4) one or more co-stimulatory domains (CSDs) and (5) an intracellular signaling domain (ISD). The specificity, sensitivity, therapeutic potency and persistence of the CAR-T-cell product is influenced by the choice of each of these components. The ABD determines the tumor antigen specificity and carries the largest variation across literature with over 64 target antigens spanning liquid and solid tumors[39]. Optimization efforts to improve tumor cell discrimination, safety or T-cell exhaustion include affinity-modulation[271] and reconfiguration of the ABD to a different format[199, 389-393]. The other CAR components are less diverse in the clinical landscape. Current CAR-T-cell products in the clinic are mostly limited to combinations of a CD8a-, CD28- or IgG-derived HD, CD8- or CD28-derived TMD, 4-1BB or CD28 CSD and a CD3ζ ISD[394].

To fill this gap, preclinical studies have started exploring alternative domains, such as ABDs taken from receptors[35, 395], ligands[392], autoantigens[391], peptides[393] and nanobodies[396], HDs derived from proteins including 4-1BB[397], CD34[46], low-affinity nerve growth factor receptor (LNGFR)[48] and members of the Siglec-family[47], TMDs isolated from proteins such as CD16 and NK cell-associated receptors[398], and ICDs among which inducible costimulator (ICOS)[52] and OX40 have been most extensively studied[41, 399].

However, these CAR domains are often studied in isolation with only one or, at most, a few candidates evaluated in parallel. Meanwhile, there are indications that certain domain combinations lead to favorable outcomes[37, 52, 400]. Current low throughput workflows are labor intensive and time-consuming, and do not allow for exhaustive evaluation of large CAR combinatorial libraries. Higher throughput screening would not only increase the number of domains that can be tested, but would also enable basic research on how the inclusion of a specific domain affects the overall outcome, which could eventually lead to the definition of basic CAR design rules. Recently, there have been a number of studies utilizing such high throughput CAR screening approaches. Here we review the growing body of high throughput CAR screening campaigns and compare the methodologies used therein.

Overview of current CAR library screening approaches

Most CAR library screening approaches use similar sequential workflows with (1) (combinatorial) CAR library generation in DNA-space, (2) transfer of the library into cells, (3) assaying and selection of responding library members, and (4) identification and validation of lead candidates (Figure 12). However, the actual implemented methods to achieve each of these steps can differ between studies depending on the intended goal.

The cloning techniques used for library assembly are often dictated by the CAR domain that is being screened (Table 8). For the ABD, scFv libraries are obtained by creating diversity in complementary determining regions (CDRs)[401-403], in entire variable chains [404, 405] or in full scFvs[406-409]. CDR diversity is generated by amplifying the variable chain of interest with degenerate primers[403], computational modeling[402] or by DNA synthesis[401]. Meanwhile, variable chain and scFv repertoires are created in vitro from human variable region banks[406] or by PCR amplification of immunoglobulin genes from healthy human peripheral blood mononuclear cells or B cells[405, 407-409]. With regard to ISD libraries, researchers mostly opt for synthesis of gene blocks encoding the library members[54, 410-412], presumably due to declining costs of DNA synthesis and the relatively low number of unique library components, though they can also be amplified from cDNA[413]. Subsequently, ISDs are PCR amplified and cloned into the acceptor vector using type IIs restriction enzymes[410, 411, 413] and/or overlap-extension PCR, Gibson Assembly, In-Fusion cloning or blunt-end ligation [50, 412, 414, 415].

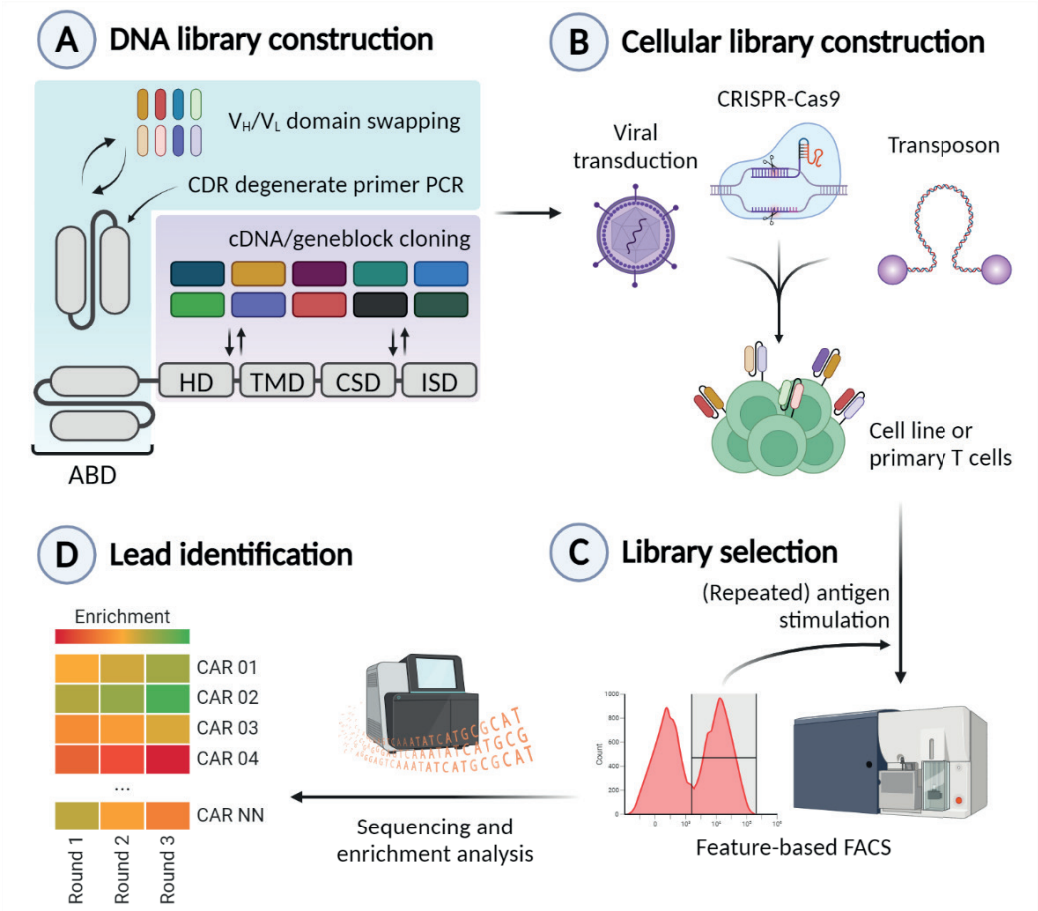

Figure 12. Generalized workflow for high throughput screening of CAR variants. (A) Diversity is created in the CAR library by means of PCR amplification with degenerate primers or permutation of domain modules. (B) Pooled library elements are integrated into cells using permanent modification methods. (C) Subsequent cycles of antigen stimulation and feature-based sorting enrich high performing library elements. (D) Enrichment over rounds of stimulation is tracked via sequencing of the pools and high performing lead candidates can be identified for further characterization. Abbreviations: ABD, antigen-binding domain. CDR, complementary determining region. CSD, co-stimulatory domain. FACS, fluorescence-activated cell sorting. HD, hinge domain. ISD, intracellular signaling domain. PCR, polymerase chain reaction. TMD, transmembrane domain. VH, variable heavy chain. VL, variable light chain. Created with BioRender.

Table 8. High throughput screening methods for the discovery of novel CAR domains.

REF	Library Variable	Library Generation	Cell Type	Modification Method	Stimulation Method	Enrichment Method
[401]	scFv (CDR)	Degenerate HDR template synthesis	B3Z cell line (IL2-EGFP reporter)	CRISPR-Cas9	Soluble rhHER2 antigen, or SKBR3 cell line	3x rhHER2 binding sort, or 3x GFP expression sort
[403]	scFv (CDR)	Degenerate primer PCR and restriction-ligation cloning	Jurkat cell line (NFkB-ECFP & NFAT-EGFP reporter)	Transposon	JeKo cell line	rhROR1 binding sort and $ECFP^+EGFP^+$ expression sort
[404]	scFv (VL/VH)	Not specified	HEK293F cell line (Gal4-VP64-BFP reporter)	Lentiviral	Healthy PBMC and $CD38^+$ K562 cell line	BFP expression sort
[405]	scFv (VL/VH)	Human B cell cDNA nested PCR and overlap extension PCR	Primary T cells	Retroviral	NY-ESO-1_{157}/HLA-A2-modified K562 or T2 cell line Raji cell line (CD19)	Repeated antigen stimulation followed by tetramer binding sort (NY-ESO-1_{157}/HLA-A2), or Repeated antigen stimulation followed by rhCD19 binding sort
[406]	scFv (full)	Degenerate primer PCR and restriction-ligation cloning	Jurkat cell line	Lentiviral	CEA-modified HeLa cell line	3x $EGFP^+CD69^+$ expression sort
[407]	scFv (full)	Human B cell cDNA PCR and restriction-ligation cloning	Jurkat cell line (NFAT-mCherry reporter)	Lentiviral	HEK293-6E cell line, or H-226 cell line, or AsPC-1 cell line	$mCherry^-$ expression sort followed by 2x $GFP^+mCherry^+$ expression sort
[408]	scFv (full)	Human PBMC cDNA, murine spleen or BM cDNA nested PCR and restriction-ligation cloning	Primary T cells; NK-92 cell line	Lentiviral	Subcutaneous mouse models with an $EGFR^+$-modified MCF-7, $HER2^+$-modified MCF-7, SW480, A549, SK-OV-3, SK-BR-3, N3 or BN16 cell line	In vivo enrichment for tumor reactive CARs of unknown specificity
[409]	scFv (full)	Phage display library PCR and restriction-ligation cloning	Jurkat cell line	Retroviral	Plate-bound CEA	3x $CD69^+$ expression sort with subsequent gDNA PCR and recloning
[410]	ISD	Gene synthesis, PCR and restriction-ligation cloning	Primary T cells	CRISPR-Cas9	SKBR3 cell line	CAR^+ expression sort
[411]	ISD	Gene synthesis, PCR and Golden Gate assembly	Primary T cells	Lentiviral	$CD19^+$-modified K562 cell line	IFN-γ^+, IL-2^+ or $CD69^+$ expression or proliferation sort

REF	Library Variable	Library Generation	Cell Type	Modification Method	Stimulation Method	Enrichment Method
[412]	ISD	Gene synthesis, PCR, overlap extension PCR and restriction-ligation cloning	Jurkat cell line	Lentiviral	rhCD19 antigen	3x $CD69^+$ and/or $CD69^+PD\text{-}1^-$ expression sort
[413]	ISD	cDNA PCR and restriction-ligation cloning	Jurkat cell line	Retroviral	Plate-bound c-Myc antibody	$CD69^+$ expression sort
[414]	ISD	Gene synthesis and In-fusion cloning	Primary T cells	Lentiviral	Nalm-6 cell line	NA
[415]	ABD, HD, ISD	Gene synthesis and restriction-ligation cloning	Jurkat cell line; Primary T cells	Retroviral	NA	$CD69^+$ expression sort Proliferation sort
[417]	ISD	Site-directed mutagenesis	Jurkat cell line (NFAT-GFP & NFkB-CFP reporter)	Retroviral	Soluble rhB7H6	GFP^+CFP^+ expression sort

Abbreviations: AA, amino acid; ABD, antigen-binding domain; BFP, blue fluorescent protein; BM, bone marrow; CD, cluster of differentiation; cDNA, complementary DNA; CDR, complementary determining region; CEA, carcinoembryonic antigen; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; HD, hinge domain; HDR, homology directed repair; HER2, Human Epidermal growth factor Receptor 2; ISD, intracellular signaling domain; ML, machine learning; NA, not applicable; NFAT, nuclear factor for the activation of T cells; NFkB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NGFR, nerve growth factor receptor; rh, recombinant human; scFv, single-chain variable fragment.

High throughput CAR-screening efforts also differ in the cell type used (Table 8). Most studies rely on cell lines because they offer a convenient unlimited supply of homogeneous cells. The Jurkat T-cell lymphoma line is often the cell line of choice due to its largely conserved T-cell signaling machinery [403, 406, 407, 409, 412, 413, 415]. While functional markers such as CD69 upregulation and IL-2 secretion are present in Jurkat cells, they show little cytotoxic capacity, do not secrete the full repertoire of primary T-cell cytokines and have higher basal signaling due to their nature as a continually dividing cell line[413, 416]. Therefore, while they can be valuable as a first step in a screening campaign, any screening effort must be validated in primary T cells. Other cell lines used for screening include the NK-cell lymphoma line NK-92[408], HEK293F[404] and the murine hybridoma line B3Z[401]. Cell lines also have the added advantage that they can be easily engineered to express a reporter gene in response to activation signals. They offer an attractive alternative to simple activation markers like CD69, which may not be sensitive enough to capture subtle differences in signaling. In contrast, multiple reporter genes driven by different transcription factors can capture a broader image of CAR-induced T-cell signaling pathways[403, 416-419]. To date, only a few groups have used primary murine or human T cells for screening purposes[50, 410, 411, 414, 415], directly assessing CAR functionality in the final cellular product.

A suitable method to genetically introduce the CAR gene is critical for proper evaluation of CAR libraries (Table 8). Candidate selection is based on the enrichment of library members that show phenotypical and/or functional superiority (see below). Incorporation of multiple CAR constructs per cell inevitably leads to co-selection of poorly functional CARs with lead candidates[403, 409, 413], causing the loss of valuable resources on unnecessary validation experiments. Consequently, transfection, electroporation, or nucleofection of transient CAR-encoding nucleic acids is not suitable for this application because of the need for high cytoplasmic concentrations of unique library elements to achieve sufficient CAR expression for functional evaluation[409]. Permanent modification methods, such as viral transduction[50, 402, 404-409, 411-413, 415], transposon systems[403] and CRISPR-Cas9[401, 410], can be carefully titrated to ensure singular or site-specific integration.

Instead of evaluating each candidate independently, most high throughput screening campaigns rely on enrichment of superior CAR constructs from a pool of constructs. To quickly eliminate oversensitive, non-specifically activating or tonic signaling constructs, some approaches incorporate an initial round of negative selection of their CAR library on antigen-negative cell lines or healthy peripheral blood mononuclear cells (PBMC)[404, 407]. Then, cellular CAR libraries are stimulated by plate-bound or soluble antigen, or antigen presenting cells (Table 8). Expression of CD69[406, 409, 411-413, 415], cytokines[411] or activation-induced reporter genes[401, 403, 404, 407] and proliferation[411, 415] are functional markers that have been used to sort for the best responders. Incorporating multiple stimulation-and-sorting rounds and/or more stringent sorting gates will increase the relative frequency of the best performers, even if

extremely rare in the initial population[409, 417, 420]. Alternatively, long-term stimulation assays rely on the outgrowth of top candidates over time because of their competitive advantage, followed by a sort via antigen or tetramer staining[405, 408]. Finally, computational approaches can assist in the selection of lead candidates by modeling for orientation, conformation, antigen-receptor interactions and changes in stability[50, 402]. Artificial intelligence has also been used for CAR selections, using sparse data to make predictions of functional outcomes of CAR designs[414]. Others have opted for the rational selection of lead candidates based on single-cell transcriptional profiling of pooled populations[410].

As a final step, the enriched library is sequenced and the relative frequency of lead candidates is determined. Those lead candidates are subsequently validated and benchmarked against the wildtype receptor or a state-of-the-art CAR construct. Although not all novel CARs identified through high throughput screening outperform their benchmark, these screening efforts can still offer valuable information on the effects induced by specific changes in the CAR building blocks and aid in future rational CAR design. Below we provide an overview of current literature related to high throughput and computational methods for the discovery of improved CAR architectures.

Screening studies of different CAR domains

Antigen-binding domain

The ABD of a CAR is typically an scFv derived from a monoclonal antibody that was acquired via hybridoma technology, or phage, bacterial or yeast display methods[409, 421]. However, these scFvs are not guaranteed to maintain desired binding properties in the context of CARs. Indeed, improper protein folding may diminish CAR expression, and antigen-independent crosslinking of CARs through the scFv can cause tonic signaling and compromise CAR functionality[409, 416, 422, 423]. Soluble or plate-bound antigen used in these enrichment platforms may not maintain the same structure as membrane-bound antigen, leading to selection of scFvs specific for epitopes that are not available on antigen-presenting cells[420], or loss of scFvs that recognize epitopes present in the membrane-bound conformation[407]. Similarly, ABD conformation and binding characteristics are also context-dependent and may not be properly translated from the antibody to the scFv typically found in a CAR, potentially changing CAR affinity and functionality [402, 421, 422, 424]. High throughput screening of scFv variants in CAR format can overcome these challenges as both target antigen and scFv conformation are selected in their clinically relevant configurations.

The ability to enrich rare events from a highly diverse population is essential when screening scFv libraries. To our knowledge, Alonso-Camino and colleagues were the first to demonstrate the feasibility of rare event enrichment of CARs with their lymphocyte display platform[420]. Three rounds of stimulation and sorting for $CD69^+$ Jurkat cells resulted in a nearly 1000-fold

increase in frequency of carcinoembryonic antigen (CEA)-specific Jurkat cells spiked into Jurkat cells expressing no or an irrelevant CAR. The same group used this procedure to isolate HeLa cell-specific scFvs from a starting scFv library of $1.5x10^5$ members[406]. Likewise, Lipowska-Bhalla et al. transduced Jurkats with a mix of viral particles spiked with a CEA-targeting CAR at low frequency (0.0001%), which could be enriched 10^5-fold in just three rounds of FACS-based selection on the top 2% CD69 expressing cells[409]. Subsequent application of this method to an scFv library saw a 65-fold enrichment of CEA-binding Jurkat cells. While sequence analysis revealed three dominant scFv clones to be present in this population, only one clone was verified to be able to recognize CEA, indicating the enriched population was still contaminated by non-binders, presumably as a result of cross-contamination during library preparation or co-selection of non-binders with binders within the same cell.

The heavy chain CDR3 loop is a major determinant of binding specificity of scFvs and therefore often targeted for optimization. Rydzek et al. obtained a 10^6 member CAR library via site-directed mutagenesis of the V_H CDR3 loop of a ROR1-specific CAR[403]. Single-cell sorting of $NFAT^+NF\kappa B^+$ Jurkat reporter cells upon ROR1-stimulation led to the selection of 15 unique clones. Similar to other reports[409], multiple genomic integrations had occurred in the best performers[403]. Expression of the wildtype receptor was probably driving the majority or entirety of the observed responses. While this study reaffirms the feasibility to pick up dominant clones present at very low frequency (0.0005%) in the initial library, it also highlights the importance of single integration events. Fluorescent reporters can also be coupled to an endogenous marker of activation, avoiding artificially enhanced signal amplification by synthetic promoters. For example, Di Roberto and colleagues linked GFP to the last exon of IL-2 separated by a P2A peptide in the B3Z murine T hybridoma cell line[401]. As such, CAR-modified B3Z cells recognizing their cognate antigen could be discriminated based on GFP-expression while leaving IL-2 secretion intact. Deep mutational scanning of the V_H CDR3 region generated 190 variants of the human epidermal growth factor receptor 2 (HER2)-specific trastuzumab scFv[401]. After three rounds of soluble antigen stimulation and sorting on activation-induced GFP, two lead candidates were detected that had a similar sensitivity to $HER2^{high}$-, but lower reactivity to $HER2^{low}$-target cells compared to the original scFv. By employing an affinity-based selection strategy against soluble HER2 in parallel to the activation-based screening, the authors were able to pinpoint residues that can be altered to modulate affinity without affecting antigen-specific signaling.

Instead of focusing on the V_H CDR3, some have pursued swapping variable chains, generating diversity in entire CDR sets. Ochi et al. interrogated sets of CDRs by generating scFv libraries by coupling V_H and $V_{L\lambda}$ or $V_{L\kappa}$ derived from healthy human B cells to a V_L or V_H of a validated functional scFv, respectively[405]. Two scFv libraries were created; one targeted to CD19 and one targeted to the New York esophageal squamous cell carcinoma 1 $(NY\text{-}ESO\text{-}1)_{157}$ peptide bound to HLA-A2. The libraries were stimulated three times and subsequently sorted for soluble CD19 or HLA-A2/NY-ESO-1_{157}-specific tetramer binders, respectively.

Newly discovered scFv variants were demonstrated to improve proliferation, reduce cytokine release, enrich naïve and memory T cells and have better in vivo tumor control[405]. Ma and coworkers took this approach even further and generated an scFv library with a diversity of 10^{11} through V_L/V_H randomization[404]. As it is not possible to reach sufficient coverage of such a large library in a cellular screen, phage display against recombinant CD38 was performed to reduce the library size to 10^6 members. This pre-enriched scFv library was subsequently screened in the form of a synNotch receptor driving a BFP reporter gene. Following an additional negative selection round against healthy PBMC, removing any hypersensitive clones, the remaining library was sorted for BFP expression after antigen challenge. The discriminatory power between healthy and tumor cells of two lead candidates, R02 and R03, was confirmed in subsequent co-cultures. Although R02 had a 28-fold lower affinity than R03, its cytokine response against $CD38^{high}$ cells was consistently higher. The faster on-rate (k_{on}) and slower off-rate (k_{off}) of R02 compared to R03 allow it to quickly engage with and release target antigen, which is implicated with the potential for improved clinical performance[38]. In a similar fashion, Fierle and colleagues used a Jurkat NFAT reporter cell line to screen a naïve scFv library of $2x10^{10}$ members by first pre-enriching the library to only 10^5 members through affinity-based phage display against their target antigen mesothelin (MSLN)[407]. When assessing scFv recognition characteristics against three isolated MSLN extracellular domains, it was discovered that the majority bound the membrane distal domain 1, whereas only one scFv bound the membrane proximal domain 3. Few scFvs also only bound to the full MSLN extracellular domain, pointing towards conformational or interdomain epitopes. Context-dependent recognition of MSLN was further demonstrated with some hits being able to bind to membrane-bound MSLN, but not soluble, bead-bound MSLN. Importantly, these screening methods inherently optimize the formation of the immune synapse while accounting for the effect of other extracellular domains of the CAR because the library is not restricted to the epitope of the wildtype scFv.

Thus far, most selection strategies were performed completely in vitro and were aimed at identifying a single lead scFv. However, immunosurveillance is achieved through immune cells with a repertoire of receptors. Fu et al. attempted to create an artificial immune system of CAR-engineered immune cells that is capable of recognizing a variety of non-self-antigens[408]. A naïve scFv library generated from B cells of 200 healthy individuals and subjected to four rounds of negative selection through in vivo phage display in NSG mice to remove any autoreactive clones before subcloning the $5x10^5$ member CAR library primary mouse T cells. epidermal growth factor receptor $(EGFR)^+$ SW480- or MCF7-bearing NSG mice treated with the CAR-T-cell library showed no tumor control for the first month, after which the majority of mice started to experience tumor growth reversal and ultimately total clearance, even in a challenging SW480 tumor model. Similar kinetics of tumor control were observed with $HER2^+$ mouse models. Formation of memory was illustrated by the absence of tumor growth in mice rechallenged with the same tumor. Most impressively, rechallenge with $EGFR^+$ SW480 of mice previously cured of

HER2+ tumors again showed initial tumor outgrowth with a rapid decline in tumor mass from the one-month timepoint onwards, suggesting long-term maintenance of diversity. This work shows that a pooled therapeutic approach is feasible, though it remains unknown whether anti-tumor responses were driven by a few dominant clones or a broader scFv repertoire.

Screening of scFvs does not have to be entirely carried out experimentally. In some cases, computational modeling can be used to improve binding characteristics. As such, Krokhotin et al. introduced single residue mutations in all six CDRs of an scFv derived from the $HER2_{369-377}$/HLA-A2-specific antibody SF2 in silico[402]. At least two hits had improved specificity and sensitivity for the $HER2_{369-377}$/HLA-A2 antigen facilitated by enhanced stability of the binding complex. Notably, using feedback loops of the experimental data, the models can be further refined. Another group used 3D modeling of the scFv for in silico mutagenesis to evaluate the effect of amino acid substitutions in framework regions on the stability of scFv, which elicits tonic signaling through antigen-independent CAR clustering[31]. FMC63 CAR destabilization by modifying key framework region residues led to severe tonic signaling and reduced functionality. Conversely, the authors could rescue a natively unstable chondroitin sulfate proteoglycan 4 (CSPG4)-targeted CAR using this method without compromising specificity. Of interest, humanization of the murine CSPG4-specific scFv with the human stable framework rFW1.4 avoided CAR aggregation and signaling in the absence of antigen.

Hinge domain

A hinge domain was initially incorporated into CARs with the aim of providing the ABD with the necessary reach and flexibility for effective binding to the target antigen and enhancing anti-tumor activity[40, 425]. Despite the established benefits of integrating a HD, there remains a lack of consensus regarding how specific properties, such as length, physical size, and binding sites precisely influence CAR function. For instance, shorter HDs tend to outperform longer ones by maintaining a tighter immune synapse upon antigen binding[42, 426], while longer HDs may provide more effective access to membrane-proximal or heavily glycosylated targets[41, 400, 427]. This was elegantly demonstrated by McComb and colleagues, who showed that progressive truncation of a CD8α HD resulted in progressive attenuation of CAR functionality[396]. Similarly, CARs designed to target a membrane-proximal epitope often exhibit superior performance compared to those directed towards a membrane-distal epitope[42, 428-430]. One possible mechanism behind this is the physical exclusion of phosphatases from the immune synapse upon antigen binding, akin to mechanisms key for endogenous TCR signaling[42, 426, 431]. If correct, the field would have to adjust its view on hinge length from the number of amino acids (AA) to the physical size as those are not always linearly correlated (e.g., a IgG4 hinge is 240AA and ~7 nm long while a CD8α hinge is 55AA and ~5 nm long[42]. Recent work by Rios et al. added valuable insights by creating a combinatorial library of different CAR domains[415]. This library included three different HDs of similar length (CD8α, CD28, and a short IgG4 hinge), two ABDs (CD19 and GD2), and five CSDs (wild-type

CD28, mutant CD28, 4-1BB, OX40, and DAP12). Consistent with previous research, their findings indicated that the ABD-HD combination significantly influenced CAR expression and CAR-T cell proliferation, expansion, and tumor control. Moreover, similar to the dependency of ICOS TMD-CSD[52], there were indications of a CD28 HD-TMD dependency for proper CAR functionality[415]. While larger HD library screening efforts are yet to be performed, the recent studies by McComb and Rios highlight the potential of higher throughput approaches in unraveling the complexities of hinge design and interdomain interactions[396, 415].

Transmembrane domain

The TMD is a short peptide sequence of 21-27AA forming a membrane spanning alpha helix. Most clinical CAR designs make use of TMDs derived from CD3ζ, CD8α or CD28, often matching the source protein of the adjacent HD or CSD, but TMDs from OX40[399], CD4[425], CD7[425], ICOS[52], CD16[398], CD27[395] and NK-cell receptors[398] have been explored as well. Based on the oligomeric state of the protein of origin, the TMD can determine CAR multimerization and may in some cases (e.g., CD8α and CD28) facilitate interactions with endogenous proteins, which can increase antigen sensitivity, but also induce tonic signaling[51, 432, 433]. To gain better control over CAR interactions, oligomeric state and geometry through the TMD, Elazar and colleagues developed a computational design approach to generate TMDs de novo that can control the structure and function of CARs[50]. They generated artificial TMDs that were predicted to form CARs in a mono-, di-, tri- or tetrameric state, called proCARs. ProCAR-T cells showed in vitro and in vivo anti-tumor potency that scales linearly with the oligomeric state, and lower inflammatory cytokine release than CARs with a natural CD28 TMD. Notably, it was only the tetrameric proCAR that matched the in vivo tumor control of CD28 TMD CARs. This study demonstrates that controllable and more predictable CAR-T responses can be achieved with computer-assisted design of the transmembrane domain.

Intracellular signaling domain

The composition and spatial configuration of the intracellular signaling domains of the CAR, which recruit signaling intermediates to produce downstream effector functions in response to antigen detection, have been found to be integral to their clinical performance. This is well demonstrated by the paradigm shift from first generation CARs, which comprise only FcRγ or CD3ζ signaling moieties and show no efficacy in a clinical setting due to poor persistence, to second generation CARs bearing an additional signaling domain—most commonly derived from co-stimulatory receptors CD28 or 4-1BB. This modification boosted CAR persistence and enabled the first demonstration of efficacy in patients, which catalyzed FDA-approval of a handful of second generation CARs targeting CD19 or BCMA.

Studies comparing CD28- and 4-1BB-based second generation CARs have yielded important insights into the functional consequences of choice of signaling domains. CD28 is known to induce a switch to aerobic glycolysis that enables rapid T cell activation, proliferation

and antitumor function[53]. 4-1BB CARs, on the other hand, signal through TRAF and have slower activation kinetics; they rely on fatty acid oxidation and exhibit enhanced mitochondrial biogenesis, which enhances T cell longevity[53]. There is some evidence to suggest that incorporating both CD28 and 4-1BB in addition to CD3ζ, commonly referred to as third generation CARs, produces a synergistic combination of both properties. However, all three signaling components of the canonical CD19-28BBζ CAR may contribute to antigen-independent constitutive signaling, which likely causes premature T cell exhaustion[27].

Incorporating signaling domains from other members of the CD28 and TNF families of receptors has produced diverse results, such as distinct cytokine secretion profiles, enhanced proliferation and persistence, lower tonic signaling, and improved in vivo efficacy[52, 399, 434]. More broadly, adding functionality from other families of signaling receptors have produced promising results. For example, use of signaling domains derived from NK cells and macrophages have yielded unique advantages over the CD28 and 4-1BB-based CARs[435, 436]. Addition of a cytokine signaling (signal 3) component could also warrant further investigation. Integrating IL-2Rβ and STAT3 signaling enhanced proliferation, cytokine polyfunctionality, and cytolytic activity upon repeated antigen exposure in addition to preventing terminal differentiation[28]. Cytokine signaling is known to have a profound effect on the differentiation status of T cells, with IL-2 promoting an effector state and IL-7 and IL-15 supporting memory[437]. Signaling also influences downstream epigenetic changes that should be taken into consideration, since certain NFAT and AP-1-mediated T cell signaling pathways reinforce epigenetic changes that stabilize an exhausted state.

Although most CARs use CD3ζ to induce T cell activation, investigation of other signaling components from the TCR-CD3 complex may be warranted. A recent study reported that not all of the CD3ζ ITAMs are required for optimal signaling in a CD19-28ζ CAR and, in fact, a CAR that only contains the membrane proximal ITAM outperformed CARs with all three[57]; additionally, they found that the distance of the ITAM from the membrane was crucial for function. Moreover, fine tuning ITAM multiplicity has demonstrated enhanced control over potency and selectivity to ligand density, with higher multiplicity resulting in an increased proportion of T cells being activated at lower antigen concentrations[44].

Duong and colleagues reported on high throughput screening of ISDs almost a decade ago[413]. Fourteen signaling domains were linked in random order and number into an HER2-specific CAR, generating a library of an estimated size of 10^8 elements. However, diversity was severely reduced in their Jurkat library ($3x10^4$), presumably due to a bias towards smaller inserts. Two rounds of stimulation through a myc-tag in the CAR and single-cell sorting for high CD69 expression identified 39 clones that showed response to antigen. Analyzing those hits for IL-2 production upon stimulation with c-myc antibody or HER2-expressing target cells revealed that the DAP10-CD3ζ-CD27 ISD combination generated the highest responses.

Although in vitro cytotoxicity was comparable to a CD28-CD3ζ control, in vivo tumor control of a subcutaneous tumor mouse model was significantly better.

Since this initial report, there were no new high throughput ISD screening studies published until 2022. In the span of a few months, multiple groups reported their findings with each their unique approach. Gordon et al. employed a pooled screening method to assemble a 700,000 member library of third generation CD19-targeted CARs comprising 87 different signaling domains and identify CARs that exhibited increased CD69 upregulation and/or decreased PD-1 expression in Jurkat T cells upon 3 rounds of serial antigen challenge with recombinant human CD19[412]. The enriched pool showed substantial enrichment of ITAM-containing and co-stimulatory domains whereas inhibitory domains were relatively de-enriched. Two hits from this screen were selected for extensive characterization. In human primary T cells, the first—harboring CD40, CD3ε ITAM, and DAP12 signaling domains—showed enhanced cytotoxicity, increased polyfunctional cytokine secretion and stemness, lower exhaustion, and better long term persistence, proliferation, and tumor control in vitro upon rechallenge than BBζ control. It was also the most transcriptionally distinct upon single cell sequencing. However, it showed similar tumor control in an in vivo xenograft model of B-ALL. The other—composed of FceR1y, OX40 and CD3ζ ITAM3 signaling domains—performed similarly to BBζ in vitro by all metrics but showed better tumor control in an in vivo model of $CD19^+$ melanoma, as was predicted by single cell sequencing gene sets.

Goodman et al. subjected a library of second generation CD19-targeted CARs utilizing one of 40 different co-stimulatory domains expressed in human primary CD4+ or CD8+ T cells to 11 challenges and subsequently selected for CD69 upregulation, IFNy or IL-2 secretion, or CellTrace dilution as a proxy for proliferation[411]. They identified BAFF-R as a highly cytotoxic hit with high IFNy secretion and an innate NK-like phenotype that showed lower proclivity to exhaustion and robust memory formation. Meanwhile CD28 and TACI showed higher degrees of tonic signaling and basal proliferation, while CD40 exhibited the lowest degree of nonspecific proliferation and outperformed 4-1BB in long term expansion upon rechallenge. PCA analysis divided the CAR domains into groups that showed 1) slow kinetics and robust long term performance, 2) faster activation kinetics and poor persistence, and 3) better long term killing, CD8 survival, and less terminal differentiation and long term contraction. The study also showed that increasing the distance of CD3ζ from the transmembrane domain decreased early activation and tonic signaling. On the other hand, they identified KLRG1 as a robust inhibitory receptor that maintains a naive state. Upon testing their novel BAFF-R CARs in a TRAC locus targeted system, these CARs performed similarly to BBζ control in a $CD19^+$ mesothelioma model but showed more robust tumor control in a $BCMA^+$ multiple myeloma model at the minimally effective dose for BBζ.

Daniels et al. sought to predict CAR design rules for increased cytotoxicity and stemness by training a neural network on enrichment data following 3 antigen challenges of human primary T cells expressing an arrayed library of 2379 CARs in which 13 signaling motifs were shuffled into 3 positions between the transmembrane domain and a CD3ζ signaling domain[414]. They determined that the PLCy-mediated LAT signaling motif and TRAF-mediated CD40 and LMP1 signaling motifs elicited high levels of activation and cytotoxicity, with the latter showing increased stemness. Design principles were established by rank ordering the CARs by cytotoxicity or stemness phenotype and then analyzing the resulting distribution after filtering for a particular motif to determine its contribution to a particular phenotype, spatial preference, and synergistic function with other motifs. This revealed that a combination of TRAF and PLCy1 binding domains produced high cytotoxicity and stemness, and predicted that the PLCy1 domain would improve 4-1BB but not CD28-bearing CARs due to complementary versus redundant signaling pathways, respectively. Addition of two PLCy1 binding domains to the 4-1BB second generation CAR was found to improve tumor control in a xenograft model of B-ALL.

To better probe functional states that capture many dimensions of effector response, Castellanos-Rueda et al. conducted single-cell RNA sequencing of primary T cells expressing a 180-member combinatorial library that incorporated two pools of ISD domains: 1) a membrane proximal pool of 15 co-stimulatory and inhibitory domains and 2) a set of single ITAM-containing domains, respectively, followed by the two membrane distal ITAMs of CD3ζ[410]. Following tumor cell co-cultures, unsupervised clustering of the transcriptional data revealed 13 unique clusters. CAR-induced clusters (CICs) were those that were relatively de-enriched in TCR-negative and unstimulated 28ζ controls, and pseudobulked PCR analysis revealed that low enrichment in CICs was correlated with memory and resting genes whereas high enrichment correlated to cytotoxic and effector genes. Ten CAR variants were selected from different phenotypic clusters to characterize relative to 28ζ and BBz. All showed activation with no significant differences in exhaustion and all showed tumor cell killing at low tumor burden, albeit with varying kinetics in vitro. Notably, 4 CAR ICD combinations (FCRL6-CD3G, CD28-FCGR2A, 4-1BB-FCER1G, and FCRL6-FCGR2A followed by truncated CD3ζ) showed potent and even enhanced tumor control at low E:T ratios. The selected CARs also showed distinct cytokine secretion profiles, though 28ζ showed the highest levels of proinflammatory cytokine secretion. In particular, CD79B was prominent in the transcriptional data—potentially owing to a proliferative advantage—while Fc or Fc-like receptors were enriched in CICs; meanwhile, CD28 and 4-1BB were enriched but individual CARs harboring these domains generally showed poor effector function both in the transcriptional data and in functional assays.

Future prospects and limitations

CARs have shown incredible efficacy in treating B-cell acute lymphoblastic leukemia, large B-cell lymphoma and multiple myeloma, but have yet to show meaningful translation to other cancer indications. Hypothesis-driven approaches to improve these therapies often rely on rational design of a handful of novel CARs followed by characterization which can be costly in terms of time, effort, and resources. Furthermore, the rules of CAR design are often not intuitive. In an effort to expedite the process of discovering next-generation CAR designs while also establishing CAR design principles, many groups have begun taking a more unbiased approach in which many potential CARs varying in some aspects of its architecture are screened simultaneously for function. This has produced several exciting new CAR compositions that show efficacy in preclinical studies, as well as identified new groups of CAR signaling domains and uncovered their impact on T cell function. Selection data has shown the capacity to power machine learning algorithms that could predict CAR function, with the potential to expand such principles to consider cancer genotype and patient specific factors such as gender, age and medical history.

Despite significant progress within this approach, some limitations should be highlighted. Present high throughput screening methodologies necessitate either the direct sequencing of domain pools or the establishment of a barcode-domain look-up table through long-read sequencing. Unfortunately, this approach can result in the loss of low-frequency library members after bottlenecking[415]. An alternative method involves serial barcoded DNA assembly, which streamlines the identification of unique library elements using only short-read sequencing techniques[415]. Nevertheless, the challenge persists in maintaining consistent barcode identities throughout the processes of cloning and viral transduction as viral vectors exhibit a susceptibility to recombination events during replication, giving rise to erroneous barcode-element combinations[438]. The utilization of viral vectors in CAR-T-cell therapy has sparked an ongoing debate, particularly in the wake of a rising number of reports on secondary malignancies post-treatment, potentially attributed to random integration events[439]. This indiscriminate integration into the genome also exerts an impact on gene expression profiles, resulting in more heterogeneous therapeutic products[440]. Non-viral, integrating cell engineering methods, exemplified by CRISPR-Cas9, present a highly specific and efficient means of generating safer and more uniform therapeutic products. For high throughput screening campaigns, these modification strategies ensure singular integration events within a predefined genomic locus, thereby mitigating confounding factors that might otherwise affect CAR performance[441, 442]. Additionally, CRISPR-based knock-in protocols have become highly efficient, obtaining around 40% CAR-expressing T cells, though cell yield and viability remain a point of attention due to the harsher transfection methods used[442]. Current high throughput CAR screening strategies employ a relatively simple selection scheme assaying a narrow part of T cell responses to CAR signaling, often based on target binding or T-cell activation. However, selecting for the best performing domains for one property does not necessarily guarantee the best performance

overall. Integrating a broader spectrum of readouts, such as cytokine release, proliferation, differentiation, degranulation or synapse formation, would provide a more complete view on CAR domain characteristics in vitro. An additional layer of information on CAR-T-cell migration, tumor infiltration, expansion, persistence and toxicity could be extracted from in vivo pooled screens. These multiparametric strategies would be particularly useful when evaluating combinatorial libraries of different CAR domains. While the focus is currently on unraveling the intricacies of each CAR domain in isolation, it is likely that that co-dependencies between CAR domains exist. For example, antigen-ABD interactions can be influenced by the choice of the HD[42, 396], CAR functionality can change based on the HD-TMD combination[415], and T-cell activation may be affected by TMD-CSD pairs[52]. The exponential increase in library size for combinatorial libraries introduces another hurdle. Currently, library size is often limited to 10^5-10^6 elements in mammalian cell screens to remain practical and maintain a decent library coverage. As datasets and computational methods continue to develop, it is possible that machine learning-aided sublibrary design could prove sufficient to train models that can predict outcomes of larger CAR domain libraries and expand the scope of the physical assays.

The application of the discussed high throughput screening and computational methods can certainly be expanded. For example, library construction can be designed to enhance any aspect of the CAR structure or similar proteins of interest as it relates to cell function, including length and rigidity of structural elements such as the HD and linkers, or mutation of individual residues within a binding or signaling domain. In addition, multicell per well screens could identify synergistic combinations of CAR therapies. CARs can also be screened in vehicles beyond T cells such as macrophages and NK cells, which may have advantages in solid tumor infiltration and allogeneic cell transfer, respectively[443, 444]. Similarly, most publications to date rely on healthy donor PBMCs rather than those derived from often heavily pre-treated patients. Finally, amino acids, sequence motifs and protein domains could be viewed as a natural language, opening the door for using deep-learning large language models for the de novo design of protein sequences with any given characteristics or functions[445]. For a comprehensive discussion on how AI can advance cellular therapies, including CAR-T-cell therapy, we refer to a review by Caponni and Daniels[446].

Conclusion

It is clear that the field has only just begun to unlock the potential of high throughput screening of CARs, with major implications in translating CAR-T cells to diseases beyond hematological malignancies such as solid tumors, autoimmunity and infectious diseases. We envision that the influx of novel domains in the CAR toolbox and the improved understanding of CAR design could fuel major advancements in personalized medicine as CAR therapies can be adjusted to challenges faced in different indications.

Acknowledgements

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K.S.G. is currently an employee and equity holder of Gingko Bioworks. M.E.B. is the inventor on patent application PCT/US2020/017794 and is a founder, consultant and equity holder of Kelonia Therapeutics and Abata Therapeutics.

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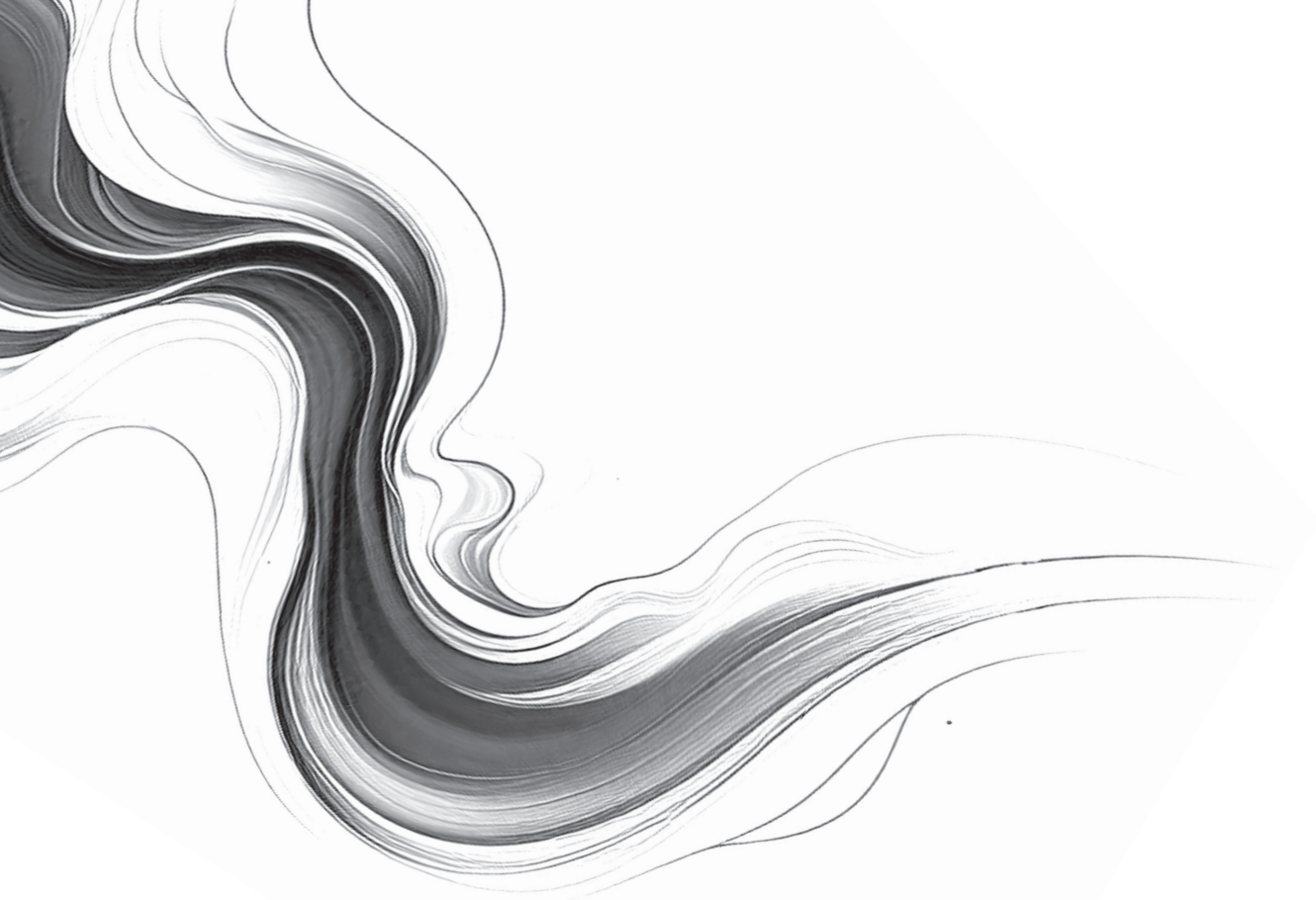

7

BULK HIGH THROUGHPUT SCREENING OF CAR HINGE DOMAINS

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Unpublished data

Research visit at the Birnbaum Lab (Massachusetts Institute of Technology, Boston, USA).
September 2022 – February 2023

Abstract

The molecular composition of chimeric antigen receptors (CARs) is a major determining factor of clinical outcome- to CAR-T-cell therapy. There is enormous potential in the large variety of proteins from which CAR domains can be derived. For the majority of the currently available CAR-T-cell products, the CAR backbone is built from only five proteins: IgG4, CD8, CD28, 4-1BB, and CD3z. Low-throughput, hypothesis-driven tests of novel domains are costly in time, effort and resources, and do not per se lead to improved outcomes. Recently developed high throughput screening (HTS) workflows facilitate simultaneous evaluation of up to 700,000 candidate domains, accelerating the discovery and characterization of novel CAR formats. Thus far, the field has focused mainly on intracellular CAR components, neglecting extracellular domains that have been shown to be crucial for CAR function, such as the hinge domain. In this explorative pilot study, we adapted a scalable HTS approach to accommodate screening of a medium size hinge domain library. We found that an activation-based selection procedure is feasible and that strict selection criteria are likely to result in faster convergence towards lead candidates. These findings provide the foundation for a confirmative study to further support lead candidate selection.

Introduction

In chimeric antigen receptor (CAR)-T-cell therapy, T cells are armed with a synthetic receptor capable of recognizing cell surface antigens resulting in the activation of the T cell and elimination of the target cell. CAR-T-cell therapy has revolutionized the treatment of relapsed/refractory B-cell hematological malignancies, where patients reached remarkable clinical responses with a single dose of CAR-T cells and remained disease-free for years following treatment[447]. Nevertheless, the relapse frequency after CAR-T-cell treatment remains high[54, 59, 447]

The composition of the CAR is known to be foundational to the strength and the longevity of the CAR-T-cell response. In this context, the intracellular co-stimulatory domain (CSD) has garnered much attention[28]. Indeed, inclusion of CD28 or 4-1BB co-stimulation into the CAR construct has markedly improved CAR-T-cell in vivo potency and persistence by modulating the cell metabolism[39, 45, 53]. Evaluation of alternative CSDs has demonstrated improved cytotoxicity, proliferation, persistence, memory formation and exhaustion resistance, which is also dependent on the spatial configuration[29, 412]. However, the progress in discovery and characterization of candidate domains has been slow as only a handful of candidates could be simultaneously assessed. More recently developed high throughput screening (HTS) strategies facilitate evaluation of large combinatorial libraries of CAR and have revealed unexpected novel CSD combinations[410-412, 414].

In comparison to the CSD, the hinge domain has received considerably less attention. Although it was long viewed as a mere structural element, it was found that the hinge plays a role in epitope binding efficiency, CAR multimerization and the antigen density threshold[448]. As such, it alters antigen sensitivity, T-cell fitness and signal transduction. In the past few years, the limited collection of established HD derived from CD8α, IgG or CD28 was slightly expanded with domains derived from IgD, CD34, LNGFR and other Siglec family members, though superior candidates have yet to be identified[41, 46-48, 397, 449].

Here, we adapted an HTS workflow for CAR CSDs developed by the Birnbaum Lab (Massachusetts Institute of Technology, Boston, USA)[412] to accommodate the evaluation and selection of novel HDs from a medium size HD library. We hypothesized that a similar activation-based selection approach could be used for HDs as HDs influence epitope binding interactions and signal transduction.

Methods

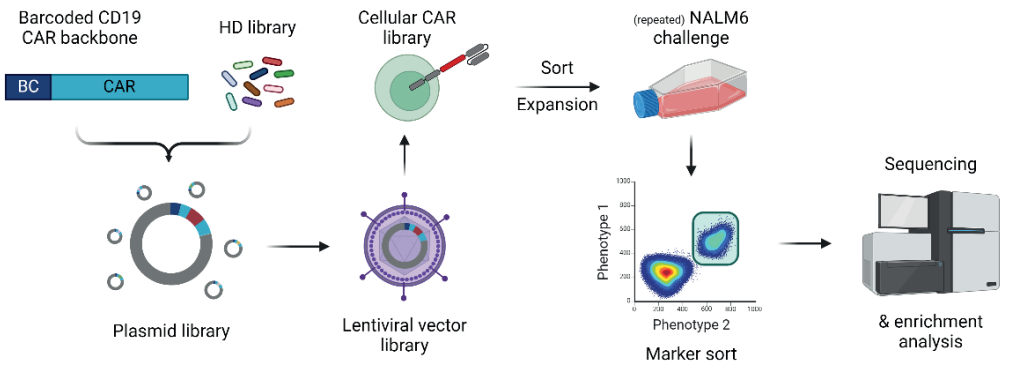

Figure 13. Overview of the workflow of this pooled screening approach for CAR HDs. HD library elements were pooled and cloned into a viral plasmid vector (plasmid library). Subsequent viral vector production and viral transduction results in a cellular CAR library. This pool of CAR-bearing cells is repeatedly challenged with target cells. Through an activation-based sort, CARs inducing high levels of activation markers are enriched. Enrichment analysis is performed based on CAR barcode counting by sequencing. Abbreviations: BC, barcode; CAR, chimeric antigen receptor; HD, hinge domain

Primary cells, cell lines and culture conditions

Clone G5 NALM6 (CRL-3273), Clone E6-1 Jurkat (TIB-152) and HEK293T (CRL-3216) cells were purchased from the American Type Culture Collection (ATCC). Jurkat-76 (J76) cells were a kind gift from Dr. Mirjam Heemskerk (Leiden University Medical Center, Leiden, The Netherlands) and modified to stably express CD8, the 1G4 TCR and a NFAT-controlled CFP reporter gene (further referred to as “Jurkat cells”). NALM6 and Jurkat cells were maintained in RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin (pen/strep; Corning). HEK293T cells were grown in DMEM (ATCC) with 10% FBS, 100 U/mL pen/strep and 25 mM HEPES (Fisher Chemical). All cell lines were maintained in logarithmic growth phase at 37 °C in a humidified atmosphere supplemented with 5% CO_2.

In silico hinge domain library generation

The basis of our HD library was built through manual curation of hinge sequences used in commercially available CAR-T-cell products and literature. Besides the fact that HDs are usually derived from the extracellular portion of a donor protein, no guidelines on hinge design are available. Additional HDs were mined from the UniProt database using a proprietary multi-step selection method to identify potential hinge domains from natural proteins.

Library construction

The envelope vector pMD2.G (VSV-G pseudotype) and the packaging plasmid psPAX2 were gifted by Didier Trono (Addgene plasmid #12259 and #12260), and the transfer plasmid pHIV-

EGFP was a gift from Bryan Welk & Zena Werb (Addgene plasmid #21373). A codon-optimized CD19 CAR comprising from 5' to 3' a Flag-tag, a 47G-4 scFv (patent US20100104509A1), a LacZ-encoding cloning site for the hinge domain, CD8α transmembrane domain and 4-1BBζ intracellular domain (based on [450]; Figure S8, Sequence S1) was cloned into the third generation lentiviral transfer vector pHIV-EGFP using Gibson Assembly, forming pHIV-CAR19LacZ. A 12 nucleotide random barcode was added to the 3'-end of the EF-1α promotor region of the vector using PCR and subsequently subcloned into pHIV-CAR19LacZ via XhoI/XbaI (Thermo Scientific) restriction digest and ligation (T4 DNA Ligase, Thermo Scientific) to produce the backbone vector pHIV-5'BC-CAR19LacZ. Hinge library elements were de novo synthesized (IDT and Twist Biosciences), pooled into three "Hinge Pools" (HP1-3) depending on hinge length at equimolar ratios, and subcloned into the backbone using the BsmBI-v2 NEBridge Golden Gate Assembly Kit (New England Biolabs)(Figure 13). To achieve a highly diverse library, the cloning product was electroporated into DH10ß electrocompetent *E. coli* cells (Thermo Scientific).

Virus production

HEK293T cells were grown to 70% confluency and transfected with transfer plasmid, psPAX2 and pMD2.g plasmid at a 28:15:5 mass ratio. For a T225 flask, 84 µg transfer plasmid, 45 µg psPAX2 and 15 µg pMD2.g were complexed with polyethyleneimine (PEI) at a 3:1 PEI:DNA mass ratio. Medium was replaced three to six hours after transfection and viral particles were harvested after 48, 72 and 96 hours. Viral supernatant was combined, filtered through a 0.45 µm low protein binding filter, and centrifuged at 100,000xg for 1.5 hours. The pellet was dissolved in serum-free OptiMEM by overnight incubation at 4°C, aliquoted and stored at -80°C.

Viral transduction

Jurkat cells were seeded at $1x10^6$ cells/mL in 1 mL complete medium supplemented with 8 µg/mL dextran (Invitrogen) per well of a 6-well culture plate. Per HP, viral stock was added to a well to reach a multiplicity of infection (MOI) of 0.1. Cells were subsequently centrifuged at 1000xg for 1.5 hours and cultured overnight at 37°C and 5% CO_2 (Figure 13). The next day, one volume of complete medium was added and cells were incubated for another two days before assessment of transduction efficiency by flow cytometry.

Flow cytometry and cell sorting

Cells were washed with 1X PBS (Sigma-Aldrich) supplemented with 0.5% bovine serum albumin (RPI) and 2 mM EDTA. They were stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain (Thermo Scientific) following manufacturer's guidelines and incubated for 15 minutes on ice before washing again. Cells were then incubated for 15 minutes on ice with anti-CD69 APC (clone FN50) or anti-Flag APC (Clone L5) antibodies purchased from Biolegend. Cells were acquired on a BD Accuri C6 or sorted with a BD FACSAria II (Figure 13).

Selection assay

All three hinge pool-transduced conditions were combined to maintain equal representation based on EGFP expression. Subsequently, cells were sorted based on EGFP expression to generate the HP_{EGFP} Jurkat cell line. For a round of selection, cells were challenged with NALM6 cells at a 1:3 effector-to-target (E:T) ratio for 24 hours and then stained for CD69 expression. Activated cells were identified and sorted as $CD69^+$/cyan fluorescent protein $(CFP)^+$ relative to the unstimulated control. Auto-activated cells were either included (relaxed selection; $pACT_{relaxed}$ Jurkat) or excluded (stringent selection; $pACT_{stringent}$ Jurkat). Sorted cells were rested without antigen and expanded for at least 5 days before subsequent rounds of selection. At least $1x10^6$ cells were sampled for NGS sequencing.

PacBio and Illumina sequencing

Sequencing was performed as described before[412]. Briefly, the PureLink Genomic DNA Mini Kit (Thermo Fisher) was used to isolate genomic DNA from selected conditions. PCR amplicons of the 5'-barcode to the hinge domain region were linked to SMRTbell adaptors using the SMRTbell Template Prep Kit 1.0 (Pacific Biosciences) and sequenced using a PacBio Sequel system. In addition, 5'-barcode regions were PCR amplified to conjugate P5 and P7 adaptor sequences and were sequenced on an Illumina MISeq system (Figure 13).

Results

In silico design of a HD library

As summarized in Table 9, 14 unique natural proteins serving as hinge sources were identified from literature. Besides immunoglobulin (Ig)-derived hinges (e.g., IgG1 and IgG4 [37, 132, 400, 451-456]), many of the currently used HDs contain protein subdomains, such as immunoglobulin (Ig)-like V-type (CD8α, CD28[45]), Ig-like C-type (e.g., CD33 and SIGL7 [47]) and cysteine-rich or pattern repeats (e.g., CD7 and CD137 [397, 425]), though some are categorized as disordered (e.g., CD34 and CD137). With the aim of discovering novel hinges, we performed a proprietary multi-step selection method in the UniProt database and defined 1805 HDs from various natural proteins.

Table 9. Summary of hinge domains identified from literature.

Ref	UniProt Accession	Donor protein	Type	Length [#AA]
[26, 43, 44, 51, 52, 135, 457]	P01732	CD8	Ig-like V-type	43-58
[43, 44, 51, 52]	P10747	CD28	Ig-like V-type	39
[451, 452, 454, 455]	P01857	IgG1	Ig	12-232
[456]	P01859	IgG2	Ig	12-228
[37, 132, 395, 400, 453, 455]	P01861	IgG4	Ig	12-240
[41]	P01880	IgD	Ig	103
[46]	P228906	CD34	Disordered	16-179
[47]	P20138	CD33	Ig-like C2-type	115
[47]	P20916	MAG	Ig-like C2-type	280
[47]	Q9Y286	SIGL7	Ig-like C2-type	204
[47]	Q9NYZ4	SIGL8	Ig-like C2-type	120
[48, 294]	P08138	LNGFR	Cysteine-rich repeats	162-222
[425]	P09564	CD7	Pattern repeats	36
[397]	Q07011	CD137	Disordered and Cysteine-rich repeats	27-163

Abbreviations: AA, amino acids; Ig, immunoglobulin; Ref, reference.

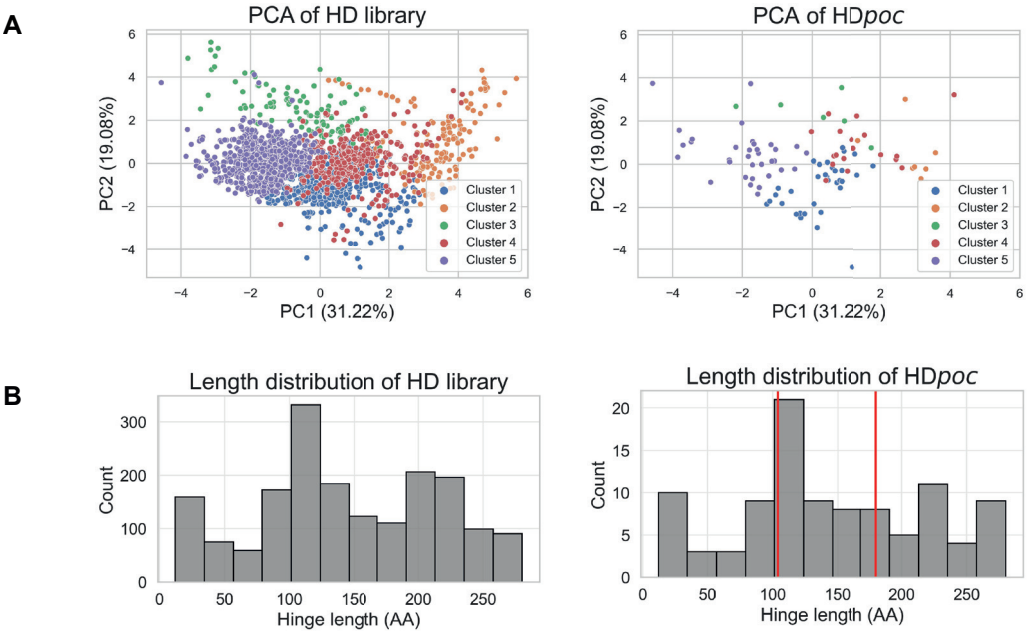

Figure 14. Hinge domain library creation. (A) Principle component analysis and k-means segmentation in five clusters of the full, 1805 elements large, HD library (left) and semi-random selection of 100 elements for the sublibrary HD_{poc} (right). (B) Size distribution of the full HD library (left) and the HD_{poc} sublibrary (right). HD_{poc} was split into three equal hinge pools (HP) based on length with cut-offs indicated with a red vertical line.

Principle component analysis (PCA) and segmentation using K-means clustering of this set of HDs revealed five clusters (Figure 14A). To achieve economic feasibility of an exploratory pilot screening campaign of HDs, 100 hinges were selected in total (called HD_{poc}), maintaining weighted representation of each cluster and subdomain type. PCA outliers might also result in outsized functional effects. Therefore, 25% of selected hinges were outliers and 75% were selected randomly. Importantly, the size distribution of HD_{poc} was highly similar to the full set of hinges (Figure 14B). Finally, 25 hinge variations of different lengths and subdomain types from HDs in literature (Table 9) were appended to HD_{poc} to form a final library of 125 candidate HDs.

Baseline distribution of HD library in transduced Jurkat cells is skewed

To minimize the impact of the length differences on the efficiency of cloning, viral packaging and viral transduction, HD_{poc} was divided into three sublibraries of equal size, called "hinge pool" (HP) 1-3, with lengths between 12-104 amino acids (AA), 105-180 AA and 181-280 AA (Figure 14B, right). The hinge pools were subcloned into a barcoded lentiviral vector containing bicistronically encoded second generation CD19-specific CAR and EGFP as detailed in the methodology. Despite our best efforts, increasing hinge length resulted in decreasing transduction efficiencies (Figure 15A). Of note, up to 70% of the total transduced cell population expressed the EGFP reporter while the CAR was not detectable ($EGFP^+CAR^-$). Indeed, HP1, HP2 and HP3 had a $EGFP^+CAR^-$:$EFGP^+CAR^+$ proportion of 25:75, 56:44 and 70:30, respectively. After combining HP Jurkat cells and sorting sequentially for EGFP (HP_{EGFP} Jurkat) and CAR^+ (HP_{CAR} Jurkat), we detected 93.6% (117/125) of hinges in the HP_{CAR} Jurkat library by sequencing, ruling out that misfolding of CARs was the cause for lack of CAR expression (Figure S6). The backbone vector for library cloning contains a LacZ gene with a premature stop codon. Contamination of the library with this vector could explain the $EGFP^+CAR^-$ population, but the absence of a LacZ signal when sequencing HP_{EGFP} Jurkat cells does not support this hypothesis (Figure S6).

Looking at the baseline distribution of hinges in HP_{EGFP} Jurkats, we observed a skew towards short hinges (Figure 15B). Some library members and subdomain-types (fibronectins and cysteine-rich repeats) were considerably overrepresented, while others were underrepresented (leucin-rich repeats) or completely lost, as shown in Figure 15C and Figure S6.

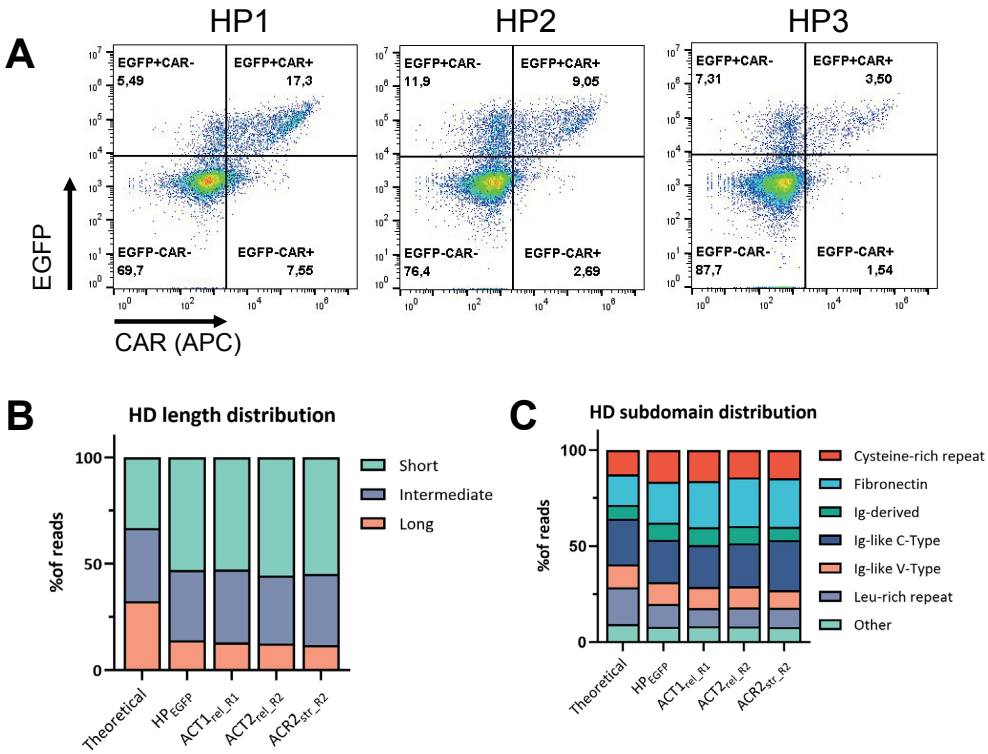

Figure 15. Evolution of the library distribution over multiple selection rounds. (A) HPs showed varying transduction efficiencies (EGFP$^+$) when transduced at a MOI of 0.1 in Jurkat E6.1 cells. (B) HD length distribution and (C) HD subdomain distribution for each selection round. Both show a skew in comparison to the theoretical distribution, which is maintained throughout the selections.

Stringent selection drives more pronounced enrichment or depletion of HD library members

For activation-based selections, HP_{EGFP} Jurkat cells were co-cultured at an E:T ratio of 1:3 with CD19$^+$ NALM6 target cells for 24h and subsequently sorted for the activation marker CD69 and NFAT-driven CFP expression. Non-stimulated HP_{EGFP} Jurkat cells were used as a negative gating control. During the first round of selection, there was no tonic signaling-driven upregulation of CD69 or CFP in unstimulated HP_{EGFP} Jurkat cells, allowing for a relaxed sorting gate on activated cells (ACT_{rel_R1} Jurkat) spanning almost two decades in each fluorescent channel (Figure S7, top). In contrast, on the second selection round, unstimulated ACT_{rel_R1} Jurkat cells had some basal activation level. Therefore, we splitted the stimulated ACT_{rel_R1} Jurkat cells into two groups where one group was sorted based on the relaxed gating strategy used in the first selection round (ACT_{rel_R2} Jurkat; Figure S7, middle) and the other group was sorted with a stringent gating strategy (ACT_{str_R2} Jurkat), excluding any tonic signaling constructs (Figure S7, bottom).

Following sorting, we PCR-amplified hinge regions and/or barcodes from genomic DNA of each of the selection rounds and counted the number of barcode-HD pairs by sequencing. Notably, HD length and subdomain distribution remained relatively unchanged across selection rounds (Figure 15B & Figure 15C). Quality control analysis revealed that the number of unique barcodes per HD (uBCs/HD) was not equally distributed across the library (Figure 16A&B). Our intended barcode coverage of the library was 400X, but we achieved 303±448 uBCs/HD (mean±SD) with a considerable number of outliers. As expected, the number of uBCs/HD strongly correlated with the number of total reads per HD (e.g., ACT_{rel_R2}, $R^2 = 0.9398$, $p < 0.0001$; Figure 16C). Therefore, it is unsurprising that a nearly identical skew towards our short hinge sublibrary was observed in terms of uBC/HD (Figure 16A). In contrast, neither uBC/HD (e.g., ACT_{rel_R2}, $R^2 = 0.02696$) or total reads per HD (e.g., ACT_{rel_R2}, $R^2 = 0.06122$) correlated with the log2-fold enrichment per HD (Figure 16D&E), suggesting that the disparity in HD barcoding does not impact the readout. Of note, the fact that the average uBC/HD decreased over different rounds demonstrates that selection is occurring (Figure 16B).

Figure 17A shows a heatmap of the Log2 fold-enrichment of HDs over the different rounds of sorting as determined by sequencing. After two rounds of relaxed sorting, only 29/125 hinges showed at least 2-fold enrichment / depletion compared to baseline. In contrast, in the alternative stringent selection applied in the second round, 12 and 29 library elements were at least 2-fold enriched and depleted, respectively, and even 1 and 9 library elements were at least 4-fold enriched or depleted. Several hinges, such as HD004, HD038, HD044 and HD095, were lost after the first selection round, though it is not unlikely they were lost due to stochasticity because of their low baseline barcode frequency (Figure S6). Notably, the selection for HD055 was improved under stringent sorting conditions compared to relaxed sorting conditions, indicating this hinge induces a stronger activation profile. In contrast, HD069 was severely penalized by more stringent selection. Another interesting case is HD090, which was strongly depleted in the first selection round and rebounded towards enrichment in the subsequent round. Looking at the enrichment of hinge subdomains and length groups, there were no significant differences over the two selection rounds (Figure 17B & Figure 17C).

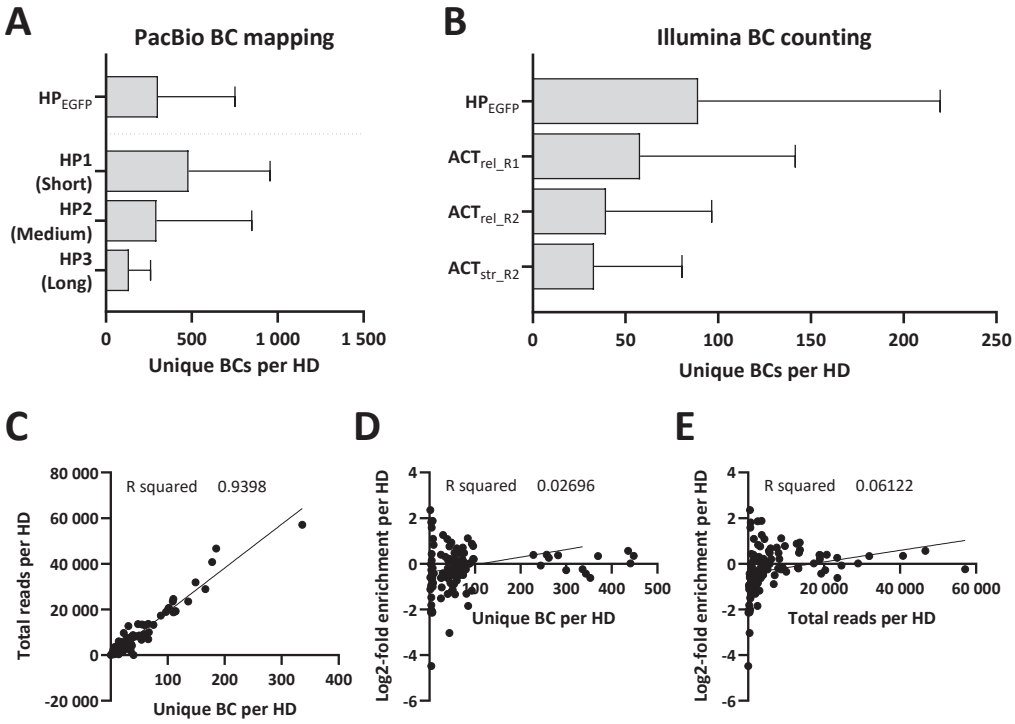

Figure 16. Barcode counting of the library across selection steps. (A) Unique BC-HD combinations identified using PacBio long-read sequencing in the baseline, EGFP-sorted HD library (HP_{EGFP}), including split between length-based hinge pools (HP1-3). (B) Unique BCs identified per HD per selection step using Illumina sequencing. Correlation between (C) total reads and unique BC per HD, (D) enrichment and unique BC per HD, and (E) enrichment and total reads per BC. Abbreviations: BC, barcode; HD, hinge domain; R#, round of selection; Rel, relaxed selection; Str, strict selection.

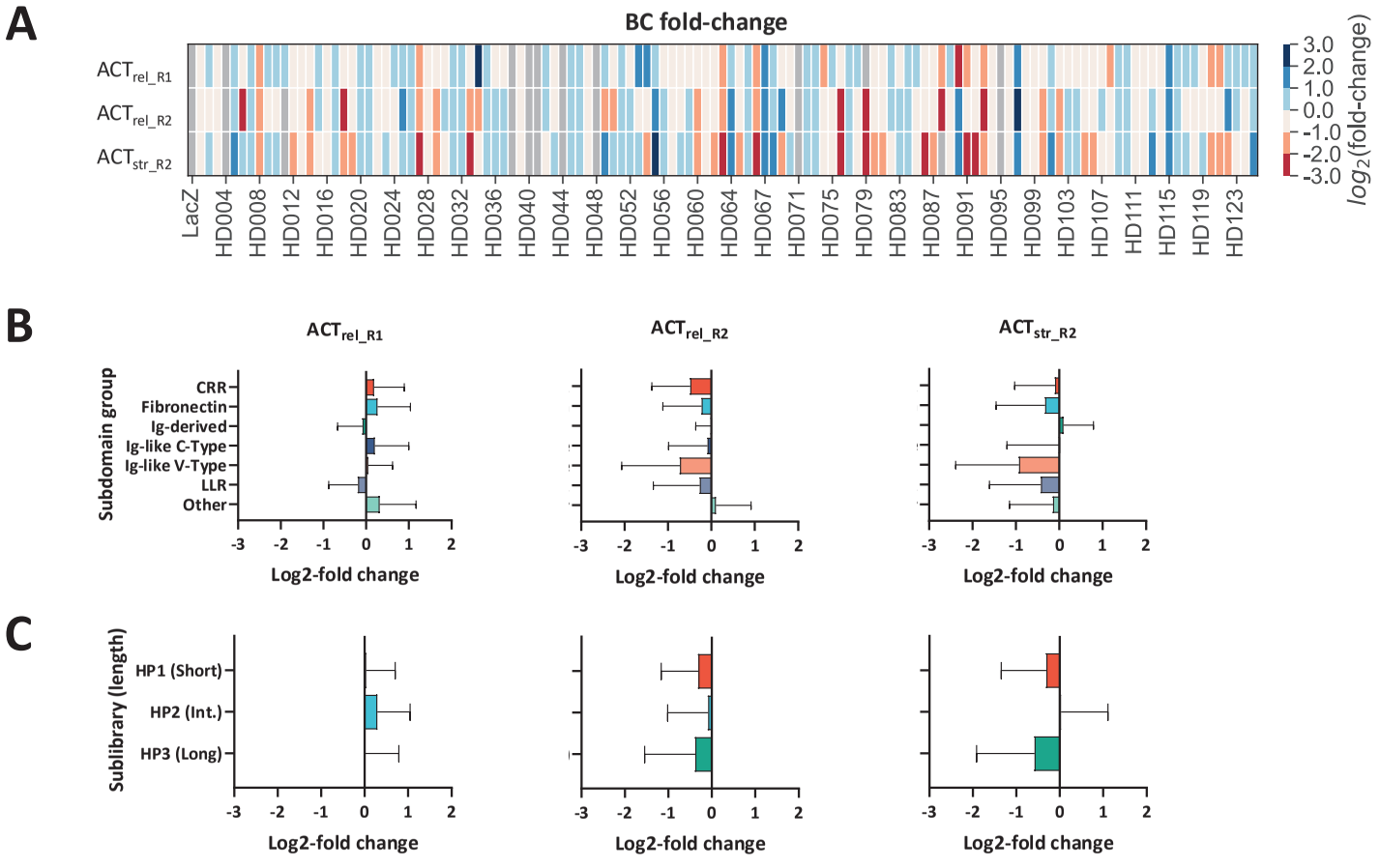

Figure 17. Enrichment of barcoded HDs over the different rounds of sorting. (A) Log2-fold enrichment (blue tints) or depletion (red tints) of HD library elements. Mean ± SD enrichment of hinge subdomain groups (B) or length-based sublibraries (C) per selection round. Abbreviations: CCR, cysteine-rich region; HP, hinge pool; Ig, immunoglobulin; Int., intermediate; LLR, leucine-rich repeat.

Discussion

CAR-T-cell therapy has become a transformative treatment for patients with B-cell lymphomas, acute lymphoblastic leukemia and multiple myeloma. The molecular design of most currently used CARs is almost standardized to a few well-studied modules derived from immune-related proteins such as IgG, CD8, CD28 and 4-1BB. Over the years, novel domains have been explored using a mostly hypothesis-driven approach, comparing a handful of new sequences against the state-of-the-art with varying degrees of success. Moving away from this time-consuming approach, multiple groups have recently reported on the development of unbiased, HTS workflows focused on the intracellular co-stimulatory and signaling domains of the CAR. This has led to the discovery of new intracellular CAR domains such as CD79B, which normally operates in the B-cell receptor complex[410], and CD40, which is the receptor of the co-stimulatory protein CD154 found mainly on CD4 T cells[412]. The approach presented by Gordon et al., named CARPOOL, appeared to be the most scalable, simultaneously assaying up to 700,000 combinations. In contrast to the intracellular domains, no extensive efforts for the discovery of other CAR domains have been reported (Chapter 6).

Here, we are the first to demonstrate the implementation of a HTS platform towards the discovery of novel CAR HDs. The CARPOOL workflow was adapted to screen a medium-sized library of 125 HDs with at least 100 HDs derived from proteins never considered before. Although this study was set up as a proof of concept, and further work is needed to solidify the results, several interesting observations were made. Despite the fact that there were no clear differences between subdomain groups, the considerable depletion of Ig-like V-type domains, akin to CD8α and CD28 HDs, is noteworthy, given their widespread use for CD19-targeted CARs[45]. However, given the very similar performance across the remaining subdomain groups, it appears that this feature has little influence on activation potency. A similar indication was found for the length-based sublibraries, although intermediate-length HDs appeared to subtly perform better for the CD19 CAR construct examined in this study. The enrichment profiles of HD055 and HD097 hint towards the importance of selection stringency as HD055 enrichment considerably improved with a stringent over a relaxed selection, whereas HD097 was more enriched under relaxed selection conditions. It appears that the magnitude of selection of HDs is lower than that of CSDs[412], which is to be expected given the less prominent role of HDs in T-cell activation compared to CSDs. It would therefore be advisable to perform additional rounds of (stringent) selection to potentially exacerbate the current observations. In this study, stringent selection criteria were also necessary because of a certain level of tonic signaling. It might be helpful to introduce at least one selection round against antigen-negative target cells to reduce background signaling to facilitate a more relaxed selection or less false positive hits.

This report involves a first implementation of the CARPOOL workflow for HD libraries and requires further optimization. The following observations can guide the future design of the full workflow.

First, we have observed a skew towards shorter hinges (HP1 sublibrary) in the HP_{EGFP} Jurkat library. As we do not have extensive sequencing data of the final sublibrary cloning products, it is impossible to rule out that this skew was already introduced during cloning. Other avenues are size-dependent differences in efficiency of viral packaging and infection. Indeed, viral packaging and infection efficiency has been shown to decrease with 10- and 2.5-fold, respectively, when the viral genome size in increased from 7kb to 10kb[458]. However, given the comparatively small viral genome size range of our sublibraries (HP1: 7.1kb-7.35kb, HP2: 7.35kb-7.6kb, and HP3: 7.6-7.9kb), we expect the size effect to be minimal. Our method for pooling the sublibraries based on total EGFP-expression has most likely caused the length skew. Following transduction, we observed that some transduced cells expressed the EGFP fluorescent marker, but not the associated CAR. More importantly, the proportion of $EGFP^+CAR^-$:$EFGP^+CAR^+$ cells dramatically differed between sublibraries: 25:75, 56:44 and 70:30 for HP1, HP2 and HP3, respectively. When our sublibraries were pooled for equal representation based on total EGFP expression, this led to a 2.5-fold bias for $EGFP^+CAR^+$ cells from HP1 versus HP3. Indeed, the theoretical proportion of $EGFP^+CAR^+$ cells of approximately 50:30:20 (HP1:HP2:HP3) based on this methodology closely resembles the observed proportions of 52.8:33.2:14 in the HP_{EGFP} Jurkat library. Future HD CARPOOL screening endeavors should therefore apply sublibrary pooling based on $\%EGFP^+CAR^+$ instead of $\%EGFP^+$ alone.

The second point of attention pertains specifically to the observation of a fraction of $EGFP^+CAR^-$ Jurkat cells. We used a bicistronic expression vector with the CAR (first gene) separated from EGFP (second gene) with an internal ribosomal entry site (IRES). As ribosome binding to the IRES is less efficient, the gene following the IRES is considerably worse translated than the first gene[459, 460]. In our case, it would be reasonable to observe CAR expression with reduced EGFP expression, though the opposite - as is the case here - is improbable. Indeed, we would expect to see a loss in library diversity in the HP_{CAR} Jurkat population if some library members disrupted proper CAR expression. Yet, we found 117/125 (93.6%) of all library members in HP_{CAR} Jurkat cells, only three fewer than in the HP_{EGFP} population as a whole (120/125; 96%). We could also not detect residual backbone vector containing the LacZ placeholder in either HP_{EGFP} and HP_{CAR} populations. Additional sequencing of the $EGFP^+CAR^-$ population would provide conclusive results in this regard. Nonetheless, incorporating a self-cleaving 2A peptide in future CARPOOL iterations would couple translation of the CAR and EGFP genes and allow for a nearly equal expression of both transgenes[461].

The third notable observation is that the number of uBCs/HD detected with PacBio sequencing of HP_{EGFP} differs considerably between library members. Unsurprisingly, this discrepancy was

maintained throughout selection rounds and may be, in part, the reason we have observed drop-outs in the first selection round of HDs with low uBCs/HD. The cause could be related to the fact that we incorporated a bottleneck during cloning to limit the theoretical barcode diversity to 400X coverage of the full library, equaling 50 000 unique barcodes. Subsequent transduction, sorting and gDNA isolation were carried out in such a way that this coverage should have been maintained. The 303±448 BCs/HD (mean±SD) of HP_{EGFP} is close to the intended 400X coverage, but there is again a notable skew towards the small HD sublibrary (481±475 uBCs/HD) and away from the long HD sublibrary (134±125 uBCs/HD). While this may be attributed to the previously discussed pooling method, we identify that 10% of HDs have more than twice the intended uBC/HD. This strengthens the case for sequencing the cloning products after assembly, before proceeding with the selection procedure. One future consideration to overcome this challenge is to bottleneck the theoretical barcode diversity to 20-40X in the final cloning step and combine this with additional post-assembly sequencing quality controls. Alternatively, combinatorial serial assembly could be adopted, as described by Rios and colleagues[415]. By barcoding each library element with a known barcode prior to library assembly, the creation of a barcode-hinge lookup table via long-read sequencing is bypassed and only barcode counting is necessary[415]. This approach would eliminate any potential PCR biases that could occur in HD libraries with larger length differences like ours.

In this report, we assessed a relatively small sublibrary of HDs. Once the CARPOOL workflow has been validated to be a practical approach for HD screening, it is possible to expand the library size to include a more diverse set of proteins of origin. However, the extent of this expansion is limited by the relatively high cost associated with the de novo synthesis of larger HD libraries. As multiple CSDs in one CAR can result in synergistic effects, building a large combinatorial library simultaneously assessing all library members at all spatial locations is a logical approach[412]. The cost per library element per datapoint decreases exponentially as combinatorial positions increase. Although it is an interesting consideration, unlike CSDs, there is currently no evidence to suggest that a multi-HD, or rather a combination of different subdomains, would result in improved functional outcomes. Focusing on the amount of data retrieved per library element would therefore be the more obvious approach. For example, assessing enrichment based on proliferation, cytokine production, and degranulation in combination with activation markers, or by studying T-cell responses through single-cell sequencing, could provide a more complete picture of each library member[410, 411]. Another option to improve the data/cost ratio is performing an initial screen of a larger HD library using CARPOOL, followed by a medium-throughput arrayed screen of lead candidates using a broader spectrum of readouts[414]. Alternatively, instead of studying HDs in isolation, novel HDs can be evaluated in combination with other CAR building blocks it is expected to be influenced by, such as the scFv or transmembrane domain[42, 415].

Conclusion

In summary, we have demonstrated the first steps into the adaptation of the CARPOOL high throughput screening workflow for HDs in the context of a CD19 CAR. Multiple challenges with regard to library cloning, transduction and selection currently prohibit making firm conclusions or the identification of clear lead candidates. Addressing these challenges in subsequent iterations of the CARPOOL workflow are necessary to improve its robustness and versatility.

Acknowledgements

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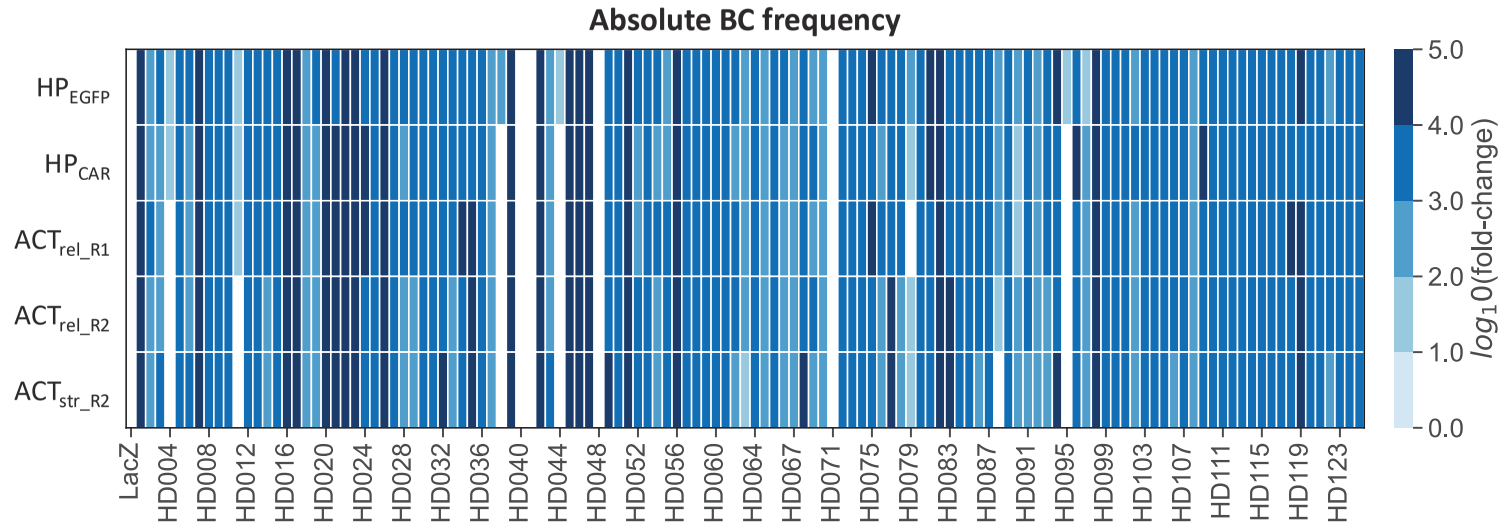


Figure S6. Absolute barcode frequency of all 125 HD$_{poc}$ library elements across selection rounds. White represents no barcode counts, light blue low counts and darker blue higher counts (log10 scale). HP$_{EGFP}$ shows the distribution before any activation-based selection took place. HP$_{CAR}$ represents HP$_{EGFP}$ sorted for CAR expression, where white datapoints demonstrate lack of CAR expression for these HDs. ACT$_{rel}$ and ACT$_{str}$ are activation-based selection rounds with relaxed or stringent selection gates, respectively. R# indicates the selection round.

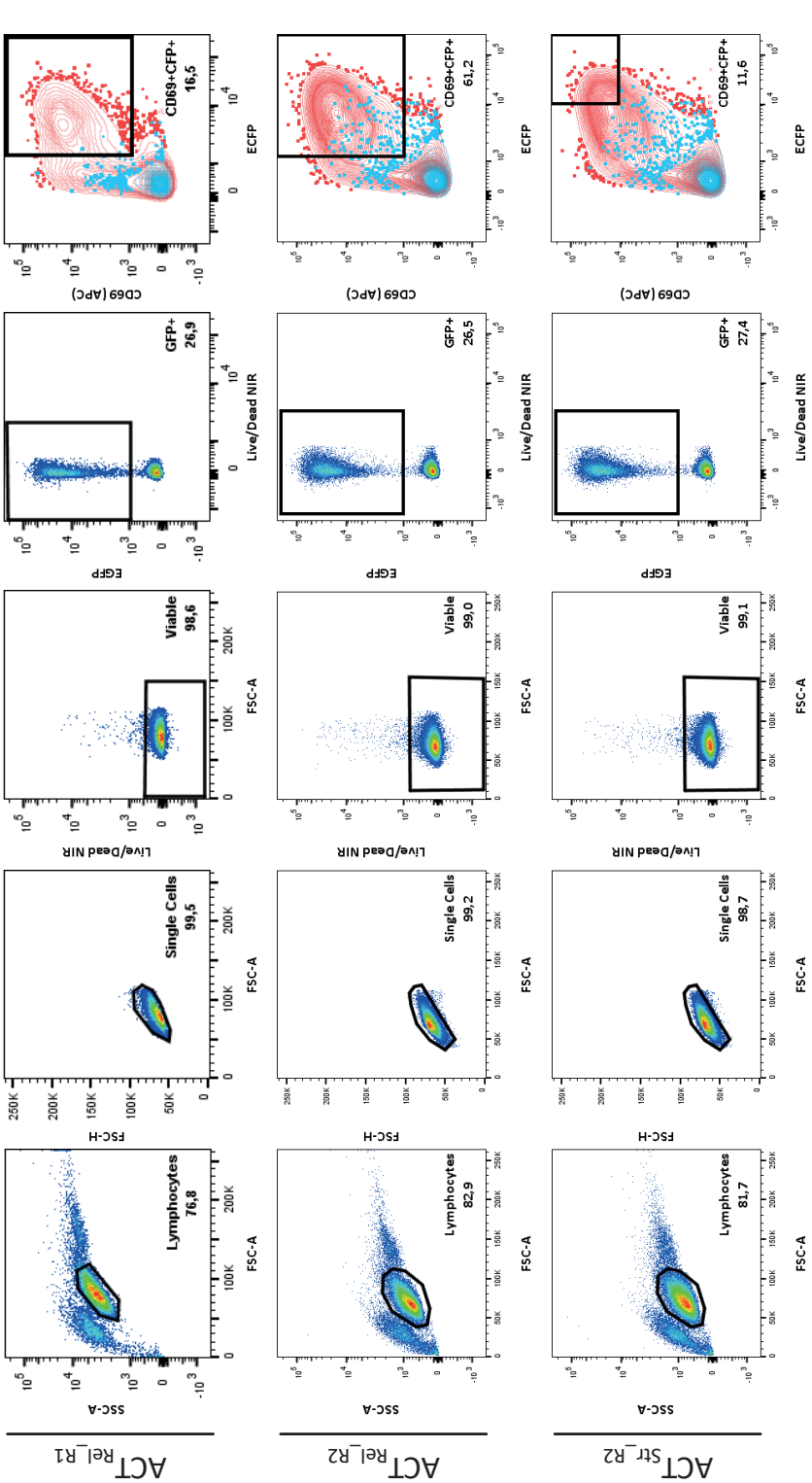


Figure S7. Gating strategy for activation-based enrichment of HD library members over two rounds. EGFP⁺ events corresponded to transduced Jurkat E6.1. CD69 and ECFP were used as activation markers. Unstimulated control (blue) is overlayed on stimulated (red) conditions in the final ECFP vs. CD69 plot. Abbreviations: ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; NIR, near-infrared; Rel_R1, relaxed gating in round 1; Rel_R2, relaxed gating in round 2; Str_R2, stringent gating in round 2.

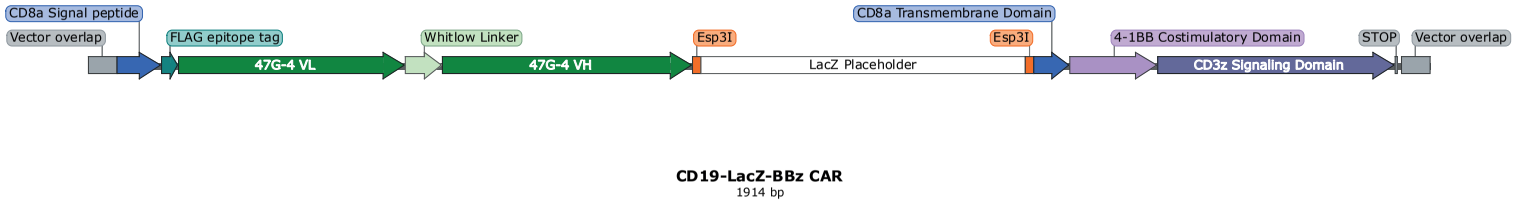
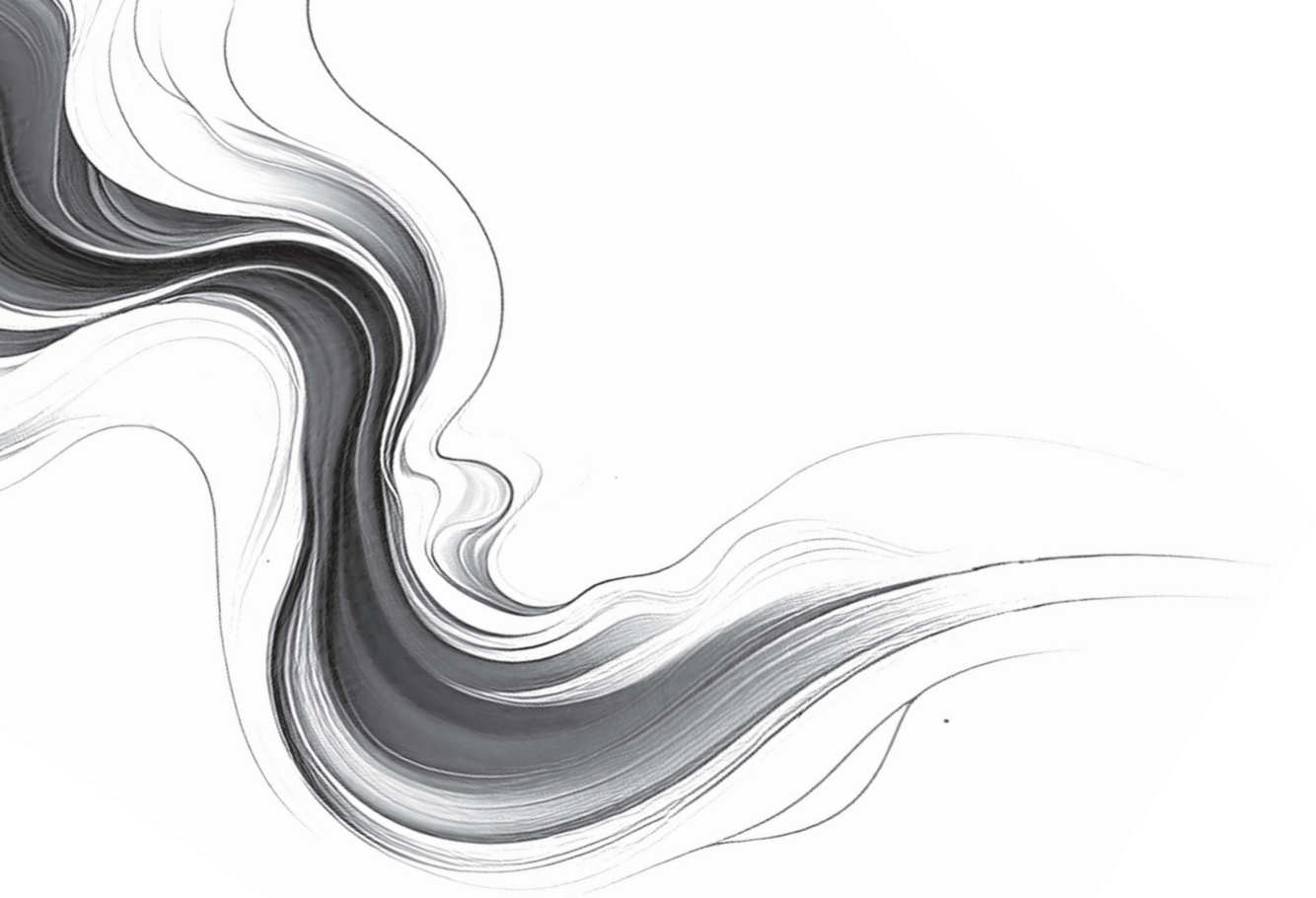


Figure S8. Map of the CD19 CAR backbone for library screening. The CD19 CAR comprises a CD8α signal peptide, a FLAG epitope tag, a CD19-specific scFv (47G-4), a LacZ placeholder flanked by outward facing Esp3I (BsmBI) restriction sites, a CD8α transmembrane domain, a 4-1BB costimulatory domain and a CD3ζ signaling domain. Vector overlaps are included to facilitate cloning in the pHIV lentiviral transfer vector.

Sequence S1. Raw sequence of the CD19 CAR backbone for library screening. Color coding matches this of Figure S8.



8

ARRAYED MEDIUM THROUGHPUT SCREENING OF CAR HINGE DOMAINS: PROTOCOL OPTIMIZATION

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Unpublished data

Abstract

Chimeric antigen receptors (CARs) are modular synthetic molecules composed of multiple protein-derived domains, each with their particular function. It is known that specific sets of CAR domains have a profound effect on therapeutic outcomes. However, as of 2024, the field's collection of domains and our understanding of how they function is severely limited. Large scale screening efforts allow exploration of the enormous diversity in properties found in natural proteins in the context of CARs. While current high throughput screening efforts are likely to provide significant expansions to the CAR domain toolbox, they are not infinitely scaleable in practice and lack deep characterization of discovered CAR domains. In contrast, arrayed screens allow for broad data generation per library element, which could be leveraged to predict outcomes for a sizeable library. Here, we report on an ongoing pilot study for the development of such arrayed screening workflow for the evaluation of novel CAR hinge domains. We highlight challenges in translating low throughput methodology to medium and high throughput workflows. The presented optimization of library cloning, plasmid and virus production, and T cell transduction and selection provide a basis for future application of the workflow on larger libraries.

Introduction

CAR-T-cell therapies have revolutionized the treatment of B-cell hematological malignancies[45]. Over the past decades, CAR design went through multiple iterations of adding or replacing building blocks by superior alternatives, depending on the intended outcome. Despite being a modular protein, the number of modules that have been studied is rather limited and, as a consequence, so is our understanding of their potential breadth of function[27]. In recent years, bulk high throughput screening has found its way to CAR-T-cells to aid in the unbiased discovery and evaluation of novel CAR domains, as reviewed in **Chapter 6**. These bulk screening campaigns are mainly reserved for scFvs or co-stimulatory domains (CSDs) and rely on the sorting-based enrichment of high performers in a chosen functional assay (e.g., activation, proliferation, cytokine production)[411, 412]. When applied to CAR domains that might induce a more subtle functional effect, such as hinge domains (HDs) or transmembrane domains (TMDs), this approach might be less successful in enriching and detecting lead candidates (as seen in **Chapter 7**). Moreover, bulk screens often employ a binary selection (in- or outside of the sorting gate), removing much of the nuance found in T-cell responses. Another challenge is that low performing library elements might persist over several rounds of selection because of dual integration with a high performing element[403], or due to paracrine effects of high performers in the bulk culture[414]. Arrayed screening offers a solution to the shortcomings of bulk screening. Although throughput can be orders of magnitude lower, individual library elements can be evaluated without the confounding factors present in bulk screens. Additionally, it is possible to obtain a continuous – instead of a binary – readout of multiple functional parameters simultaneously, providing large amounts of nuanced information per library element. That information does not only describe the element in question, but may also allow us to understand the relationship between domain sequences and functional performance, and subsequently to define generalized design rules for those CAR domains. To the best of our knowledge, there has only been one study by Daniels et al. that successfully utilized the arrayed screen approach to produce a prediction model for signaling motifs found in CAR CSDs[414]. Building further on our findings from a bulk screen of HDs (**Chapter 7**), we used the study by Daniels and colleagues as a basis to develop and fine-tune the arrayed screen workflow for HDs.

Methods

Primary cells, cell lines and culture conditions

Clone E6-1 Jurkat (TIB-152) was purchased from the American Type Culture Collection (ATCC). The Lenti-X 293T cell line was purchased from Takara Bio. The Jurkat cell line was maintained in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Gibco). The Lenti-X 293T cell line was grown in DMEM (Life Technologies) with 10% FBS

(Gibco). All cell lines were maintained in 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 following the approval by the Ethics Committee of the Antwerp University Hospital and the University of Antwerp (reference number 20/09/098). Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare), and CD8 and/or CD4 T cells were selected using human CD8 or human CD4 magnetic microbeads, respectively, for magnetic-activated cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotec).

In silico hinge domain library generation

The basis of our HD library was built through manual curation of hinge sequences used in commercially available CAR-T-cell products and literature (**Chapter 7**). Using a proprietary multi-step selection method, we identified potential hinge domains from natural proteins in the UniProt database. The 48 best and 48 worst performing CARs in a bulk high throughput screen (**Chapter 7**) were selected to maximize the expected functional diversity for future machine learning applications and to facilitate arrayed screening in 96-well plate format.

Library construction

The envelope vector pMD2.G (VSV-G pseudotype) and the packaging plasmid psPAX2 were gifted by Didier Trono (Addgene plasmid #12259 and #12260. A codon-optimized CD19 CAR comprising from 5' to 3' a Flag-tag, a 47G-4 scFv (patent US20100104509A1), LacZ encoding placeholder for the HD, CD8α TMD and 4-1BBζ intracellular domain (based on [450]; Figure S8, Sequence S1) was de novo synthesized and cloned upstream of a T2A-Puromycin resistance gene into the lentiviral transfer vector pLV by VectorBuilder, forming pLV-CAR19LacZ. Hinge library elements were de novo synthesized (IDT and Twist Biosciences) and subcloned into the backbone using the BsmBI-v2 NEBridge Golden Gate Assembly Kit (New England Biolabs) in an array of up to 96 wells in a PCR plate. Final products were transformed into NEB Stable Competent *E. coli* (New England Biolabs) in a 96 well PCR plate and plated on 6-well culture plates containing LB agar (Sigma-Aldrich) supplemented with 100 µg/mL carbenicillin, 1 mM IPTG and 80 µg/mL X-Gal (all three Thermo Scientific). Single white colonies were picked and grown in lysogeny broth (LB) or terrific broth (TB) (both Sigma-Aldrich) with 100 µg/mL carbenicillin under the indicated conditions. Subsequent plasmid isolation was performed using the NucleoSpin Plasmid Transfection-grade kit (Macherey-Nagel). Sequences were confirmed through Nanopore sequencing by the Neuromics Support Facility (VIB, University of Antwerp, Belgium).

Virus production

The day before transfection, Lenti-X 293T cells were seeded in a 96-well flat-bottom culture plate in IMDM with 10% FBS to reach 50-80% confluency after 24h. For primary T-cell transduction, the medium was additionally supplemented with 1 µg/mL NA/LE anti-CD28 (BD), 50 IU/mL IL-2 (ImmunoTools) and 10 ng/mL IL-15 (ImmunoTools). Transfer plasmid, psPAX2 and

pMD2.g were mixed at a 28:15:5 mass ratio and complexed with polyethyleneimine (PEI) at a 3:1 PEI:DNA mass ratio or Lipofectamine 3000 (Thermo Scientific) according to manufacturer's instructions. Medium was replaced three to six hours after transfection. Virus was harvested after 48 hours and optionally 72 hours, and transferred to the target T cells immediately.

Viral transduction

Jurkat E6-1 cells were seeded at $1x10^6$ cells/mL in complete medium supplemented with 8 µg/mL dextran (Invitrogen) per well of a 96-well flat-bottom culture plate. Indicated volumes of viral supernatant were transferred from the viral production plate to the culture plate. Cells were subsequently centrifuged at 1000xg for 1.5 hours and cultured overnight at 37°C and 5% CO_2. The next day, half of the volume was replaced with fresh complete medium and cells were incubated for another two days before assessment of transduction efficiency by flow cytometry. For primary human T cells, a pre-activation step is required. A 96-well flat-bottom culture plate was coated overnight at 4°C with 50 µL/well of 5 µg/mL NA/LE anti-CD3 antibody (BD). The 96-well plate was washed thrice with 1X PBS. Subsequently, T cells were resuspended in 200 µL complete medium per well, supplemented with 1 µg/mL NA/LE anti-CD28, 50 IU/mL IL-2 and 10 ng/mL IL-15. After 24h pre-activation, up to half of the culture medium was removed and replaced by viral supernatant to reach a final volume of 200 µL. Dextran was added at a concentration of 8 µg/mL in each well. Transduced primary T cells were incubated at 37°C, 5% CO_2 for three days prior to assessment of transduction efficiency by flow cytometry. To enrich for CAR-expressing T cells, cells were incubated in the presence of puromycin at indicated concentrations and durations.

Flow cytometry

For cell counting, 25 µL of cell culture and 25 µL of FACS buffer [FACSFlow sheath fluid (BD Biosciences), 0.1% bovine serum albumin (BSA) (Sigma-Aldrich), 0.05% sodium azide (Merck)] + 0.5 µL propidium iodide (PI; Life Technologies) were mixed in a 384-well flat-bottom plate. Wells were acquired in high throughput with absolute count enabled on the Novocyte Quanteon with 10 µL of volume set at the stopping condition. Absolute counts were calculated from viable cells and were converted to cell concentrations by dividing by the measured volume. When determining CAR expression, cells were washed once with FACS buffer and stained with anti-Flag PE-Cy7 (Clone L5) for 15 minutes at room temperature followed by another wash. Cells were incubated for 10 minutes with 7-AAD (BD)), prior to acquisition on the Novocyte Quanteon. For the full arrayed screen, harvested cells were washed once with FACS buffer and incubated with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen) in 1X PBS following manufacturer's guidelines. After subsequent washing with FACS buffer, cells were stained with an antibody cocktail for 15 minutes at room temperature, followed by another wash and acquisition on the Novocyte Quanteon. Antibodies included anti-CD8 Pacific Blue (Clone RPA-T8), anti-CD45RA BV605 (Clone HI100), anti-PD-1 BV711 (Clone EH12.2H7), anti-CD4 BV785 (Clone RPA-T4), anti-CCR7 APC (Clone G043H7), anti-CD3 Alexa Fluor 700 (Clone UCHT1), anti-CD45 APC-

Fire750 (Clone 2D1), anti-CD95 PE (Clone DX2), anti-TIM-3 PE-Cy7 (Clone F38-2E2), and were purchased from Biolegend.

Results

Plasmid library production can be scaled down to 96-well format

Arrayed library cloning requires high efficiency and consistent cloning of library elements into the desired viral transfer vector. Golden gate assembly does provide a potent and seamless method for cloning[462]. Our acceptor vector contains a LacZ gene as a placeholder for the CAR HD and is flanked by outward facing BsmBI restriction sites. Similarly, inward facing BsmBI restriction sites flank our HD gene blocks. Careful sequence design allows for seamless insertion of the HD into the vector. Successful HD insertion will result in white colonies on appropriate selection plates. To reduce costs, we serially diluted the BsmBI-v2 NEBridge Golden Gate Enzyme Mix while keeping other components constant. Figure 18A illustrates the quasi-perfect cloning efficiency of a CD8α HD into the vector with no observed blue colonies. The presence of white colonies on the negative control (NC) plate, however, indicated some re-ligation of the vector after restriction digest.

Following cloning, constructs were transformed into NEBStable Chemocompetent *E. coli* according to the manufacturer's instructions. To allow a 96-well PCR-plate format, only 200 µL of recovery medium was added after the heat-shock. Six-well cell culture plates are an economic alternative to 10 cm Petri dishes, requiring only 3 mL of selective LB agar while having sufficient surface area for colony picking. Transformed bacteria were 25X – 100X diluted and 25 µL of each mixture was plated in duplicate on a 6-well plate. The wells of the 25X (uncountable) and 50X (220±28 white colonies) dilution were very densely populated impedes accurate colony picking. In contrast, the 100X dilution resulted in 125±35 white colonies with appropriate inter-colony distance for colony selection (Figure 18B).

To maximize throughput to facilitate arrayed screens, plasmid production should be performed in 96-well format as well. Though, the small well format poses some challenges to reach plasmid yields of at least 5 µg – an amount needed for sequencing and virus production. Indeed, plasmid yield is determined by several factors, among which plasmid copy number, culture volume, oxygen-exchange rate and culture medium composition[463-466]. Therefore, we equipped our vector with a pBR322-derived high copy number origin of replication. Furthermore, oxygen transfer rates (OTRs) are affected by the size of the liquid column (and shaking speed). Perhaps counterintuitively, Lara and colleagues found that limiting the oxygen transfer rate led to higher plasmid yield[466], peaking at 10 mmol O_2 L^{-1} h^{-1}. Hence, we tested three culture volumes (1000 µL, 750 µL and 500 µL) at 300 rpm (20 mm shaking diameter) to achieve OTRs of approximately 3-12 mmol O_2 L^{-1} h^{-1}, respectively[464]. Finally, the amount of

yeast extract as a carbon source was found to affect plasmid yield[463]. Consequently, we also compared TB medium, which is rich in yeast extract, to LB medium. Figure 18C shows a larger plasmid yield for LB when increasing the volume (0.63±0.15 µg from 500 µL vs 1.66±0.01 µg from 1000 µL); not only because of the more abundant nutrients, but perhaps also due to the reduced OTR. However, this observation was not recapitulated with TB as plasmid yield was equal across conditions. In addition, the intended yield of 5 µg was not reached. In a second set of experiments, we compared 96-well deep-well culture plate with 1.5 mL of medium to a 24-well deep-well culture plate with 4 mL of medium (as to not oversaturate the plasmid isolation columns). Remarkably, this time TB outperformed LB in 96-well format (6.84±1.07 µg and 4.40±0.39 µg, respectively) and exceeded the desired yield of 5 µg (Figure 18D). Plasmid yield from the 24-well plate reached the maximum yield of the columns at 30 µg (Figure 18E).

Taken together, we demonstrate that plasmid library cloning, bacterial transformation and viral transfer plasmid production can be scaled to 96-well format while providing sufficiently high plasmid quantities for subsequent viral particle production.

Choice of transfection reagent and seeding density seem somewhat affected by viral genome size in small scale virus production

For virus production, producer cells should have a confluency between 50-80% at the time of transfection to allow for some additional growth during incubation. We created a 2-fold dilution series of Lenti-X 293T producer cells in a 96-well flat-bottom culture plate, ranging from 5-80x10^3 cells per well. After 24 hours of incubation, confluency was assessed visually (Figure 19A). The highest seeding density (80x10^3 cells) reached full confluency, whereas 5-10x10^3 cells resulted in minimal surface coverage. We estimate that 20x10^3 and 40x10^3 cells per well provide a confluency of 30-40% and 60-70%, respectively. For the comparison of transfection reagents, these two cell densities were used.

Similar to plasmid production, achieving high viral titers in small production volumes is essential. At the same time, this should be balanced with economic feasibility. We therefore compared the polymer-based polyethyleneimine (PEI) and lipid-based Lipofectamine 3000 (further references as Lipofectamine) as transfection reagents. A mix of transfer plasmid, packaging plasmid psPAX2 and envelope plasmid pMD2.g (VSV-G pseudotype), totaling 100 ng DNA, were complexed with transfection reagents at room temperature as previously described (PEI; [412]) or according to manufacturer's instructions (Lipofectamine). To assess the effects of size differences within the HD library, we evaluated pLV transfer plasmids containing a short HD (CD8A, 55 amino acids [AA]) or a long HD (HD065A, 235 AA, see **Chapter 7**).

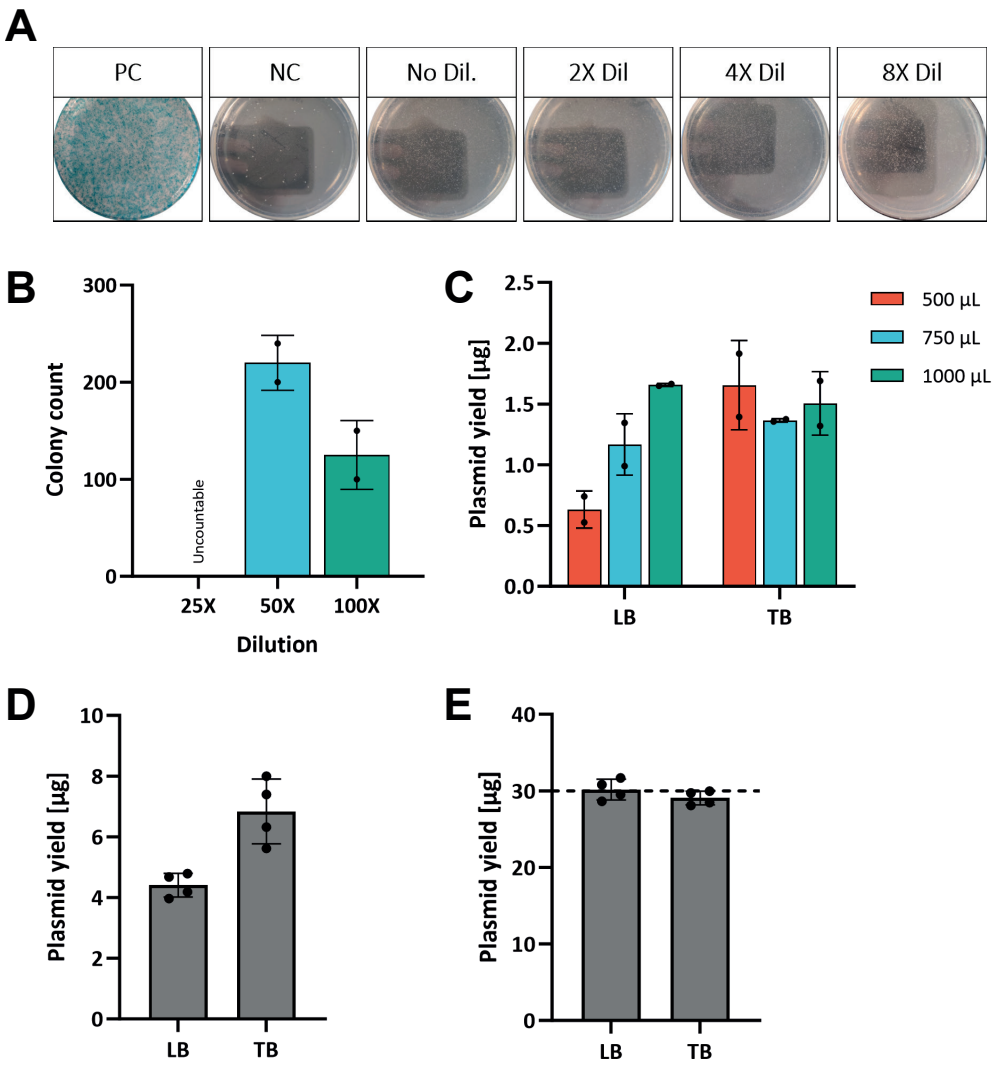

Figure 18. Optimization of arrayed cloning and plasmid production. (A) Blue/white screening of transformed bacteria with plasmid that was cloned through a serial dilution of the BsmBI-v2 NEBridge Golden Gate Enzyme Mix. (B) Dilution series of transformants plated on 6-well selection plates (n=2). (C & D) Plasmid yield from 24 hour 96-well (500-1500 µL; n=2-4), or (E) 24-well bacterial cultures (4 mL; n=4). Abbreviations: Dil., dilution; LB, lysogeny broth; NC, negative control; PC, positive control; TB, terrific broth.

Furthermore, 72 hours following transfection, a 10-fold dilution series of the viral supernatant was made to spinfect Jurkat E6.1 cells. With regard to viability, we only noticed a decrease for the highest virus concentration (100 µL), with the most pronounced effect seen for the short HD virus produced from $40x10^3$ Lenti-X 293T cells: 85.4±0.7%, 77.4±1.3% and 56.2±4.4% for control, PEI and Lipofectamine, respectively (Figure 19B, top row). It is possible that the application of a higher viral load causes the drop in viability. Indeed, the short HD consistently shows higher viral

titers than the long HD (Figure 19B, middle row), calculated based on the CAR-expression on the Jurkat E6.1 cells in the linear range of viral transduction (Figure 19B, bottom row). Increasing the viral genome size with the long HD reduced viral titer between 1.04-2.91-fold. Notably, doubling the amount of seeded Lenti-X 293T cells only resulted in a 1.04-1.36-fold increase in titer for the short HD, whereas a 1.72-2.79-fold rise for the long HD was seen. Taken together, in a small scale format, high viral titers can be achieved using both PEI and Lipofectamine 3000 with minimal differences between the two transfection reagents. Lenti-X 293T confluency prior to transduction seemed of particular importance for larger viral genomes.

Puromycin selection ensures pure CAR-T cultures at the cost of cell viability

From the results above, we chose to proceed with seeding of $40x10^3$ Lenti-X 293T producer cells as the obtained confluency sits at the higher end of the desired range. We continued the comparison between PEI and Lipofectamine in order to determine whether we were able to achieve high transduction rates of primary T cells with the lower titer obtained with PEI. We tested a range of T cell seeding densities with the aim of finding the concentration that allows for high CAR-expression, sufficient T cell expansion and adequate viability. With titers between $3.36\text{-}5.90x10^6$ transducing units (TU)/mL (Figure S9), we reached a multiplicity of infection (MOI) of 0.875-5.9 by transferring 100 µL of viral supernatant to $1x10^5$, $2x10^5$ or $4x10^5$ T cells (1:1 CD8:CD4-ratio). Four days after transduction, CAR-transduced T cells were purified by puromycin selection. In line with literature[467], we determined a concentration of 2.5 µg/mL to be optimal to remove >99.5% of untransduced cells over three days (Figure S10).

Prior to puromycin selection, both control and transduced cells experienced a decline in viability with increasing cell density, indicating that the T cell density is too high (Figure 20, top row – left). As expected, higher MOI resulted in higher transduction efficiencies, reaching approximately 40% CAR-expression in CD8 and CD4 T cells at a $1x10^5$ cells seeding density and decreasing to approximately 10% CAR^+ cells for the $4x10^5$ cells condition (Figure 20, middle and bottom row – left). Interestingly, following puromycin selection, viability of each of the cultures (Figure 20, top row – right) showed great similarity to the fraction of CAR-expressing cells before selection. This implies that the proportion of transduced and non-transduced cells remained the same, indicating that expansion of transduced cells during the selection procedure is limited. Nonetheless, CAR-T cells consistently reached >80% purity with the exception of CAR^+ CD8 T cells at a seeding density of $4x10^5$ cells (Figure 20, middle and bottom row – right).

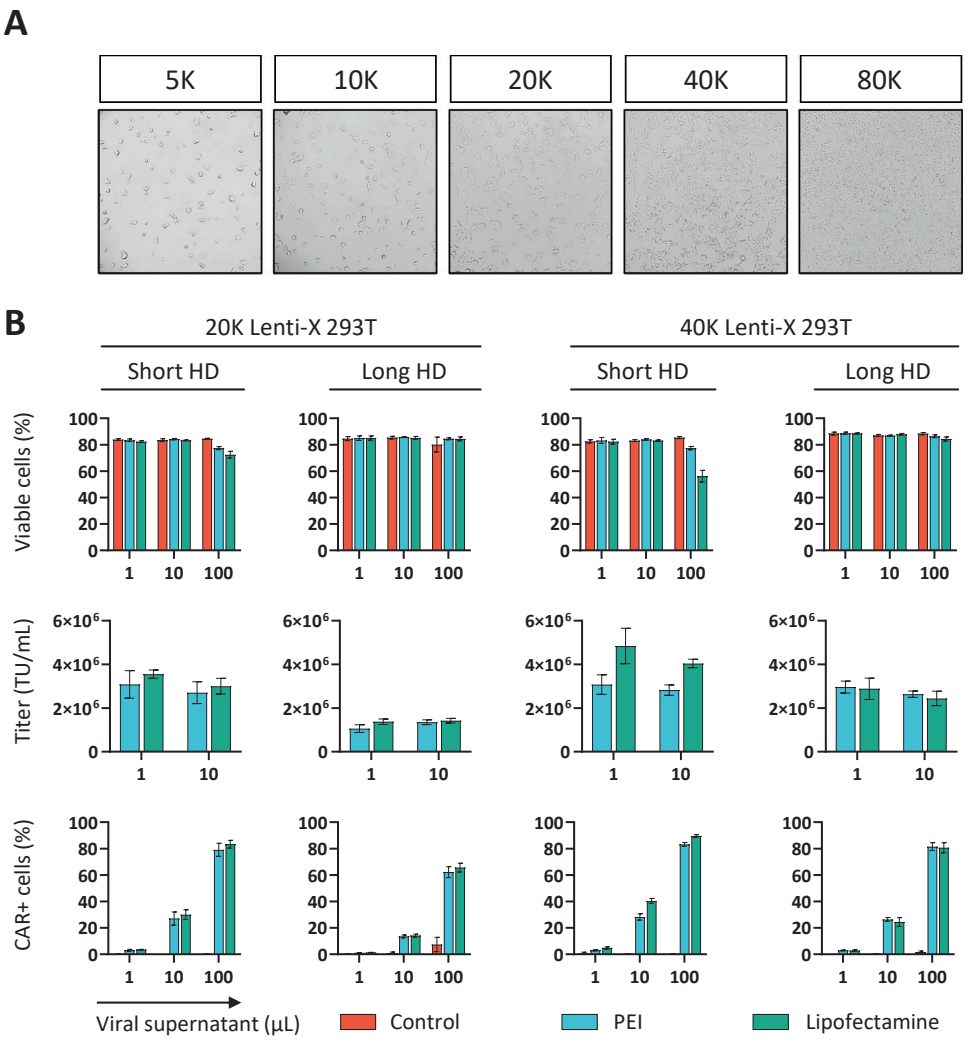

Figure 19. Optimization of arrayed transfection, virus production, and transduction. (A) Microscopic evaluation of confluency of a serial dilution of Lenti-X 293T cells in 96-well flat-bottom plates. Numbers indicate total seeded viable cells per well. (B) Viability (top), viral titer (middle), and CAR-expression (bottom) after transduction of Jurkat E6.1 with increasing volumes of viral supernatant. Titer was calculated based on transduction efficiencies within the linear range of transduction events versus signal, which is considered to be between 5-40% reporter gene expression. Virus particles encoding CARs with either a short or long HD were produced with PEI (blue) or Lipofectamine (green) in $20x10^3$ (left half) or $40x10^3$ (right half) Lenti-X 293T cells. Data represented as mean±SD of three technical replicates.

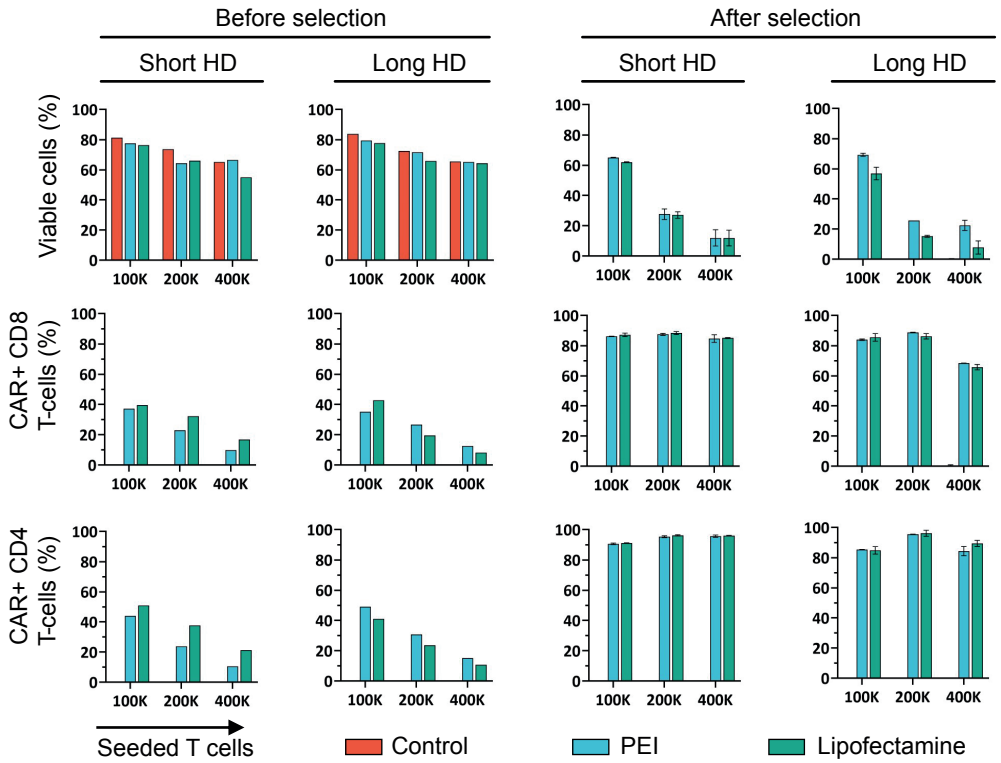

Figure 20. Optimization of arrayed transduction and purification of primary T cells. T cells (1:1 CD8:CD4-ratio) seeded at various densities were transduced with 100 µL of viral supernatant (encoding CARs with a short or long HD) that was produced with either PEI (blue) or Lipofectamine 3000 (green). Viability (top), %CAR+ CD8 T cells (middle) and %CAR+ CD4 T cells (bottom) of transduced T cells were assessed before (left half) and after (right half) puromycin-selection.

Optimizing co-culture setup and maintenance

We aim to use functional data generated with the arrayed screen to develop a prediction model for CAR HDs. To reduce noise, confounding variables such as cross-well variations in volume and seeding density should be minimized. The edge effect in 96-well culture plates, where evaporation of medium varies between the center and the edges of the plates, is a known phenomenon that can cause artifacts in functional readouts[468]. We evaluated two approaches to minimize the edge effect: (1) maintaining a saturated humidity around the culture plate using an additional water-filled container (“saturated box”), and (2) facilitating equal evaporation by covering culture plates with sealing membranes instead of plate lids. We gravimetrically measured the remaining volume of 200 µL cultures after 3-4 days of incubation, a typical duration of longer-term cultures (Figure 21A). The saturated box showed considerably less total evaporation than the two sealing membranes with a median final culture volume of 185 µL vs. 146 µL and 119 µL, respectively (Figure 21B). More importantly, the remaining culture volume with sealing membranes had a normal distribution, while that of the

saturated box did not (Figure 21C). We can therefore conclude that sealing membranes are the preferred method to cover culture plates for long-term cultures.

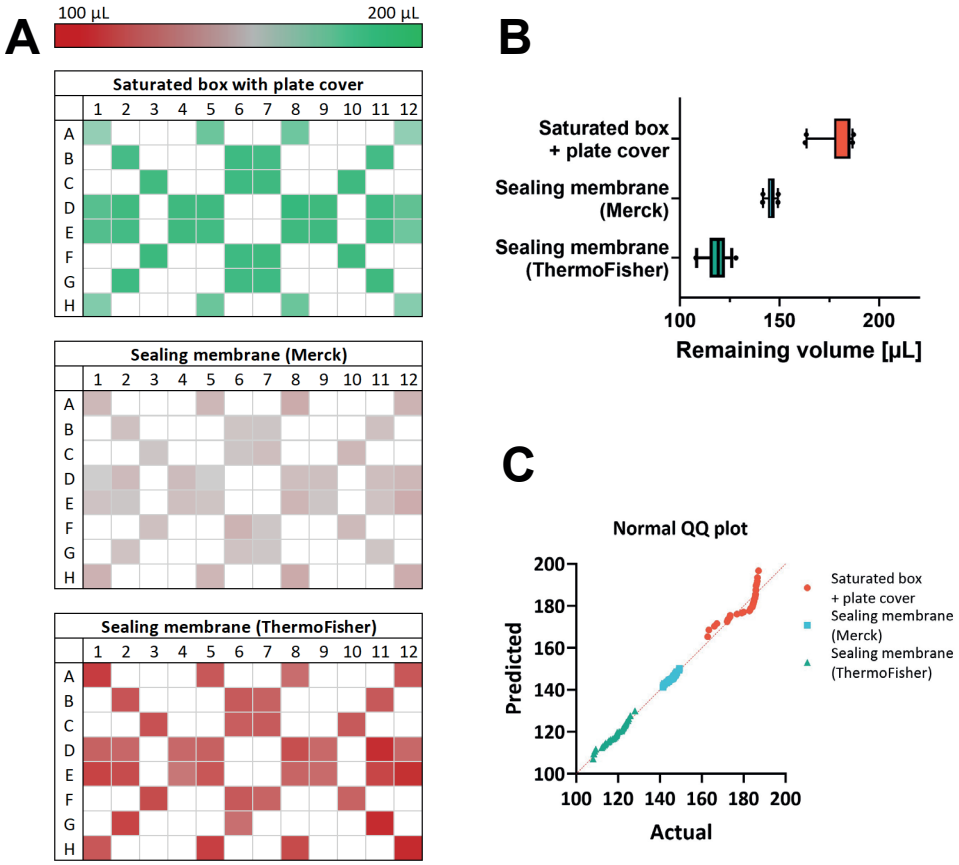

Figure 21. Removing the edge-effect in 96-well cell culture plates with membrane seals. (A) Gravimetric evaluation of well volumes using a grid pattern (n=39-40). (B) Box-and-whiskers plot (median, 5-95% percentile) and (C) normality test of measured remaining volumes after 3-4 days of incubation.

Setting up co-cultures following T-cell transduction and selection requires normalization of the number of T cells per condition. In our lab, cell counting is normally done with an automated cell counter, the Horiba ABX Micros ES 60, but the throughput is limited to approximately 50 samples per hour. We considered two flow cytometry (FCM)-based alternatives that could increase throughput to more than 150 samples per hour: (1) the absolute counting feature in non-pressure-based systems, like the Novocyte Quanteon, and (2) counting beads. Expanding T cells in 96-well plates were counted using these three methods. We found no statistical difference in cell concentrations obtained with the automated cell counter (1.93±0.22 x10^6 cells/mL) and the absolute counting feature of the FCM (1.92±0.21 x10^6 cells/mL; Figure 22A). In contrast, counting beads significantly overestimated cells counts by 23% (2.36±0.27 x10^6 cells/mL; p = 0.0021).

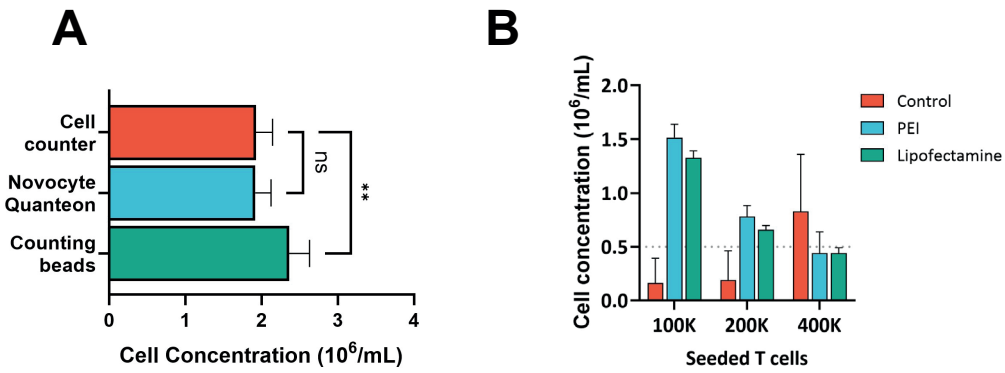

Figure 22. Comparison of automated counting methods (A) and application to transduced primary T cells after puromycin selection (B). Results are represented as the mean±SD of 2 biological replicates in quadruplicate (A) or 3 technical replicates (B). Statistical analysis in (A) was performed using ANOVA with Dunnetts correction for multiple comparisons. **, $p < 0.01$. Abbreviations: ns, not significant; PEI, polyethylenimine.

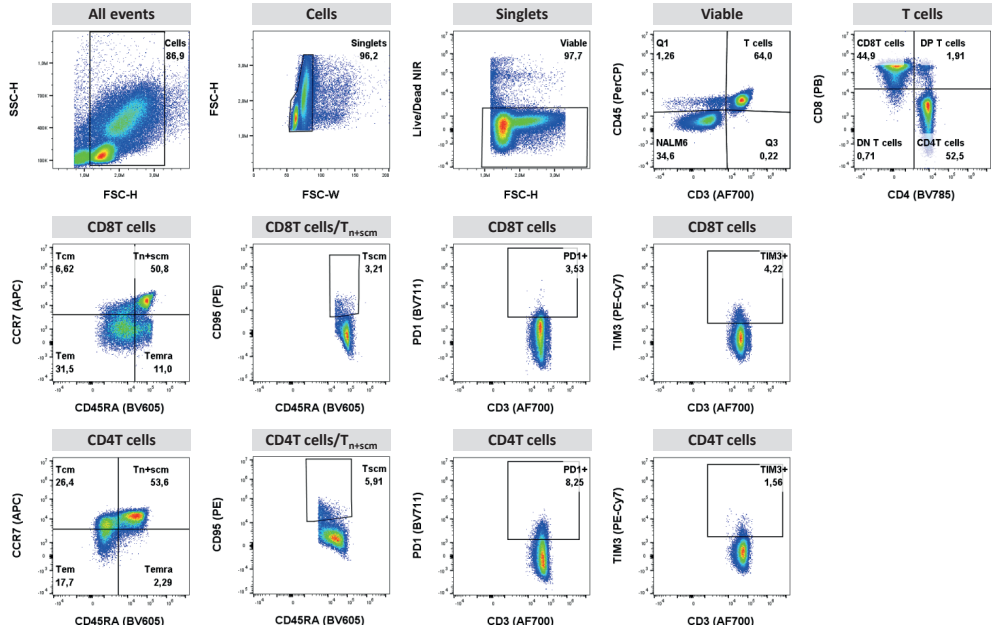

Figure 23. Gating strategy of a 10-color panel for the measurement of cytotoxicity, differentiation and exhaustion. Abbreviations: DN, double negative; DP, double positive; Tcm, central memory T cells; Tem, effector memory T cells; Temra, effector memory T cells re-expressing CD45RA; Tn, naïve T cells; Tscm, stem cell memory T cells.

Finally, we developed a 10-color fluorescent panel capable of assessing cytotoxicity (target cell absolute count), T cell proliferation (T cell absolute count), T cell differentiation (CD45RA, CCR7 and CD95) and T cell exhaustion (PD-1 and TIM-3). Figure 23 displays the suggested gating strategy for a test sample in which unmodified T cells were co-cultured with NALM6 target cells. Future experiments with co-culture of CD19 CAR-T cells and NALM6 should validate the robustness of this panel in the presence of proliferating cells and more cell debris.

Arrayed screen trial run

To put the above findings to the test, we performed a first arrayed screen run of 96 different HDs. Based on blue/white screening, arrayed golden gate assembly of all library members was successful (data not shown). A single white colony was picked for each of the plasmids and cultured for 24 hours in 4 mL TB medium in 24 wells in an attempt to maximize plasmid yield. However, 96-well plasmid preparation resulted in poor plasmid recovery with large inter-well variation (31.49±10.65 ng/µL; Figure 24A & Figure 24B). Given the high turbidity prior to processing and the hampered flow-through of samples, we expect that excessive cellular material causes clogging of the columns with low plasmid recovery as a result. Regardless, we were able to verify correct cloning of 88/96 HDs by Nanopore sequencing and we had sufficient material to continue with viral transduction. For each of the constructs, we produced virus by seeding $40x10^3$ Lenti-X 293T 24 hours prior to transfection with Lipofectamine. We transduced $1x10^5$ pre-activated T cells (1:1 CD8:CD4-ratio) for four days and performed another three day 2.5 µg/mL puromycin selection. Figure 24C shows a mean cell concentration of 0.035±0.039 $x10^6$ T cells/mL, which is too low to perform any functional analysis as at least $5x10^4$ viable T cells are recommended. Instead, we harvested all wells with at least 0.025 $x10^6$ T cells/mL and assessed viability and CAR expression. Unfortunately, the average viability across wells was only 32.91±23.47% (Figure 24D), so we assessed CAR expression only on those samples exceeding 30% viability to assure a sufficiently large sample size (>7 500 events). CAR expression also displayed large variation (Figure 24E), spanning the full range of 0 – 100% for CD4 T cells.

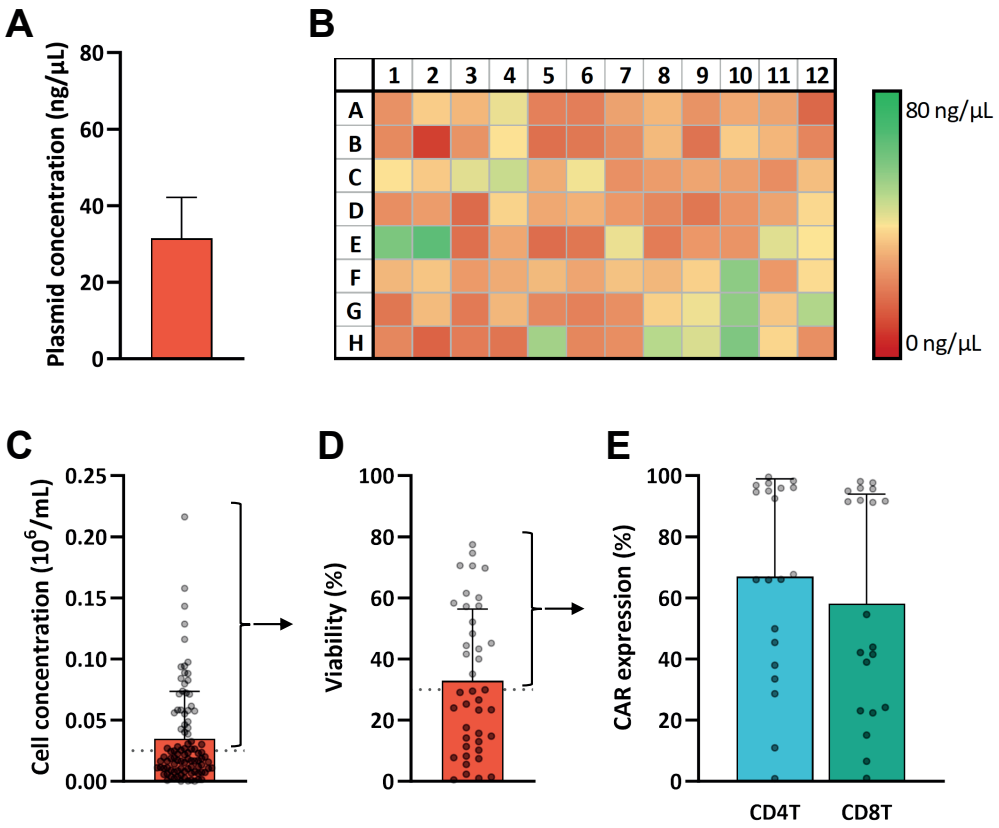

Figure 24. Results from a trial run using optimized workflow parameters. Average (A) and individual well (B) plasmid concentrations of a 96-well plate containing a HD library. Viral particles were produced with these plasmids and used for primary T cell transduction (1:1 CD8:CD4-ratio). Automated cell counting (C) and flow cytometric assessment of viability (D) and CAR-expression (E) of T cells. Dotted lines indicate cut-off values. Results are represented as the mean±SD of 96 (A, C), 41 (D) and 20 (E) wells.

Discussion

In this study, we provided optimized parameters for library assembly, plasmid production, virus production, and T cell expansion and selection. We applied these findings to a pilot medium throughput arrayed screening campaign for a set of 96 HDs, and identified several challenges associated with the translation of low throughput methods to a medium throughput workflow.

A consequence of upscaling throughput is volume reduction and the associated requirement of higher growth densities and yields. Plasmid yields with carbon-rich TB medium can reach 11.4 µg/mL of culture in a medium scale well-aerated fermentation process, as reported by Danguah et al[463]. Furthermore, Lara and colleagues could obtain plasmid yields of 29.0 µg/mL of culture under a limited OTR of 30 mmol/L^{-1}h^{-1}[466]. In fact, decreasing OTR to 10

mmol/$L^{-1}h^{-1}$ could further improve the yield, but results in a slight decrease in DNA quality. Duetz et al. have previously defined the relationship between culture volume, orbital diameter, shaking frequency and OTR, which we used as a reference for our experiments[465]. For example, when culturing 1 mL in a 96-well deep-well plate at 300 rpm with 20 mm orbital diameter, we would expect to reach an OTR of 7-12 mmol/$L^{-1}h^{-1}$, in line with the lowest OTR tested by Lara et al.[464, 466]. However, with 1.5-4.5 µg/mL culture volume (Figure 18) we were unable to reach their reported yields. Our 24-well deep-well cultures (4 mL, 300 rpm, 20 mm, <24 mmol/$L^{-1}h^{-1}$) allowed for sufficiently high plasmid yields (>7.5 µg/mL of culture), but the format is not compatible with standard multi-channels, hampering scalability of the arrayed screen.

A point of attention in our data is the discrepancy in viral titer between viral transfer plasmids with the short HD (~7.2kb viral genome size) and long HD (~7.7kb viral genome size)[458]. This was expected to some extent as transfection efficiency is known to inversely correlate with viral genome size with a 3-fold decrease in functional titer per kb increase in genome size,[458] which equates to a 1.6-fold decrease in our case. Though, given the wide range (1.04-2.91-fold decrease) of our results, it is not possible to rule out that other factors are in play. While viral titer may differ greatly, it appears that the transduction efficiency is affected to a lesser extent if high MOIs are used, reaching a fairly consistent 35-45% CAR+ T cells (Figure 20). It remains to be seen if this extrapolates to the wide variety of HDs in our library.

Puromycin selection could alleviate the concern of variability in CAR expression. Indeed, we observed an enrichment to >80% CAR+ T cells after applying selective pressure during our optimizations, though this could not be consistently replicated in our arrayed screen trial run. One challenge we identified is the apparent stalling of T-cell expansion during puromycin selection, which could jeopardize the ability to perform downstream functional assays. In addition, the large amount of dead cells and cell debris brings difficulties determining viable T cell count in higher throughput (Figure 22). It is also unknown if this cell debris will affect functional readouts, and the concern is that - since we intend to train a predictive model with these data - a “rubbish in, rubbish out” scenario will occur. Therefore, it might not only be more convenient, but also better to discontinue the puromycin selection step and instead perform a correction for differences in CAR expression during downstream analysis.

We compared two commonly used transfection reagents for virus production, PEI and Lipofectamine 3000. Although viral titer was meaningfully higher for Lipofectamine in a few cases (Figure 19 and Figure S9), differences between transduction efficiencies were less pronounced (Figure 19 and Figure 20). For our arrayed screen trial run, we have used Lipofectamine, but given that PEI is up to 168 times more affordable per 96-well plate, we will opt for the use of PEI in the future instead.

Conclusion

In summary, we provide the foundation for an arrayed screening workflow for the evaluation of HD libraries in which we optimized molecular cloning, clone selection, viral production and transduction. Additional work is required with regard to plasmid production and CAR-T-cell selection before a successful validation run can be performed. Once complete, we believe this method can provide tremendous insight in hinge functionality in the context of CARs.

Acknowledgements

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Supplementary material

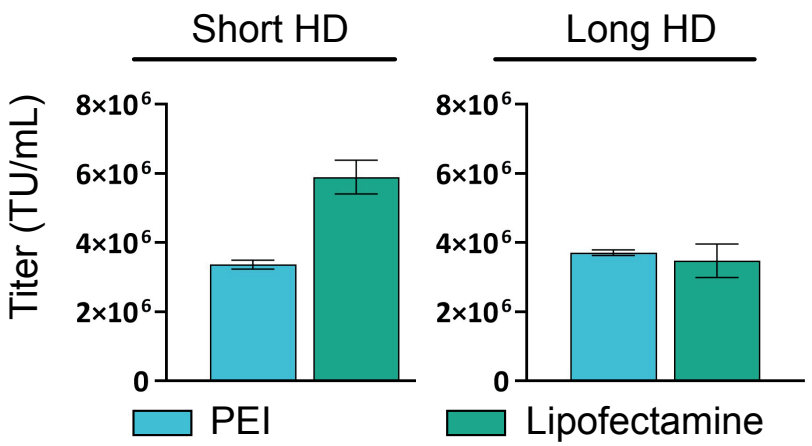


Figure S9. Titer of viral supernatant used in primary T cell transductions (Figure 20). The titer was determined by transduction efficiency of Jurkat E6.1 as assessed by flow cytometry. Data represented as mean ± SD of three technical replicates.

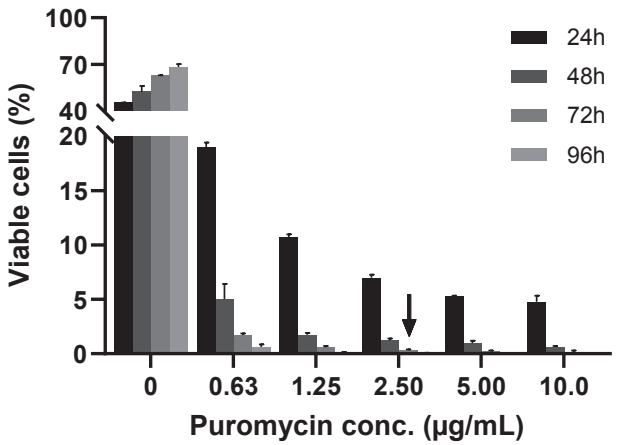
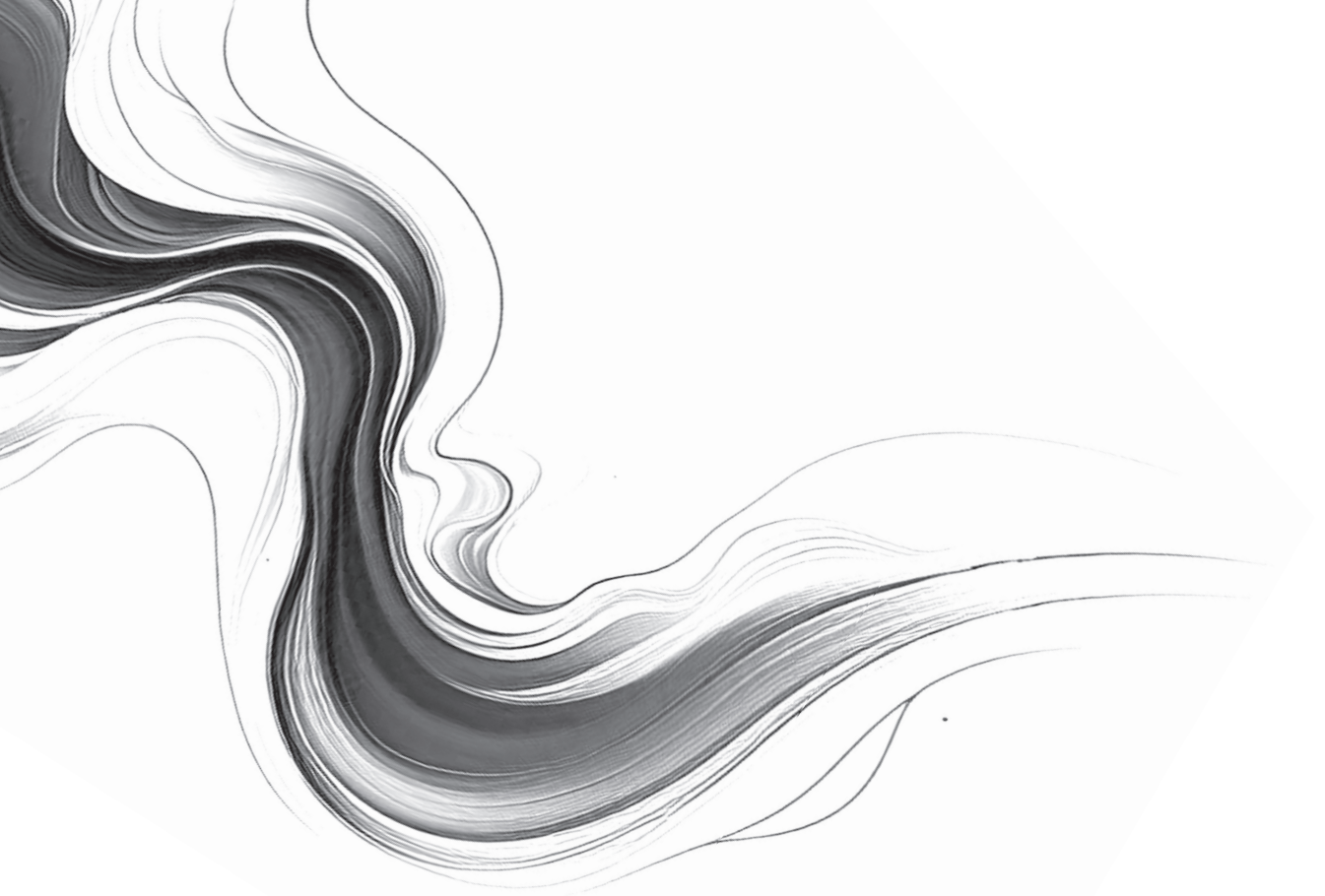


Figure S10. Titer of viral supernatant used in primary T cell transductions (Figure 20). The titer was determined by transduction efficiency of Jurkat E6.1 as assessed by flow cytometry. Data represented as mean ± SD of three technical replicates.



9

DISCUSSION AND FUTURE PERSPECTIVES

CAR-T-cell therapy has transformed the treatment landscape of B-cell hematological malignancies, but additional innovation is required to achieve an optimal outcome for more patients and to broaden the application in other cancers. In this PhD dissertation, I have identified patient and product characteristics, such as age, prior lines of treatment and CAR design, that result in favorable therapeutic outcomes in MM (**Chapter 3**). To solidify our conclusions, the analysis should be repeated on a larger data set. Additionally, although others have partly corroborated our results[387], it would be of interest to perform an identical subgroup analysis for other indications to discover if more of our findings are generalizable to other cancer types. I also demonstrated the feasibility and potency of dual-specific BCMA/CD19 CAR-NK-92 (**Chapter 5**). Efficient targeting of multiple antigens reduces the likelihood of antigen escape. Ideally, the chosen combination of antigens should result in deeper and longer responses, and improve clinical response rates. Although BCMA and CD19 show some level of co-expression in NHL and MM patients, there are likely yet to be discovered antigen combinations that have more stable and consistent co-expression patterns across patients. Integrating gene expression and surfaceomics data on tumor and healthy tissue could soon reveal interesting novel target combinations[469-471]. In the chapter, I also highlight the use of an unrestricted cell source, like NK-92 cells, eliminateing logistical and economical challenges faced with primary T cells. The speed of iterations possible with the mRNA modification strategy used in the chapter is useful for preclinical development of improved CAR-NK-92 therapies. In addition, when evaluating novel target combinations, transient CAR expression provides an extra layer of safety against unexpected complications. Yet, once therapeutic safety has been established, a permanently modified CAR-NK-92 cell line would likely be more economical for clinical development. Furthermore, I presented the early development of bulk (**Chapter 7**) and arrayed (**Chapter 8**) screening methods with a unique focus on the CAR HD. The presented results are still crude and the methods should be further refined to be more robust. The inclusion of multiple features on top of the commonly used T-cell activation would be helpful in narrowing down the number of hits and provides a deeper understanding of the CAR domains.

It is clear that the cellular and molecular modular nature of CAR therapy offers immense opportunities. Indeed, CAR therapy might have the versatility that is required to combat a diverse and complex disease like cancer, but many aspects remain to be elucidated. Below, I discuss the current state of the field, by providing a 2024 update on clinical CD19 and BCMA CAR-T developments for B-cell hematological. Next, four key outstanding questions will be discussed that need to be addressed in order to unlock the full therapeutic potential of CAR therapy for cancer.

Clinical developments of CD19 and BCMA CAR-T cells: a 2024 update

The field of CAR-T-cell therapy is evolving rapidly. Anno 2024, there are six commercial therapeutic products available for the treatment of B-cell lymphoma, B-cell leukemia and MM. Long-term follow-up of the pivotal studies discussed in **Chapter 2** demonstrate durable responses in a large subset of NHL patients treated with CD19 CAR-T-cell therapies (Table 10). At a median follow-up of 63.1 months in the ZUMA-1 trial, the 5-year progression-free survival (PFS) and overall survival (OS) were estimated to be 31.8% and 42.6%, respectively, which is a substantial improvement over the 20% 2-year OS within the historical control (SCHOLAR-1) [11, 472]. Despite lower initial response rates of 53% in the JULIET trial (tisa-cel), the median DOR was not reached at a median follow-up period of 40 months[473]. Whereas median PFS and OS for all patients was only 2.9 and 11.1 months, respectively, they were not reached for patients with a complete response. Notably, a meta-analysis of real-world outcomes for the treatment of LBCL with axi-cel and tisa-cel showed consistent results with the pivotal trials in terms of effectiveness and safety[474]. A two-year follow-up of the TRANSCEND NHL 001 trial showed an impressive median DOR of 23.1 months and estimated 2-year PFS and OS of 40.6% and 50.5%, respectively, closely resembling the results of the 2-year interim analysis of ZUMA-1[82, 475]. Based on the results of this trial, liso-cel was approved by the FDA (2021) and EMA (2022) for the treatment of adult DLBCL patients with at least two prior lines of treatment. Commercial use of CD19 CAR-T-cell products have since been successfully expanded to other indications, such as adult relapsed/refractory (R/R) mantle cell lymphoma (MCL)(ZUMA-2)[476], B-cell ALL (ZUMA-3)[477], FL (ZUMA-5 and ELARA)[478, 479], and CLL and small lymphocytic leukemia (TRANSCEND CLL 004)[480]. Furthermore, axi-cel, liso-cel and brexucabtagene autoleucel (brexu-cel; which harbors the same CAR construct as axi-cel, but has a different manufacturing method) have been approved as second line therapies for adult R/R DLBCL, FL, MCL and B-ALL[476, 477, 481, 482].

BCMA-targeted CAR-T-cell therapies have also advanced significantly since the publication of **Chapter 3** in 2020. Ide-cel (bb2121) has received EMA and FDA approval for fourth (EMA) and fifth (FDA) line treatment of R/R MM patients based on the results of the KarMMa[327]. The last update was published in 2021 and reports more complete data at a median follow-up of 13.3 months, showing a 8.8 months median PFS and 19.4 months median OS[327]. In contrast, a five-year follow-up of the LEGEND-2 trial of cilta-cel (LCAR-B38) was recently published[483]. The trial delivered impressive results with a median DOR of 23.3 months, and 5-year PFS and OS estimates of 21% and 49%, respectively. The USA/Japan-centered counterpart, CARTITUDE-1, is yet to report on longer term clinical outcomes, though results are promising given that median DOR, PFS and OS have not been reached at a median follow-up of 27.7 months[484]. Based on interim results of both trials, cilta-cel was market-approved as a fifth line treatment for R/R MM patients in the USA and Europe in 2022. Furthermore, recent

outcomes of the KarMMa-3 and CARTITUDE-4 studies supported expansion of these market authorizations of ide-cel and cilta-cel as a third and second line treatment, respectively[61, 485]. We are awaiting the first results of phase 3 clinical trials for the evaluation of axi-cel (ZUMA-23) and cilta-cel (CARTITUDE-5 and -6) as frontline therapies for high-risk B-cell lymphomas and transplant (in)eligible newly diagnosed MM, respectively. A full overview of commercialized CAR-T-cell products and their approval dates can be consulted in Table 11.

Table 10. Long-term efficacy data of CD19-targeted CAR-T-cell therapies axi-cel, tisa-cel and liso-cel in NHL.

Cell Product	**axi-cel**	**tisa-cel**	**liso-cel**
Trial [REF.]	**ZUMA-1[469]**	**JULIET[470]**	**TRANSCEND NHL 001[472]**
N enrolled (infused)	111 (101)	167 (115)	270 (270)
N response-evaluable	101	115	257
Best ORR (CR)	83% (58%)	53% (39%)	73% (53%)
Median DoR	11.1 *(4.2–51.3 mo)*	Not reached *(10.0 mo–n.e.)*	23.1 *(8.6 mo–n.e.)*
Median PFS	5.9 mo *(3.3–15.0 mo)*	2.9 mo *(2.3–5.2 mo)* Not reached for CR	6.8 mo *(3.3–12.7)*
PFS rate	5y PFS est.: 31.8%	NR	2y PFS est.: 40.6%
Median OS	25.8 mo *(12.8 mo–n.e.)* *Not reached for CR*	11.1 mo *(6.6–23.9 mo)* *No reached for CR*	27.3 mo *(16.2–45.6 mo)*
OS rate	5y OS: 42.6% 5y OS CR: 64.4%	NR	2y OS est.: 50.5%

Axi-cel, axicabtagene ciloleucel; CR, complete response; DoR, duration of response; Est., estimated; liso-cel, lisocabtagene maraleucel; mo, months; N, number; n.e., not estimable; NHL, non-Hodgkin lymphoma; NR, not reported; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; PR, partial response; ref., reference; tisa-cel, tisagenlecleucel.

The increase in number of clinical trials on CAR-T cells registered on ClinicalTrials.gov from 1087 at the end of 2022[490] to 1601 as of May 2024 is illustrative of the booming field. With 174 trials, BCMA CAR-T-cell therapies comprise almost 11% of all trials. To provide a high-level update of the trials included in our meta-analysis of **Chapter 3**, I performed the same search strategy on PubMed. As such, 43 unique studies covering 28 BCMA CAR-T-cell products were identified with 1806 patients, representing a 2.8-fold increase compared to the report of 2020. Of the 27 studies included in **Chapter 3**, nine were not updated, whereas 18 published a follow-up report since our analysis. Eleven additional trials were initiated of products that were already in the clinic, dominated by ide-cel (four studies) and cilta-cel (three studies), and five new products were brought to the clinic. Our meta-analysis indicated that the incorporation of a VHH-derived ABD and 4-1BB CSD, and CAR-T-cell production with lentiviral vectors had a positive effect on clinical response rates and PFS (**Chapter 3**). In addition, a VHH-derived

ABD and fewer prior lines of treatment were associated with less CAR-T-related toxicities. It is difficult to assess whether these findings have been incorporated into new clinical trial designs given the long lead times prior to clinical trial initiation and reporting of results. Using the large body of new datapoints, it could be of interest to reperform the meta-analysis to improve the confidence in our recommendations or gain additional insights. Table 12 provides an updated summary of clinical trial outcomes from full articles only, excluding conference abstracts and press releases.

Table 11. Overview of pivotal trial studies, follow-up studies, and FDA and EMA approval dates of commercialized CAR-T-cell products in 2024. Last search performed on 30 April 2024.

Product	Indication	PLT	Pivotal trial [Ref]	Follow-up	FDA approval	EMA approval
CD19						
Lisocabtagene maraleucel	Adult R/R DLBCL	=> 2	TRANSCEND NHL 001 [325]	[475]	2021	2022
	Adult R/R DLBCL	=> 1	TRANSFORM [486]	[481]	2022	2023
	Adult R/R CLL / SLL		TRANSCEND CLL 004 [480]	NA	2024	NA
Tisagenlecleucel	Adult R/R DLBCL	=> 2	JULIET [83]	[473]	2018	2018
	Young adult R/R B-ALL	ND	ELIANA [95]	[487]	2017	2018
	Adult R/R FL	=> 2	ELARA [478]	NA	2022	2022
Brexucabtagene autoleucel	R/R MCL	=> 1	ZUMA-2 [328]	[476]	2020	2020
	Adult R/R B-ALL	=> 1	ZUMA-3 [477]	NA	2021	2022
Axicabtagene ciloleucel	Adult R/R DLBCL	=> 2	ZUMA-1 [82]	[472]	2017	2018
	Adult grade 3B FL	=> 2	ZUMA-5 [479]	[488]	2021	2022
	Adult R/R DLBCL	=> 1	ZUMA-7 [489]	[482]	2022	2022
BCMA						
Idecabtagene vicleucel	R/R MM	=> 4	KarMMa [327]	NA	2021	2021
	R/R MM	=> 2	KarMMa-3 [485]	NA	2024	2024
Ciltacabtagene autoleucel	R/R MM	=> 4	LEGEND-2 [107]	[483]	2022	2022
	R/R MM	=> 4	CARTITUDE-1 [326]	[390]	2022	2022
	R/R MM	=> 1	CARTITUDE-4 [61]	NA	2024	2024*

Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; BCMA, B-cell maturation antigen; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; EMA, European Medicines Agency; FDA, US Food and Drug Administration; FL, follicular lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; NA, not available; ND, not disclosed; PLT, prior lines of treatment; R/R, relapsed/refractory; Ref, reference; SLL, small lymphocytic leukemia.

Table 12. Summary of clinical trial outcomes reports of BCMA CAR-T-cell products between 2020-2024. Only full text reports were included. Last search performed on 30 April 2024.

Ref	Trial	Product name	Pts 2020	Pts 2024	ORR	CR	mFO (mo)	mDOR (mo)	mPFS (mo)	mOS (mo)	CRS 1-2	CRS 3-4	NT 1-2	NT 3-4
[491]	NCT04093596 (UNIVERSAL)	ALLO-715	NA	43	56%	19%	10.2	ND	ND	ND	53%	2%	14%	0%
[492]	NCT04155749 (iMMagine-1)	Anitocabtagene autoleucel (CART-ddBCMA)	NA	12	100%	75%	12.6	NR	NR	NR	92%	8%	17%	0%
[493]	ChiCTR2000033567	BC19	NA	50	92%	60%	11.0	NR	19.7	19.7	84%	8%	4%	0%
[494]	NCT03661554	BCMA nanoantibody	9	34	88%	56%	12.5	ND	12.1	NR	82%	3%	0%	0%
[495]	ChiCTR-OPC-16009113	BCMA-CAR T	28	30	90%	43%	12.6	NR	5.2	14.0	80%	17%	3%	0%
[364]	ChiCTR-1800018143	BM38 CAR	22	23	87%	52%	9.0	NR	17.2	NR	65%	22%	0%	0%
[496]	NCT03815383	C-CAR088	5	31	96%	57%	9.4	NR	NR	NR	84%	10%	3%	0%
[497]	ChiCTR-OIC-17011272	CD19 & BCMA CAR-T	21	62	92%	60%	21.3	20.3	18.3	NR	85%	10%	8%	3%
[483]	NCT03090659 (LEGEND-2)	Ciltacabtagene autoleucel (LCAR-B38M)	74	74	95%	73%	65.4	23.3	18.0	55.8	92%	9%	1%	0%
[484]	NCT03548207 (CARTITUDE-1)	Ciltacabtagene autoleucel (LCAR-B38M)	29	97	97%	82%	27.7	NR	NR	NR	91%	4%	11%	9%
[498]	NCT04133636 (CARTITUDE-2)	Ciltacabtagene autoleucel	NA	20	60%	30%	11.3	11.5	9.1	NR	60%	0%	10%	10%
[61]	NCT04181827 (CARTITUDE-4)	Ciltacabtagene autoleucel	NA	208	85%	73%	15.9	NR	NR	NR	75%	1%	4%	1%
[499]	NCT03758417 (CARTIFAN-1)	Ciltacabtagene autoleucel	NA	48	90%	77%	18.0	NR	NR	NR	63%	35%	0%	0%
[500]	ChiCTR-1800018137	CT103A	18	18	100%	72%	13.0	10.7	NR	NR	67%	28%	0%	0%
[501]	NCT03502577	FCARH143+GSI	10	18	89%	56%	36.0	14.4	11.0	42.0	83%	11%	28%	11%
[502]	NCT03093168	HRAIN BCMA-CART	49	49	78%	47%	14.0	NR	10.0	29.0	29%	6%	0%	0%
[503]	NCT02658929	Idecabtagene vicleucel (bb2121)	43	62	76%	39%	18.1	10.3	8.8	34.2	69%	7%	34%	2%

Ref	Trial	Product name	Pts 2020	Pts 2024	ORR	CR	mFO (mo)	mDOR (mo)	mPFS (mo)	mOS (mo)	CRS 1-2	CRS 3-4	NT 1-2	NT 3-4
[327]	NCT03361748 (KarMMa)	Idecabtagene vicleucel	NA	128	73%	33%	13.3	10.7	8.8	19.4	80%	4%	15%	3%
[485]	NCT03651128 (KarMMa-3)	Idecabtagene vicleucel	NA	254	71%	39%	18.6	14.8	13.3	NR	84%	4%	12%	3%
[504]	NCT03318861	KITE-585	NA	14	7%	0%	12.0	NR	NR	NR	21%	0%	21%	0%
[339]	NCT03196414	SZ-MM-CART01	29	10	90%	40%	20.0	ND	ND	ND	89%	11%	11%	0%
[505]	NCT03455972	SZ-MM-CART02	32	11	100%	100%	42.0	ND	NR	NR	100%	0%	0%	0%

Abbreviations: CR, complete response rate; CRS, cytokine release syndrome; GSI, gamma-secretase inhibitor; mDOR, median duration of response; mFO, median follow-up; mo, months; mOS, median overall survival; mPFS, median progression-free survival; NA, not available; ND, not disclosed; NR, not reached; NT, neurotoxicity; ORR, objective response rate; Pts, number of patients; Ref, reference.

CAR-T-cell therapies for MM directed towards antigens other than BCMA were discussed in **Chapter 4**. Unfortunately, updated results for trials described in Table 7 were not available in May 2024. Similarly, availability of data for targets that had pending clinical trials in 2020 is highly variable and often limited to conference abstracts (Table 13). The most promising alternative target is GPRC5D with at least five ongoing trials as a monotherapy. Between 83-100% of patients responds to the therapy and 17-64% achieve CR[506-510]. A study investigating dual targeting of GPRC5D and BCMA is under way. Based on a small study (n=9), efficacy of monotherapy of CD38 CAR-T cells appears limited with an ORR of only 33% and no CRs[511]. Only studies investigating dual targeting of CD38 with BCMA demonstrate good results, with ORR and CR ranging from 87-91% and 52-81%, respectively[364, 512, 513]. However, given the limited results as a monotherapy[511], it is possible that observed responses are mainly driven by the BCMA CAR. One SLAMF7 CAR-T-cell trial was completed, but showed no effect in 10 patients (NCT03958656). In contrast, a bispecific CAR for SLAMF7 and BCMA was able to induce a response in 81% of patients and a complete response in 38%[514]. Evaluation of AUTO2[389], an APRIL-based CAR targeting TACI and BCMA, and CD56- (NCT03473457, NCT03473496) and CD44v6- (EudraCT 2018-000813-19) targeted CAR-T-cell was terminated due to poor responses. Other trials on CD38, LewisY and NY-ESO-1 have been terminated or have an unknown status (Table 13).

At least 12 studies investigating BCMA/CD19 dual targeting in 347 R/R MM patients have published their results[515]. Meta-analysis of these data[515] showed improved ORR (94% vs 80.5%), CR (50% vs 44.8%) and median PFS (12.97 vs 12.2 months), but also higher rates of cytokine release syndrome (98% vs 80.3%) and equal occurrence of neurotoxicity (10% vs 10.5%) compared to our analysis on BCMA CAR-T-cell monotherapy in **Chapter 3**. This further encourages the pursuit of BCMA/CD19 dual targeting strategies, such as our work in **Chapter 5**, but also highlights that additional research is needed to minimize the trade-off between therapeutic potency and associated side-effects.

Table 13. Overview of clinical trials of non-BCMA CAR-T-cell therapies in multiple myeloma. Last search performed on 30 April 2024.

Ref	Target	Trial	Pts	ORR	CR	Status
[511]	CD38	NCT03464916	9	33%	0%	Completed
[513]	CD38 + BCMA	ChiCTR1800017051	22	91%	55%	Completed
[364]	CD38 + BCMA	ChiCTR1800018143	23	87%	52%	Completed
-	CD38 + BCMA	NCT03767751	-	-	-	Unknown
[512]	CD38 + BCMA + NY-ESO-1	ChiCTR1900026286	16	88%	81%	Unknown
-	CD38 + BCMA + NY-ESO-1	NCT03638206	-	-	-	Unknown
-	CD38 + Other	NCT03125577	-	-	-	Unknown
-	CD38 + Other	NCT03271632	-	-	-	Unknown
-	CD38 + Other	NCT03473496	-	-	-	Terminated
-	CD44v6	EudraCT 2018-000813-19	-	-	-	Terminated
-	CD56	NCT03473457	-	-	-	Terminated
-	CD56	NCT03473496	-	-	-	Terminated
[507]	GPRC5D	NCT05739188	7	86%	43%	Ongoing
[510]	GPRC5D	NCT05016778	10	100%	60%	Ongoing
[508]	GPRC5D	NCT04555551	18	83%	17%	Ongoing
[509]	GPRC5D	ChiCTR2100048888	33	91%	64%	Ongoing
[506]	GPRC5D	NCT04674813	70	86%	38%	Ongoing
-	GPRC5D + BCMA	NCT05431608	-	-	-	Ongoing
-	LewisY	NCT01716364	-	-	-	Unknown
-	NY-ESO-1	NCT03638206	-	-	-	Unknown
-	SLAMF7	NCT03958656	10	0%	ND	Completed
-	SLAMF7	NCT04499339	-	-	-	Ongoing
-	SLAMF7	NCT06185751	-	-	-	Ongoing
-	SLAMF7	NCT03710421	-	-	-	Ongoing
-	SLAMF7	NCT04541368	-	-	-	Ongoing
-	SLAMF7	NCT04142619	-	-	-	Terminated
[514]	SLAMF7 + BCMA	NCT04662099	16	81%	38%	Ongoing
-	SLAMF7 + BCMA	NCT05950113	-	-	-	Ongoing
-	SLAMF7 + BCMA	NCT04156269	-	-	-	Unknown
-	TACI + BCMA	NCT04657861	-	-	-	Ongoing
-	TACI + BCMA	NCT05020444	-	-	-	Ongoing
[389]	TACI + BCMA	NCT03287804	11	45%	0%	Terminated

Abbreviations: BCMA, B-cell maturation antigen; CR, complete response rate; GPRC5D, G protein–coupled receptor class C group 5 member D; NY-ESO-1, New York esophageal squamous cell carcinoma 1; ORR, objective response rate; Pts, patients; Ref, reference; SLAMF7, signaling lymphocytic activation molecule family member 7; TACI, Transmembrane activator and CAML interactor.

Question 1: What is the way forward in tackling relapse in CAR-T-cell therapy?

Disease relapse is a persistent problem in CAR-T-cell therapy. The cause for these relapses can be found both on the level of the tumor and the CAR-T cell. Antigen escape, where the target antigen is downregulated or lost on tumor cells, originates from clonal selection of genetic alterations during treatment, or from enzymatic release of the antigen. Controlling the latter is actively under investigation with gamma secretase inhibition in BCMA CAR-T-cell therapy[64, 186]. The options at the level of the CAR are more plentiful. As myself (**Chapter 5**) and others (**Chapter 3**) have demonstrated, a multi-target strategy can be an effective way to prevent antigen escape. In that regard, OR-gated approaches such as bicistronically expressed CARs[65, 450], tandemCARs[326, 366], loopCARs[516] and pooled CARs[358, 517] have been explored. Proper target selection is essential as the risk for off-tumor toxicities is heightened with each additional target. One appealing alternative would be a modular CAR system where antigen detection and signaling is separated: a target module in the form of an antibody or small molecule detects the antigen of choice, and the CAR-T-cell recognize a universal marker linked to the target module[518]. As such, the target module can be infused separately from the CAR-T-cell product, providing flexibility in the case of antigen escape and an additional layer of safety due to the clearance of the target module over time. Relapses can also occur while antigen is still present because of a lack of long-term CAR-T-cell persistence. CAR-T-cell therapy is currently only considered following at least one, in many cases multiple, prior treatments, which can be detrimental for the quality of the leukapheresis product. Early clinical results on the use of CAR-T-cells in combination with first-line treatment are promising and a first phase 3 randomized trial comparing CD19 CAR-T-cell therapy with the standard of care is on its way[62, 63]. Furthermore, central memory and stem cell memory T cells are known to have an enhanced capacity for self-renewal and are therefore studied as superior cell sources over bulk T cells[519, 520]. Another intriguing avenue to improve the quality and the availability of CAR-T-cell products is the use of healthy donor, universal T cells. These allogeneic T cells require knock-out (KO) of the T-cell receptor to avoid graft-versus-host disease (GvHD). Additional KO of β_2 microglobulin to remove HLA-class I expression on T cells has been considered to overcome potential host-versus-graft disease (HvGD)[521]. However, this introduces the problem of NK-mediated HvGD due to “lack of self”[522]. Alternatively, CD52 KO CAR-T cells can be combined with anti-CD52 monoclonal antibody therapy in order to deplete host T cells while leaving edited CAR-T cells intact[521]. Early-phase clinical trials in B-ALL, AML and MM have demonstrated safe use of allogeneic CAR-T cells with varying degrees of therapeutic responses[491, 523, 524].

Question 2: Which cell source is most suitable for CAR therapy?

Although CAR therapy has been mostly studied in the context of T cells, there is a growing number of viable alternatives. For one, NK cells prove to be a suitable candidate because of their natural anti-tumor activity through their suite of activating and inhibitory receptors, lack of GvHD because of the absence of a TCR, and favorable safety profile in terms of CRS and neurotoxicity[525]. Besides peripheral blood, the main origin of NK cells are umbilical cord blood (UCB) and cell lines. UCB contains up to 30% NK cells, that can be modified, readily expanded using feeder cells, cryopreserved and administered as an off-the-shelf product[526, 527]. NK-92 cells are a cell line with unlimited proliferative capacity that has a high natural cytotoxic potential and is easy to manipulate, as we have also demonstrated in **Chapter 5**. Their main drawback is the need for lethal irradiation to prevent uncontrolled proliferation. While this limits their potential for long-term, memory-driven responses, the self-limiting nature of the therapy could lend itself to quicker and safer testing of novel CAR iterations.

Gamma-delta (γδ)T cells are innate-like T cells that comprise up to 5% of the T cells in the peripheral blood[240]. Using GMP-compliant protocols, they are easily expandable to clinically relevant numbers. Their target recognition is MHC-independent, obviating the risk for GvHD, making them suitable for allogeneic treatments. Furthermore, as a bridge between the innate and adoptive immune system, they have been found to cross-present antigens[240], allowing them to initiate a broader immune response towards cancer cells, which is particularly useful in heterogeneous malignancies, such as solid tumors. Broad clinical application of CAR-γδ T cells is currently hampered by their reduced persistence in vivo, limited tumor infiltration, and our limited understanding of how γδ T cells respond to sustained (tonic) CAR signaling[528].

Macrophages are long-lived phagocytic cells of the immune system and were only recently considered for CAR engineering[529], in particular for solid tumors. In addition to phagocytosis of tumor cells, they are known to remodel the hostile tumor microenvironment by secretion of large amounts of pro-inflammatory cytokines and subsequent repolarization of tumor-associated macrophages to favorable M1 macrophages[530]. Similar to γδT cells, macrophages also involve the endogenous innate and adaptive immune system through antigen presentation. Potential limitations to achieve widescale clinical application are their resistance to gene engineering and their limited proliferative capacity.

This could be resolved by using induced pluripotent stem cells (iPSC). Indeed, iPSC are capable of essentially limitless propagation and can be differentiated into any specialized cell type. In fact, iPSC can be readily differentiated into T cells, NK cells and macrophages and have been used for CAR engineering[398, 531, 532]. This opens up interesting possibilities, such as building an artificial immune system with cells of both the innate and adaptive immune

system, modified with cell type-specific CARs or other augmentations to combat tumors in multiple ways. Some potential limitations of iPSC-derived CAR therapies are that they require a lengthy and complex manufacturing protocol including feeder cells, and several media and sera of various sources, complicating broader clinical application[533]. In addition, the timing of CAR-transduction is of importance, as CAR-mediated signaling can unpredictably affect the iPSC differentiation process[534].

Question 3: What are essential considerations in CAR design?

The modularity of CAR molecules provides opportunities for fine-tuning of cellular responses, but simultaneously introduces an immense level of complexity. Below, I attempt to briefly summarize the considerations per CAR domain and speculate on how they can be unified to achieve the best possible outcomes.

In the case of the ABD, it is common belief that a low nanomolar-range affinity is required to obtain a potent therapeutic. In contrast to this, a recent retrospective analysis of 38 published clinical trials of CAR-T cells in solid malignancies revealed that moderate affinity CARs (20-100 nM) resulted in almost 7-fold higher response rates compared to high affinity CARs (<20 nM) due to shorter binding kinetics[38]. Early clinical results of affinity-modulated CD19 CAR-T-cells have shown outstanding responses and persistence in pediatric B-ALL[133]. In addition to potency, discrimination between antigen-high tumor cells and antigen-low healthy cells can be improved through affinity modulation[271]. The origin and size of the ABD should also be considered. For example, nanobodies are small, single domain antibodies that possess a long, flexible CDR3, allowing them to reach epitopes that are not accessible to conventional scFvs[535]. Interestingly, in **Chapter 3**, we found that nanobody-based BCMA CAR-T-cell products had greater clinical potency than human(ized) scFv-based CAR-T-cells. Despite their camelid origin, nanobody CARs have not been found to be immunogenic, in contrast to scFvs from murine origin[536]. ABDs are commonly obtained by immunization of animals and subsequent phage display against the antigen of interest. However, the ABD is not assessed in its intended configuration (that is, in a CAR), and the target antigen is not displayed in its cellular context. High throughput workflows as those discussed in **Chapter 6** do screen scFvs in an appropriate context and could aid in the discovery of novel tumor-specific ABDs with a desirable affinity, size and epitope location[404, 407, 408].

In contrast to the ABD and the CSD, the HD remains generally poorly investigated. Hinge length has been shown to facilitate binding of otherwise sterically inaccessible epitopes[41]. Moreover, in combination with epitope location relative to the target cell membrane, the HD is able to modulate T-cell responses by adjusting the size of the immune synapse[42, 396]. The sequence of particular hinge elements can prove to be both detrimental or useful. On one

hand, it is known that HDs sourced from IgG require point mutations to disrupt their affinity for the Fc-receptors of endogenous NK cells that result in clearance of the CAR-T cells[37]. On the other hand, CAR-T cell detection and purification with GMP-grade beads can be enabled by including a natural epitope in the HD, replacing epitope tags or bicistronic truncated reporter proteins[46]. Non-IgG-based hinges include CD8α and CD28, which are the HDs that are predominantly found in commercial CAR-T products. Due to its propensity to form dimers, the CD28 HD decreases the antigen density threshold in comparison to the CD8α HD[44]. However, this was paired with increased activation-induced cell death and cytokine secretion, which could be a concern with regard to CRS. Due to the limited number of known HDs, it is difficult to tell if those findings are generalizable. In **Chapter 7**, I provided preliminary evidence that activation-based bulk high throughput screens can be used to discover novel HDs. Yet, a deeper understanding is required to better grasp the functional implications of hinge length, subdomains, and perhaps the crude AA sequence. I, therefore, delivered the first steps in developing an arrayed screen for the long-term phenotypical and functional characterization in **Chapter 8**.

The TMD is potentially the only domain for which interdependency with other domains was detected thus far. Indeed, the TMD interacts with endogenous proteins that can affect signal transduction[50, 52]. As such, the ICOS TMD was found to be essential for ICOS co-stimulation when the ICOS CSD was positioned membrane-proximally in the CAR construct[52]. The TMD also regulates CAR multimerization. Owing to its small size, it is probably the only computationally modeled CAR domain – a feature that has enabled the de novo creation of TMDs that tightly control the CAR's valency and the associated increase in cytotoxic potency and cytokine secretion[50]. Further exploration of how and which endogenous membrane proteins associate with the TMD could allow us to exploit these interactions to strengthen or broaden the CAR signal transduction pathway.

The diversity of signal transducing molecules that could be incorporated in the CAR cytoplasmic domains is immense. This diversity not only highlights the potential for fine-tuning CAR T cell responses but also emphasizes the complexity of optimizing their functionality. The two main areas that require further investigation are the number and the spatial localization of cytoplasmic domains within the CAR structure. Second generation CARs with a single CSD represent the majority of CARs under clinical investigation. Third generation CARs (with multiple CSDs) should in theory outperform second generation CARs because of enhanced T cell responses, but there is no conclusive evidence to support this thus far[27]. This could be indicative of the severe impact that the arrangement of these domains can have. Indeed, it has been proposed that the performance of CD28 and ICOS CSD is enhanced when they are positioned in proximity to membrane-proximal signaling kinase Lck[52, 537]. Similarly, in a pooled screen of combinatorial CSD libraries, enrichment of CD40 and FcεRI CSDs was concentrated in the membrane proximal position, whereas CD3ε ITAM and DAP12 were

concentrated in the membrane distal position, indicating a spatial preference for superior T-cell activation[412]. Furthermore, Daniels and colleagues showed that these positional effects could be brought back to the linear signaling motifs present in the natural CSDs[414]. Interestingly, it is expected that the linear signaling motifs can be configured into novel custom CSDs to achieve the desired functional outcome.

It needs to be noted that with a few exceptions, CAR domains have been studied in isolation. It is entirely possible that certain hinges negate or supercharge the effects of cytoplasmic domains. Unfortunately, the combinatorial space for a classical third generation CAR with eight variables (VH-linker-VL-HD-TMD-CSD1-CSD2-ISD) is unimaginable to practically cover with today's technologies. As a surrogate, several researchers have made the analogy between proteins and language, where subdomains or even single amino acids are seen as words that form protein sentences[414, 445]. Using a relatively limited dataset that can be generated with high throughput methodologies, it is theoretically possible to train a model that is able to predict outcomes of unseen CAR combinations, or even build CAR sequences from scratch[445].

Question 4: How can CAR therapy be made more accessible?

The impressive clinical results with CAR-T-cell therapies are unfortunately also associated with a significant cost. Prices for commercial CAR-T-cell products range from $373 000 and $475 000, excluding any charges related to pre-, peri- and post-infusion procedures, and management of adverse events. These additional charges can add up to almost $161 000, with adverse event management comprising over 70% of the total[538, 539]. Despite the large cost, current estimates are that CAR-T-cell therapy still provides a favorable cost-effectiveness ratio, contingent on longer follow-up data and the results of direct comparisons to the standard of care[540]. We note that although these calculations are based on US healthcare estimates, they are largely generalizable to Europe[541]. In the future, this cost-effectiveness can be further improved by reducing the upfront cost and minimizing the occurrence of adverse events. For instance, larger scale, centralized production of off-the-shelf CAR-T-cell products through cell sources discussed above could considerably discount the pricing of the cell product. Alternatively, decentralized, automated, closed-loop manufacturing in an academic non-profit setting could reduce the production cost dramatically to $60 000 - $80 000 per product[542]. Moreover, employing plasmid-based engineering strategies, such as transposons or CRISPR, over lentivirus would offer an additional $20 000 reduction. In addition to improving affordability, limiting therapy associated adverse events by careful selection of the cellular vehicle and CAR composition could allow for outpatient treatment and consequently enhance the quality of life.

9

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Curriculum vitae

Gils Roex
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Nationality: Belgian
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Professional experience

Scientist Cell Therapy — ***Mechelen, Belgium***
Galapagos nv — Jun 2024 - Current

PhD Candidate / FWO Strategic Basic Research Fellowship — ***Antwerp, Belgium***
University of Antwerp — *Sep 2018 – May 2024*

Co-founder ViviGate (Startup) — ***Leuven, Belgium***
KU Leuven / RVO-Society — *2016 – 2018*

Intern Medical Device Development (Industry internship) — ***Wijnegem, Belgium***
Novosanis — *Jul 2016 – Aug 2016*

Education

MSc. Bioscience Engineering: Cell and Gene Technology — **Leuven, Belgium**
KU Leuven — *Sep 2016 – Jun 2018*

BSc. Bioscience Engineering: Cell and Gene Biotechnology — **Antwerp, Belgium**
UAntwerpen — *Sep 2013 – Jun 2016*

Scientific publications

Van den Eynde A, Gehrcken L, Verhezen T, Lau HW, Hermans C, Lambrechts H, Flieswasser T, Quatannens D, **Roex G**, Zwaenepoel K, Marcq E, Joye P, Cardenas De La Hoz E, Deben C, Gasparini A, Montay-Gruel P, Le Compte M, Lion E, Lardon F, Van Laere S, Siozopoulou V, Campillo-Davo D, De Waele J, Pauwels P, Jacobs J, Smits E, Van Audenaerde JRM. IL-15-secreting CAR natural killer cells directed toward the pan-cancer target CD70 eliminate both cancer cells and cancer-associated fibroblasts. Journal of Hematology & Oncology **2024**; 17: 8

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Roex G, Campillo-Davo D, Flumens D, Shaw PAG, Krekelbergh L, De Reu H, Berneman ZN, Lion E, Anguille S. Two for one: Targeting BCMA and CD19 in B-cell malignancies with off-the-shelf dual-CAR NK-92 cells. Journal of Translational Medicine **2022**; 20: 124

Campillo-Davo D, De Laere M, **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.

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. Journal of Hematology & Oncology **2020**; 13: 164

Roex G, Feys T, Beguin Y, Kerre T, Poiré X, Lewalle P, Vandenberghe P, Bron D, Anguille S. Chimeric Antigen Receptor-T-Cell Therapy for B-Cell Hematological Malignancies: An Update of the Pivotal Clinical Trial Data. Pharmaceutics **2020**; 12(2): 194.

Campillo-Davo D, Versteven M, **Roex G**, De Reu H, van der Heijden S, Anguille S. 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.

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.

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.

Campillo 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): A8-A9.

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 gd T cell-based anti-cancer immunotherapy. Cytokine & Growth Factor Reviews **2018**; 41: 54-64.

International research stays

Research Fellowship — ***Cambridge, USA***
Birnbaum Lab, Massachusetts Institute of Technology (MIT) — *Sep 2022 – Mar 2023*

Research Fellowship — ***Shanghai, P.R. China***
Biotherapy Research Center, Fudan University — *Oct 2018 – Dec 2018*

Oral and poster presentations

International Conference on Lymphocyte Engineering — ***Munich, Germany***
First author poster presentation — *Apr 2022*

NVC – BSAC Joint Congress — ***Breda, The Netherlands***
First author speaker — *Nov 2019*

ESH – Immune and Cellular Therapies — ***Mandelieu-La-Napoule, France***
First author poster presentation — *Sep 2019*

10th International Cellular Therapy Symposium — ***Erlangen, Germany***
Second author poster presentation — *Mar 2019*

Scientific awards & grants

Gilead Award — ***Belgian Hematology Society***
€25 000

Travel Grant Long Stay Abroad — ***Fonds Wetenschappelijk Onderzoek***
Research fellowship at the Birnbaum Lab, MIT, USA — €10 000

Travel Fellowship — **European Association for Cancer Research**
Research fellowship at the Birnbaum Lab, MIT, USA — *€2 700*

Research Mobility Grant — ***European Hematology Association***
Research fellowship at the Birnbaum Lab, MIT, USA — *€2 800*

Strategic Basic Research Fellowship — ***Fonds Wetenschappelijk Onderzoek***
Aspirant fellowship renewal — *2 years*

Aanmoedigingsbeurs — ***Legaat Stichting Rosa Blanckaert***
€2 500

Strategic Basic Research Fellowship — ***Fonds Wetenschappelijk Onderzoek***
Aspirant fellowship — *2 years*

ImmunoTools Special Award — ***ImmunoTools***
Reagents

Travel Grant — ***European Society of Haematology***
ESH – Immune and Cellular Therapies — *€500*

International Research Visit Support ***OJO, UAntwerpen***
Research fellowship at the BRC, Fudan University, P.R. China *€1 200*

Student mentorship

Jolien Op de Beeck ***Antwerp, Belgium***
Master student *Sep 2021 – Jun 2022*

Jona Verstraelen ***Antwerp, Belgium***
Bachelor student *Nov 2021 – May 2022*

Tine Pinxteren ***Antwerp, Belgium***
Bachelor student *Nov 2021 – May 2022*

Emma De Geest ***Antwerp, Belgium***
Master student *Sep 2020 – Jun 2021*

Laurens Krekelbergh ***Antwerp, Belgium***
Voluntary internship *Aug 2020*

Eveline Mermans ***Antwerp, Belgium***
Voluntary internship *Aug 2020*

Extracurricular Activities

SciMingo – Science Figured Out ***Belgium***
https://bit.ly/SciMingoOnYouTube *Sep 2022*

PRESS>SPEAK science communication contest ***Antwerp, Belgium***
University of Antwerp / Wetenschap aan de stroom *Feb 2022*

Full Member Of The Scientific Research Commission ***Antwerp, Belgium***
Faculty of Medicine of the University of Antwerp *Mar 2020 – May 2024*

President of ALABO VZW ***Antwerp, Belgium***
Department of Bioscience Engineering (UAntwerpen) *Jan 2021 – Jan 2024*

Full board member of ALABO VZW ***Antwerp, Belgium***
Department of Bioscience Engineering (UAntwerpen) *Jan 2018 – Jan 2024*

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Producten die op zijn? Nieuwe assays delen of samen uitwerken? Samen op congres of naar een sportevenement? Daarvoor kon ik bij mijn CORE-collega's terecht: **Jonas, Astrid, Sanne, Hasan, Jasper V, Hanne V, Jorrit, Tias, Evelien, Ho Wa, Delphine, Yannick, Julie**, en alle anderen! Bedankt voor alle topmomenten, groot en klein!

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Gils