“Success is the ability to go from failure to failure without losing your enthusiasm”

Winston Churchill

Harnessing gamma delta T cells, interleukin-15 and CD56 in immunotherapeutic strategies combating leukemia

Heleen Van Acker

Harnessing gamma delta T cells, interleukin-15 and CD56 in immunotherapeutic strategies combating leukemia:
a functional and mechanistic characterization

Proefschrift voorgelegd tot het behalen van de graad van
doctor in de medische wetenschappen
aan de Universiteit Antwerpen te verdedigen door

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Not all those who wander are lost – J.R.R. Tolkien

SUMMARY

The past decades our vastly growing, comprehensive knowledge on the intricacies of the human immune system and its active role in cancer development, progression and control has boosted the development of immunotherapeutic approaches to treat cancer. Consequently, as the magnitude of the cancer immunotherapy field steadily increases, orthogonal approaches are required to drive further advancement. This PhD thesis therefore focused on four promising aspects/types of immunotherapy being; gamma delta (γδ) T cells, interleukin (IL)-15, dendritic cell (DC) vaccination and the molecule CD56.

γδ T cells – Being an evolutionarily ancient white blood cell type, typified by the expression of a γδ T cell receptor (TCR), γδ T cells straddle the innate and adaptive arms of the immune system. As opposed to classic αβ T cells, they (I) sense target antigens independent of major histocompatibility complex (MHC) molecules or conversely, react to MHC molecules without peptide, (II) carry out sturdy (innate-like) cytotoxic activities mediated by several different effector mechanisms, (III) are able to take up large particulates, including bacteria, and (IV) can act as professional antigen-presenting cells. The 'stress sensing' capacities of γδ T cells, in combination with their ability to produce various cytokines and interact with several immune cells, have resulted in a keen interest in their use as immunotherapeutic, not least in cancer.[1,2]

Interleukin-15 – Ranked third on the cancer immunotherapy trials network (CITN) priority list of immunotherapy agents,[3] the pleiotropic cytokine IL-15 has a lot of potential to boost the anti-tumor immune response and help cure cancer. This is also reflected by the results of the first in human clinical trial of recombinant IL-15[4] and of the IL-15 superagonist complex ALT-803[5]; showing both a favorable safety profile together with proliferation and activation of natural killers (NK) cells and T cells.

Dendritic cells - DCs are the most potent professional antigen-presenting cells of the immune system, occupying a crucial position in the induction of adaptive immune responses.[6] Furthermore, innate immune functioning can as well be directed by DCs. Namely, a crosstalk has been established between both DCs and NK cells, and DCs and γδ T cells.[1,7] To date, it is ascertained that DC-based vaccination is safe, well-tolerated and capable of inducing anti-tumoral immune responses. Objective clinical responses are, however, amenable to substantial improvement; for example by the implementation of next-generation DC vaccines with improved immunogenicity.[6]

CD56 - The neural cell adhesion molecule, also known as CD56, is the archetypal phenotypic marker of NK cells but can actually be expressed by many more immune cells, including αβ T cells, γδ T cells, DCs, and monocytes. Common to all these CD56-expressing cell types are

strong immunostimulatory effector functions, including T helper type (Th)1 cytokine production and an efficient cytotoxic capacity.[8]

Chapter 1 provides a coherent review on the effects of the different cytokines of the common gamma-chain receptor family – IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 – on γδ T cells, in view of boosting γδ T cells as a therapeutic target in cancer immunotherapy. From the gathered reports, we conclude that IL-15-activated γδ T cells will arise as strong candidates for future cancer immunotherapies, holding the key to unlock cancer immune escape and resistance in both active and passive strategies.

One of the reports that supports this statement is discussed in **chapter 2**, where we characterize the sheer effect of IL-15 on untouched and isolated γδ T cells and translate these results into a γδ T cell expansion protocol for adoptive transfer. We report that the addition of IL-15 to γδ T cell cultures results in a more activated phenotype, a higher proliferative capacity, a more pronounced Th1 polarization and an increased cytotoxic capacity of γδ T cells. Moreover, γδ T cell expansion starting with peripheral blood mononuclear cells (PBMC) from healthy individuals and acute myeloid leukemia (AML) patients is boosted in the presence of IL-15, whereby the anti-tumor properties of the γδ T cells are strengthened as well.

Given the amount of different types of malignancies, possible stages of disease and concomitant treatments, as well as the inherent diversity between patients as individuals, it will probably be necessary to develop personalized immunotherapeutic strategies and combine different immunotherapies to achieve a maximal effect. Consequently, we sought to translate the above findings into the field of dendritic cell vaccination; more specifically, if our new IL-15 DC vaccine could harness autologous γδ T cells in their anti-tumor immune response.

Building on this hypothesis, **chapter 3** discusses our knowledge on DC-mediated γδ T cell activation, the mechanisms behind the cell-to-cell interactions and its therapeutic potential for implementation in DC-based cancer immunotherapy. In this regard, consideration should be given to the fact that conventional monocyte-derived DCs (i.e. IL-4 DCs) generally lack the ability to stimulate (patients') γδ T cells, and that additional/alternative signals are required.

To affirm that IL-15 DCs could represent an improved therapeutic vaccine component with regard to immunostimulatory activity and activation of both innate and adaptive anti-tumor arms, we first looked in **chapter 4** at their capacity to recruit immune cells, including γδ T cells. Namely, vaccine DCs should come in contact with the necessary effector cells to

perform their directing and activating functions. Our results show that IL-15 DCs are superior to IL-4 DCs in terms of attraction of all important anti-tumor effector lymphocytes and that at least γδ T cells and NK cells exhibit enhanced cytotoxic function upon migration. Furthermore, our data demonstrate involvement of the CCL4-CCR5 signaling pathway in the improved capacity of IL-15 DCs to recruit anti-tumor immune effector lymphocytes, by means of increased expression and secretion of CCL4 by IL-15 DCs.

In **chapter 5** we were able to establish that the application of IL-15-secreting DC subsets could render DC-based anti-cancer vaccines more effective through, among others, the involvement of γδ T cells in the anti-leukemic immune response. We demonstrated that IL-15 DCs induce the upregulation of cytotoxicity-associated and co-stimulatory molecules on the γδ T cell surface, but not of co-inhibitory molecules, incite γδ T cell proliferation and stimulate their IFN-γ production in a malign environment. Moreover, the innate cytotoxic capacity of γδ T cells is significantly enhanced upon interaction with IL-15 DCs, both towards leukemic cell lines and allogeneic primary AML blasts. Finally, we addressed soluble IL-15 secreted by IL-15 DCs as the main mechanism behind the IL-15 DC-mediated γδ T cell activation.

In the previous chapters, relatively little attention was being paid to CD56 and its expression profile. Nevertheless, during this thesis we gradually realized that this molecule might hold more information than initially anticipated. For example, in chapter 2 we observed that the proportion of $CD56^+$ γδ T cells appeared to be determined by their level of activation. Moreover, we have previously shown that a considerable fraction of the IL-15 DCs unexpectedly beard this surface marker as well. Hence, we give an overview of our current knowledge on the expression of CD56 in the hematopoietic system in **chapter 6**.

In **chapter 7** we looked in depth into the expression and functional role of CD56 on immune cells and were able to demonstrate for the first time a direct involvement of CD56 in NK cell / γδ T cell / IL-15 DC-mediated tumor cell lysis. We also detected a potential crosstalk mechanism, being CD56 as a co-stimulatory molecule, between IL-15 DCs and CD8 T cells. Furthermore, we are the first to describe and visualize the CD56 homophilic interaction between cancer cells among themselves as well as between immune cells and malignant cells. Finally, we confirmed our hypothesis that IL-15 stimulation directly leads to CD56 upregulation, which occurs mainly through the phosphoinositide 3-kinase (PI3K) pathway.

Considering the importance of phosphoantigens in the stimulation of γδ T cells, we discuss the rationale to use bisphosphonates in cancer patients and review the clinical position of bisphosphonates in the treatment of hematological and solid malignancies in the **addendum** chapter.

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SAMENVATTING

De afgelopen twintig jaar is onze kennis van het immuunsysteem enorm toegenomen. Een belangrijke stap in dit leerproces was de wetenschap dat ons afweersysteem een actieve rol speelt in de ontwikkeling, progressie en afweer van kanker. Deze kennis heeft namelijk geleid tot een cruciale impuls in de ontwikkeling van verschillende immuungebaseerde therapieën. Sindsdien neemt de omvang van het domein van de kankerimmuuntherapie gestaag toe en is het de kunst om deze vooruitgang te blijven voortstuwen. In deze doctoraatsthesis focussen we op vier veelbelovende aspecten/types van immuuntherapie, zijnde; gamma delta (γδ) T-cellen, interleukine (IL)-15, dendritische cel (DC)-vaccinatie en de molecule CD56.

γδ T-cellen – γδ T-cellen vormen een eeuwenoud immuuncel-subtype gekenmerkt door de expressie van een γδ T-cel receptor. Het feit dat deze cellen moeilijk te definiëren zijn en zowel kenmerken bezitten van het aangeboren als van het verworven immuunsysteem, maakt hen uniek. In tegenstelling tot klassieke αβ T-cellen zijn γδ T-cellen bijvoorbeeld in staat om antigenen te herkennen die niet gepresenteerd worden via moleculen van het majeure histocompatibiliteitscomplex. Verder bezitten γδ T-cellen verschillende effector-mechanismen, waardoor ze (I) een sterke (directe) cytotoxische activiteit kunnen uitoefenen ten opzichte van maligne cellen, (II) grote partikels kunnen opnemen, waaronder bacteriën, alsook (III) optreden als antigeen-presenterende cellen. Naast het feit dat ze de capaciteit hebben om verschillende cytokines te produceren en te interageren met verscheidene immuuncellen, is hun vermogen om stress-signalen op te pikken uit hun omgeving en hierop in te spelen zeer interessant voor hun gebruik in immuuntherapie, niet in het minst in kanker.[1,2]

Interleukine-15 – De derde plaats van IL-15 op de lijst van prioritaire immunotherapeutica van het 'Cancer Immunotherapy Trials Network (CITN)' weerspiegelt het potentieel van dit pleiotroop cytokine om de antitumor immuunrespons te versterken en kanker te bestrijden.[3] Zo werden er zeer mooie resultaten behaald in de eerste klinische trial met IL-15,[4] alsook in deze met de IL-15 superagonist ALT-803.[5] Beide studies konden eveneens een gunstig veiligheidsprofiel voorleggen van de geteste molecule en detecteerden bovendien activatie en proliferatie van 'natural killer (NK)' cellen en T cellen.

Dendritische cellen – Een sleutelrol voor het opwekken van een antigeen-specifieke immuunrespons is weggelegd voor DCs. De hoofdfunctie van deze cellen bestaat erin om antigenen op te nemen, te verwerken en te presenteren aan T-cellen en op deze manier een adaptieve immuunrespons op gang te brengen.[6] Daarnaast kan ook het functioneren van het aangeboren immuunsysteem gestuurd worden door DCs. Er is namelijk een wisselwerking aangetoond tussen zowel DCs en NK cellen, als DCs en γδ T-cellen.[1,7] Uit de resultaten van klinische studies die DC-vaccinatie onderzoeken, komt duidelijk naar voren

dat behandeling met DCs veilig is voor de patiënt, goed verdragen wordt en bovendien een effectieve antitumor immuunrespons induceert. Objectieve klinische responsen blijven echter beperkt en de algemene consensus luidt dat er absoluut nog ruimte is voor verbetering; bijvoorbeeld door de implementatie van een volgende generatie DC-vaccins met verbeterde immunogeniciteit.[6]

CD56 – De neurale celadhesiemolecule, ook bekend als CD56, is de archetypische fenotypische merker van NK cellen, maar wordt in werkelijkheid door veel meer immuuncellen tot expressie gebracht, waaronder αβ T-cellen, γδ T-cellen, DCs en monocyten. Gemeenschappelijk voor al deze $CD56^+$ celtypes zijn sterke immuun-stimulerende effectorfuncties, waaronder T helper 1 cytokineproductie en een efficiënte cytotoxische activiteit.[8]

In **hoofdstuk 1** wordt een overzicht gegeven van de effecten van de verschillende cytokines van de 'common gamma-chain receptor' familie – IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 – op γδ T-cellen, met het oog op het stimuleren van γδ T-cellen voor therapeutische doeleinden in kanker. Uit de verzamelde informatie kunnen we concluderen dat IL-15 geactiveerde γδ T-cellen sterke kandidaten zijn voor het gebruik in kankerimmuuntherapie, mede dankzij hun potentie tot het opheffen van immuun-ontsnappingsmechanismen van maligne cellen en het opnieuw ontketenen van de antitumor effectorfuncties van het immuunsysteem.

Eén van de onderzoeksartikels die deze stelling onderbouwt, wordt besproken in **hoofdstuk 2**. Hierin karakteriseren we het effect van IL-15 op verse, geïsoleerde γδ T-cellen en worden de bevindingen vertaald naar een klinisch protocol voor γδ T-celexpansie voor adoptieve transfer. We rapporteren dat het toevoegen van IL-15 aan geïsoleerde γδ T-celculturen resulteert in een geactiveerd fenotype, hogere proliferatieve capaciteit, een meer uitgesproken T-helper 1 polarisatie en versterkte cytotoxische capaciteit van γδ T-cellen. Bovendien worden de expansie en antitumor eigenschappen van γδ T-cellen voor adoptieve celtherapie bevorderd door het toevoegen van IL-15, dit startende met perifeer bloed mononucleaire cellen van zowel gezonde donoren als van acute myeloïde leukemie (AML-)patiënten.

Gezien de hoeveelheid aan types maligniteiten, ziektestadia en behandelingen, bovenop de reeds inherente diversiteit tussen patiënten als individuen, zal het waarschijnlijk noodzakelijk zijn om gepersonaliseerde immuuntherapieën te ontwikkelen, alsook met elkaar te combineren, om het effect van kankerimmuuntherapie te maximaliseren. Vandaar onze interesse om te onderzoeken of we bovenstaande bevindingen konden vertalen naar het onderzoeksgebied van DC-vaccinatie; meer specifiek, of ons nieuw IL-15 DC vaccin in staat is om γδ T-cellen te betrekken in hun antitumor immuunrespons.

In navolging hiervan bediscussiëren we in **hoofdstuk 3** onze kennis van DC-gemedieerde γδ T-celactivatie, de mechanismen die deze cel-celinteractie ondersteunen en het therapeutisch potentieel van γδ T-celactivatie voor implementatie in DC-gebaseerde immuuntherapie voor kanker. Hierbij dient opgemerkt te worden dat conventionele monocyt-afgeleide DCs, zijnde IL-4 DCs, doorgaans niet in staat zijn om (patiënt) γδ T-cellen te stimuleren, en dat additionele / alternatieve signalen vereist zijn om dit te bereiken.

Om te bevestigen dat IL-15 DCs, ten opzichte van de IL-4 DCs, een verbeterd therapeutisch vaccin vormen - met betrekking tot immuunstimulerende werking en activatie van beide armen van het antitumor immuunsysteem - hebben we in **hoofdstuk 4** in eerste instantie gekeken naar de capaciteit van IL-15 DCs om immuuncellen aan te trekken, waaronder ook γδ T-cellen. Dit is belangrijk, aangezien vaccin DCs in contact moeten komen met de nodige effectorcellen om hun regisserende en activerende functies te kunnen uitoefenen. Onze resultaten hebben aangetoond dat IL-15 DCs superieur zijn aan de IL-4 DCs op het gebied van het aantrekken van alle belangrijke antitumor effectorlymfocyten en dat in ieder geval de gemigreerde γδ T-cellen en NK cellen een verhoogde cytotoxische activiteit vertonen. Verder geven onze resultaten aan dat de CCL4-CCR5 signaaltransductieroute betrokken is in het versterkte vermogen van de IL-15 DCs om antitumor effectorlymfocyten te rekruteren en dit door een verhoogde expressie en secretie van CCL4 door de IL-15 DCs.

In **hoofdstuk 5** hebben we experimenteel preklinisch kunnen vaststellen dat het implementeren van IL-15 secreterende DC subsets in vaccins de effectiviteit van deze behandelingsvorm kan verhogen, mede door het betrekken van γδ T-cellen in de antitumor immuunrespons. Meer specifiek induceren IL-15 DCs cytotoxiciteit-geassocieerde en co-stimulerende moleculen op het celoppervlak van γδ T-cellen, maar hebben ze geen invloed op de expressie van co-inhibitoire moleculen, zetten ze γδ T aan tot proliferatie en stimuleren ze hun IFN-γ productie in een maligne omgeving. Verder wordt ook de innate cytotoxische capaciteit van γδ T-cellen verhoogd door IL-15 DCs, zowel ten opzichte van leukemische cellijnen als allogene primaire AML blasten. Tot slot tonen we aan dat gesecreteerd IL-15 door de IL-15 DCs het voornaamste mechanisme is achter de IL-15 DC-gemedieerde γδ T-celactivatie.

Naast γδ T cellen en IL-15, is een belangrijke focus van dit doctoraatsonderzoek de CD56 molecule. Deze molecule komt in de verschillende hoofdstukken aan bod. Bijvoorbeeld in hoofdstuk 2 zagen we dat het aandeel van $CD56^+$ γδ T cellen lijkt te worden bepaald door de graad van γδ T celactivatie. Bovendien hebben we reeds in voorafgaand onderzoek aangetoond dat een belangrijk deel van de IL-15 DCs CD56 tot expressie brengen. In **hoofdstuk 6** geven we een overzicht van onze huidige kennis omtrent de expressie van CD56 in het hematopoëtische systeem.

In **hoofdstuk 7** onderzochten we de expressie en functie van de CD56 molecule op immuuncellen. Zo zijn we de eerste groep die heeft kunnen aantonen dat CD56 een directe rol speelt in de tumorceldodend vermogen van NK cellen, γδ T-cellen en IL-15 DCs. Verder detecteerden we ook een directe wisselwerking tussen IL-15 DCs en CD8 T-cellen door middel van CD56 als co-stimulerende molecule. Bovendien beschrijven en visualiseren we voor de eerste keer de homodimere CD56 interactie, enerzijds tussen kankercellen onderling en anderzijds tussen immuuncellen en tumorcellen. Tenslotte bevestigden we onze hypothese dat IL-15 stimulatie direct aanleiding geeft tot een verhoging van CD56 expressie.

Rekening houdend met het belang van fosfoantigenen bij de *in vitro* en *in vivo* stimulatie van γδ T-cellen, bespreken we in het **addendum** hoofdstuk de rationale achter het gebruik van bisfosfonaten bij kankerpatiënten en bekijken we het klinisch gebruik van deze groep geneesmiddelen in de behandeling van zowel hematologische maligniteiten als solide tumoren.

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GENERAL INTRODUCTION

CANCER IMMUNOTHERAPY: THE ACUTE MYELOID LEUKEMIA CASE

Cancer is a growing and global problem. When looking at developed regions, more than a quarter of the total amount of deaths in the European Union (2014) were attributed to cancer (Eurostat) and the '3rd edition of Global cancer facts' of the American Cancer Society points at cancer as the second leading cause of death in high-income countries (following cardiovascular diseases). Worldwide cancer deaths are projected to increase by 60% by 2030 purely due to the growth and aging of the population, thereby even omitting the rise in cancer burden resulting from the growing problem of adapting the unhealthy lifestyle of high-income countries including poor diet, physical inactivity, psychological / emotional stress and smoking.[1] Despite recent successes in the field of cancer therapy, there is still a great deal to be desired in the effective treatment of disseminated cancer.

The origin of the field of cancer immunotherapy is founded in the notion that the immune system not only protects the body from infection, but also plays a major role in preventing malignant cells from dividing and metastasizing. It can be traced back to William Coley who, in the 1890s, observed that potentially fatal bacterial infections could induce an effective anti-tumor response in patients with partially resected tumors.[2] It would take another 19 years till Paul Ehrlich proposed the cancer immunosurveillance hypothesis, conveying the theory that the host defense eliminates malignant cells before they can cause harm.[3] Since then we have witnessed huge developments in the field. Immunotherapy is the first broadly successful strategy to treat metastatic cancer and has even become standard treatment in some cancer types alongside surgery, chemotherapy and radiotherapy. Newer types of immune-based treatments are being studied every day, pushing the still-nascent field forward, and the prospects are that they will impact how we treat cancer in the future even more. The latter is reflected by the fact that the 2018 Nobel Prize in Physiology or Medicine has been awarded to Prof. James P. Allison and Prof. Tasuku Honjo for their groundbreaking contributions to the field of cancer immunotherapy.

In order to design effective immunotherapies, it is essential to understand how cancer interacts with the immune system and where it goes wrong. Reference can be made to the process of cancer immunoediting, consisting of three phases.[4] The first phase is the elimination stage or the classic concept of immune surveillance, whereby the innate and adaptive immune system join forces to eliminate cancer cells. Sporadic tumor cells that manage to survive immune destruction may then enter an equilibrium phase, in which tumor cells persist as dormant cells and steadily modulate their immunogenicity. In the final escape phase, immunologically sculpted tumors start to develop progressively, become clinically apparent and establish an immunosuppressive microenvironment. Recently, it became manifest that, in addition to classic immune escape mechanisms, nutrient dearth and metabolic stress in tumors also impair immune cell anti-tumor immunity.[5] Immune cell recovery therefore requires a multifaceted approach.

The aim of cancer immunotherapy is to restore or strengthen the power of the anti-tumor immune response. Different approaches are being investigated with this end goal, though protocols vary largely from one another. This creates a range of possible therapeutic methods, each with its own benefits and disadvantages.[6] Well-known strategies include the use of therapeutic antibodies (e.g. rituximab), cytokines, immune checkpoint modulators (e.g. nivolumab), oncolytic virus therapy and adoptive cell transfer, including the use of T cells genetically engineered to express T cell receptors (TCRs) or chimeric antigen receptors (CARs). In this PhD thesis, however, we will focus on DC vaccination and the use of γδ T cells.

Dendritic cells

DCs are known as the orchestrators of the human immune system, activating both the innate and adaptive immune system. They are best recognized for their antigen-presenting capacity, being the capture, processing and presentation of antigens to T cells stimulating antigen-specific immunity. As such, they are the only antigen-presenting cells (APCs) of the human immune system endowed with the potential to prime naïve T cells into antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) or T helper cells. Therefore, DCs are regarded as ideal candidates to generate cancer vaccines.[7,8]

To date, different cell sources and manufacturing processes have been explored to obtain DCs for vaccination. Initially, native blood DCs were used. However, low prevalence of circulating DCs and the complex procedure to obtain them, led to disuse. Hence, most clinical experience has been obtained with DCs cultured *ex vivo* from monocytes, being precursor cells available in much greater quantities. The gold-standard monocyte-derived interleukin (IL)-4 DC vaccine is produced by isolating $CD14^+$ monocytes from leukapheresis products and by culturing them for five days in medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4.[7] The activation/maturation of these immature DCs is usually achieved by adding a mixture of pro-inflammatory cytokines (Jonuleit cocktail) to the cultures.[9] Looking back at two decades of DC therapy for cancer, it can be ascertained that DC-based vaccination is safe, well-tolerated and capable of inducing anti-tumoral immune responses.[8,10] By way of example, investigating the effects of DC vaccination in AML patients at very high risk of relapse, our phase I/II clinical trial demonstrated the feasibility and safety of DC vaccination, and indicated already its immunogenicity.[11] Following from this, we recently reported the results of a phase II clinical study, showing improved overall survival rates correlated with T cell responses.[12] However, when we consider the bigger picture, clinical responses are still limited to a small number of patients,[8] stressing the need for further improvement of the DC vaccine preparation protocol.

Many research teams, including ours, embarked on a search for more immunostimulatory DCs, resulting in a lot of different approaches and protocols.[7,8] Hence, given the extensive state-of-the-art literature on the topic, we will not aim to provide an exhaustive compilation of available protocols. We will rather touch on some salient examples in the field that will provide a frame of reference to the performed research. One such aspect is the use of other stimulatory signals, apart from cytokines, to induce DC maturation. DCs are indeed rather refractory to the maturation inducing effects of the Jonuleit cocktail. However, different toll-like receptor (TLR) ligands, including poly(I:C) (TLR3)[13], LPS (TLR4),[14] and R848 (TLR7/8),[15] are able to generate strong danger signals for the induction of DC maturation. Another way to functionally activate DCs is the use of immunogenic apoptosis,[16-18] or the exposure of DCs to tumor-cell lysates.[19,20] The electroporation of immature DCs with a mixture of mRNA, coding for a constitutively active TLR4, CD40L and CD70 (TriMix), incites DC maturation as well.[21] The latter already touches on the use of different methods to improve the DC-T cell interaction by redirecting the balance between activating and inhibitory signals.[22-24] Looking into different DC subsets as vehicles for vaccination is of interest too. By way of example, the Langerhans cell-phenotype of DCs has been ascribed with superior immunostimulatory properties.[15,25-27] In this regard, we designed a novel protocol in which IL-15 and a R848-based maturation cocktail were used, generating so called (Langerin$^+$ and CD56$^+$) IL-15 DCs; investigated in this PhD thesis.[28]

γδ T cells

The primary objective of currently available cancer immunotherapeutics is to harness the adaptive (antigen)-specific immune response against cancer.[29] Additionally, preclinical data show that when innate immunotherapy is successfully applied, this leads to subsequent long-term adaptive cancer immunity.[30-32] Hence, cancer immunologists have to go beyond αβ T cell activation. For example, γδ T cells arise from the same common multipotent double negative precursor than αβ T cells, but evolve into immune cells with entirely different characteristics. Lineage commitment is completed by the DN3 stage. However, mechanisms that direct developing human T cells to become either αβ or γδ lineage cells, still need to be determined. One favored model is the TCR signal strength model.[33-36]

γδ T cells, an enigmatic and versatile T cell subtype bridging innate and adaptive immunity, are of interest for the sake of their immunostimulatory properties and their ability to recognize and eradicate tumor cells. In particular their ability to sense metabolic changes and identify antigens out of the context of classical major histocompatibility complex (MHC) molecules has raised expectations for their use in therapeutic anti-cancer applications.[37] This is further strengthened by the fact that intratumoral γδ T cells have emerged as the most favorable prognostic immune population among many cancer types, including hematologic malignancies .[38]

In humans, there are three major subsets of γδ T cells identified by their variable (V)δ chain, namely Vδ1, Vδ2 and Vδ3 T cells. However, it is still not clear which subset of γδ T cells is more effective. It might well be that different subsets are necessary for different purposes**.** Vδ1 T cells recognize various stress-related antigens, mostly uncharacterized, and are predominant in the thymus and peripheral tissues.[39] Apart from their ability to act in an innate like fashion, similar to NK cells, Vδ1 T cells also exhibit an adaptive immunobiology. For example, they undergo profound and highly focused clonal expansion in response to specific immune challenges.[40,41] Moreover, tumor-reactive Vδ1 T cells can persist in the circulation for many years.[42] The most abundant subset of circulating γδ T cells is the Vδ2 subset (preferentially paired to the Vγ9 chain), which is mainly activated by phosphoantigens, i.e. metabolic intermediates of the isoprenoid biosynthesis.[43] It concerns, amongst others, isopentenyl pyrophosphate (IPP), produced in eukaryotic cells via the mevalonate pathway. Under physiologic conditions, levels of IPP do not activate γδ T cells. However, cancer cells display significant metabolic abnormalities, including the accumulation of mevalonate metabolites, which does provide a powerful danger signal recognized by γδ T cells.[44] Finally, the Vδ3 T cell subset is enriched in the liver and is generally more common in patients with some viral infections and leukemia.[45]

Leukemia

Leukemia refers to a group of hematopoietic malignancies, characterized by the accumulation of clonal progenitor cells arrested in their ability to differentiate into mature blood cells. The four main types of leukemia are defined based on their rate of progression (acute vs chronic) and their hematopoietic cell-lineage origin (myeloid vs lymphocytic).

AML is chosen as the primarily studied cancer type model in this thesis. It concerns a clonal neoplasm derived from myeloid progenitor cells, compromising normal hematopoiesis and thereby causing anemia, infections and/or bleedings. The high amount of leukemic cells can cause pain in bones, joints or in both. AML is the most common form of leukemia among adults and accounts for the largest number of annual deaths from leukemia.[46] Pursuant to the 2007-2013 statistics of the Surveillance, Epidemiology and End Results (SEER) program, the 5-year overall survival of AML patients is only 26.9%, with even worse prospects for AML patients over 65 years.

AML is commonly treated with chemotherapy, which consists of two sequential phases: the induction phase (aimed at achieving a status of complete remission, i.e. <5% leukemic cells in the bone marrow) and the consolidation phase (aimed at maintaining complete remission). This general therapeutic strategy in patients with AML has not changed substantially in more than 30 years. Continuous-infusion cytarabine with an anthracycline

remains the mainstay of induction therapy. Standard post-remission strategies include conventional chemotherapy as well as hematopoietic stem cell transplantation (HSCT). Despite a high complete remission rate with this therapeutic strategy, the majority of AML patients will eventually relapse. This is due to the persistence of a small population of residual leukemic (stem) cells, a condition designated as minimal residual disease (MRD).[47]

New treatment options are therefore urgently warranted. Last year the FDA approved four new drugs for the treatment of AML: (I) CPX-351, a liposomal formulation of cytarabine and daunorubicin, (II) midostaurin, an intensive chemotherapeutic for newly diagnosed FLT3-mutated AML, (III) gemtuzumab ozogomycin, an anti-CD33-conjugated anthracycline (calicheamicin) for newly diagnosed and relapsed $CD33^+$ AML and (IV) enasidenib, an IDH-2 inhibitor for adults with relapsed and refractory AML with an IDH-2 mutation.[48] However, none is specific for the eradication of MRD.

Increasing attention is focusing on identifying suitable immunotherapeutic strategies for AML. Analogous to solid tumors, AML creates an immunosuppressive microenvironment, whereby both innate and adaptive immune responses are profoundly deregulated.[49] Allogeneic HSCT and donor lymphocyte infusions have shown efficacy against MRD due to a graft-versus-leukemia effect of donor T lymphocytes, but are accompanied by morbidity and mortality. This restricts their use in the large proportion of elderly AML patients, who represent the majority of AML patients.[50-52] Yet, these findings demonstrate the role of an effective anti-leukemia immune response in relapse prevention or control, thereby stimulating the development of different strategies aimed at establishing potent autologous anti-leukemia immunity in the adjuvant setting for AML. Of interest, γδ T cells exert a graft-versus-leukemia effect as well. But, in contrast to αβ T cells, they do not show alloreactivity and therefore do not induce graft-versus-host disease.[53]

Immunotherapeutic approaches, currently being investigated but not further discussed in this thesis, include for example the use of DNA vaccines.[54] The use of isolated immunogenic tumor-associated antigen epitopes, as peptide vaccines, is another potentially promising method to prolong disease-free survival in AML patients. It generates an anti-leukemic immune response in patients against e.g. PRAME, proteinase-1, RHAMM and Wilms' tumor 1 peptides.[55-59] Furthermore, (conjugated) antibodies are being investigated as blast-targeting therapy, primarily focusing on exploiting CD33, CD123 and CD56.[60] Moreover, radioimmunotherapy through targeted antibodies is currently exploited as an alternative to antibodies conjugated to toxins.[61] Lastly, the adoptive transfer of CAR- and TCR-engineered T cells could tackle the issue of relapse due to MRD in the future.[62,63] Hopefully the next few years will lift the veil and show us which of these approaches will become general clinical practice.

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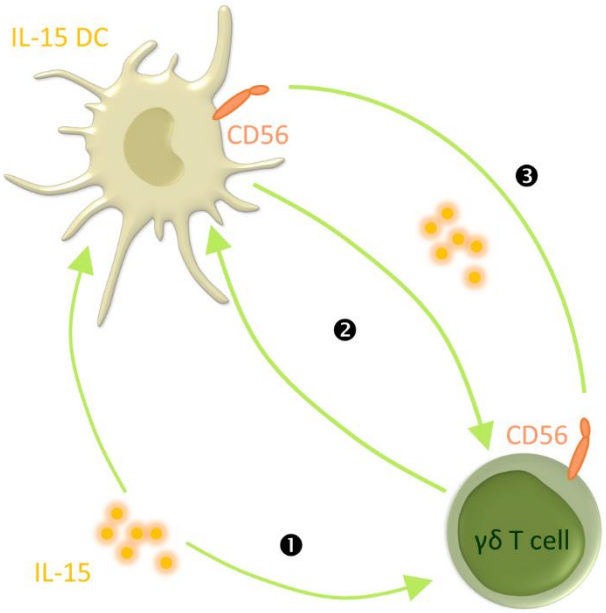
AIMS AND SCOPE

In this PhD project we hypothesize that IL-15 and IL-15 DCs are superior in the activation of immune cells and the harnessing of immune effector cells in the fight against cancer and this in comparison to the currently used standards IL-2 and IL-4 DCs, respectively. We presume that IL-15 leads to the upregulation of CD56 on immune cells, and postulate that CD56 expression not only indicates a desirable activation state, but also play a direct role in the activation of other immune cells and the killing of tumor cells.



Graphical objectives overview

Objective 1. Study the immunostimulatory effect of IL-15 on γδ T cells.

Chapter 1 – Chapter 2

Objective 2. Delineate the cross-talk between CD56^{+} IL-15 DCs and γδ T cells.

Chapter 3 – Chapter 5

- Part 1. Establish if γδ T cells are recruited by IL-15 DCs, creating the opportunity for vaccine-mediated γδ T cell activation.
- Part 2. Investigate the capacity of IL-15 DCs to harness γδ T cells in the anti-tumor immune response.

Objective 3. Unravel the role of CD56 on immune cells and elucidate if IL-15 stimulation directly leads to CD56 upregulation.

Chapter 6 – Chapter 7

CHAPTER 1:

THE ROLE OF THE COMMON GAMMA-CHAIN FAMILY CYTOKINES IN γδ T CELL-BASED ANTI-CANCER IMMUNOTHERAPY

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ABSTRACT

Cytokines of the common gamma-chain receptor family, comprising IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, are vital with respect to organizing and sustaining healthy immune cell functions. Supporting the anti-cancer immune response, these cytokines inspire great interest for their use as vaccine adjuvants and cancer immunotherapies. It is against this background that γδ T cells, as special-force soldiers and natural contributors of the tumor immunosurveillance, also received a lot of attention the last decade. As γδ T cell-based cancer trials are coming of age, this present review focusses on the effects of the different cytokines of the common gamma-chain receptor family on γδ T cells with respect to boosting γδ T cells as a therapeutic target in cancer immunotherapy. This review also gathers data that IL-15 in particular exhibits key features for augmenting the anti-tumor activity of effector killer γδ T cells whilst overcoming the myriad of immune escape mechanisms used by cancer cells.

1. INTRODUCTION

γδ T cells are a conserved population of innate lymphocytes taking part in numerous immune responses during tissue homeostasis, infectious disease, autoimmune disease, inflammation, transplantation and tumor surveillance. Since their accidental discovery in the 1980s, while characterizing the alpha beta (αβ) TCR genes,[1] γδ T cells remain an enigmatic immune cell subset which has not yet revealed all his secrets. They are very effective in thwarting most attempts at defining their exact role in the immune system. Depending on the particular context, γδ T cells share attributes of the adaptive or innate immune system, or both.[2-5] In humans, there are three major subsets of γδ T cells identified by Vδ chain, namely Vδ1, Vδ2 and Vδ3 T cells. These subsets are largely non-overlapping in function as they differ in their tissue localization and antigen recognition. Vδ1 T cells recognize various stress-related antigens, mostly uncharacterized, and are predominant in the thymus and peripheral tissues.[6] The most abundant subset of circulating γδ T cells is the Vδ2 subset (preferentially paired to the Vγ9 chain), which is mainly activated by phosphoantigens, i.e. metabolic intermediates of the isoprenoid biosynthesis.[7] The two most studied phosphoantigens are (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), produced by the non-mevalonate pathway of many bacteria and protozoan parasites, and IPP, which is produced in eukaryotic cells through the mevalonate pathway. Finally, the Vδ3 T cell subset is enriched in the liver and is generally more common in patients with some viral infections and leukemia.[8] In the present review, we focus on the effects of the different cytokines of the common gamma-chain receptor family on γδ T cells, in view of boosting γδ T cells as a therapeutic target in cancer immunotherapy.

2. γδ T CELLS IN CANCER

γδ T cells are an imperative part of the tumor immune microenvironment and are known to affect the anti-tumor immune response in a wide variety of tumors.[9] This is reflected, *inter alia*, by the fact that γδ T cells are able to infiltrate many types of tumors, including breast cancer,[10] colorectal cancer,[11] lung cancer[12] and melanoma.[13] In addition, a recent study analyzing the gene expression profiles of 18,000 samples derived from 39 different cancer types, both solid tumors and hematopoietic malignancies, identified tumor-infiltrating γδ T cells as the most significant favorable cancer-wide prognostic signature.[14] Furthermore, patients with a high γδ T cell immune reconstitution (after HSCT) show significantly improved long-term disease-free survival rates as compared to patients with

low or normal γδ T cell counts.[15-17] This can be attributed to the plethora of protective properties of γδ T cells; production of abundant pro-inflammatory cytokines, including interferon (IFN)-γ and tumor necrosis factor (TNF)-α, MHC-independent recognition of antigens, direct lysis of malignant cells, antibody-dependent cellular cytotoxicity (ADCC), establishment of a memory response and even antigen presentation.[18] Unfortunately, cancer patients often have exhausted and/or reduced numbers of γδ T cells.[19,20] On the other hand, evidence is emerging that certain subsets of γδ cells can perform regulatory undertakings, among others, by means of programmed cell death (PD)-1/PD-L1 immune checkpoint inhibition, regulatory T cell (Treg) and Th2 cell-like activity, and IL-17a production.[21-24] Therefore, in view of the use of γδ T cells as an anti-cancer therapeutic, their high functional plasticity is a hurdle to take. Namely, exposure of γδ T cells to polarizing factors present in the tumor microenvironment might induce the polarization of γδ T cells from a desirable Th1 to an unfavorable Treg and Th17 type.[25-27] On the other hand, the reverse polarization can be seen as well. For example, suppressive γδ T cells can revert to a more pro-inflammatory phenotype after TLR ligand encounter.[28,29] As a result, in the context of cancer immunotherapy, it will be vital to harness γδ T cells (back) into the anti-tumor immune response and to consolidate their functional polarization to effector γδ T cells for their use as an immunotherapeutic tool.

3. γδ T CELLS FOR CANCER IMMUNOTHERAPY

Several biotech companies aiming at bringing γδ T cells into the clinic recently saw the light of day, including Gadeta (Utrecht, The Netherlands), GammaDelta Therapeutics (London, UK), Incysus (Hamilton, Bermuda), Lymphact (Coimbra, Portugal) and TC BioPharm (Holytown, UK). Indeed, targeting γδ T cells for the modulation of anti-tumor immune responses holds many promises and (commercial) benefits. A key feature of γδ T cells is that they can perceive cancer as a metabolic disorder, through sensing altered lipid pathways, providing an alternative mechanism for detecting and eliminating malignant cells.[30] Since there is no requirement of specific tumor-associated antigens and co-stimulatory molecules for γδ T cell activation, unlike for classic αβ T cells, γδ T cells are still able to recognize cancer cells with loss of tumor MHC class I expression and other defects in antigen presentation. This offers stirring prospects for novel universal (off-the-shelf) cancer immunotherapies. A further advantage of γδ T cells, in addition to their aforementioned robust anti-tumor effects, is their chemoresistancy. This leaves γδ T cells ideal candidates for combinatorial chemoimmunotherapy, two approaches long considered mutually exclusive.[31-33]

γδ T cell cancer immunotherapy can be divided into two main approaches; *(1) in vivo* expansion of γδ T cells using primarily combinations of aminobisphosphonates and IL-2, and *(2)* the adoptive transfer of *ex vivo* activated and expanded γδ T cells.[7,18] These approaches are continually further developed and refined, comprising the use of CAR engineered γδ T cells[34] and TCR transduced cells.[35] γδ T cell immunotherapy has already been assessed against a variety of solid and hematological malignancies in early phase clinical trials, reviewed by Fisher *et al*..[36] Recent clinical trials investigating γδ T cells as immunotherapeutic are summarized in table 1-2. These trials established γδ T cell immunotherapy as feasible, i.e. they can be easily expanded both *in vivo* and *in vitro*, safe, and able to induce anti-tumor immunity. To further advance the field, large phase 3 clinical trials should now be initiated to look directly at the effectivity of γδ T cell immunotherapy (as compared to the standard treatment of care).

4. THE COMMON GAMMA-CHAIN RECEPTOR FAMILY CYTOKINES

The common gamma-chain receptor family includes six members, namely IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. These cytokines, also referred to as IL-2 family cytokines, all signal through receptor complexes that contain the common cytokine receptor gamma-chain subunit paired with a distinctive cytokine-specific subunit (Figure 1). IL-2 and IL-15, however, have an additional common IL-2/IL-15Rβ unit, resulting in a heterotrimeric complex. Signaling after binding to their receptor is channeled to three major pathways, namely *(1)* the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway, *(2)* the PI3K–Akt pathway and *(3)* the mitogen-activated protein kinase (MAPK) pathway. The common gamma-chain cytokines are indispensable in the functioning of the immune system, holding decisive functions in the development, homeostasis, proliferation, survival and functioning of various cell lineages of both the innate and adaptive immune system.[37,38] From this, it is apparent that the common gamma-chain cytokine family is exploited in many immune-related conditions.

Currently, the use of these cytokines to harness the immune system in the fight against malignant cells is one of the most important areas of cancer immunotherapy research. Consistent with this, the function and proliferative capacity of γδ T cells heavily relies on the actions of such cytokines, for which IL-2 is by far the most studied cytokine, albeit with limitations that ultimately might be averted through the use of other cytokines of the same family. Below we will discuss each cytokine of the γ_c receptor family in more detail, especially with regard to its use for γδ T cell immunotherapy (Table 3; *vide infra*).

Table 1. Clinical trials aimed at the *in vivo* expansion of γδ T cells

Paper	Disease	n	Ph	IL-2	Schedule	aBP or PAg	Schedule	Courses	Additional treatments
Pressey JG. *et al.* 2016 [39]	Refractory neuroblastoma	4	1	3 or $6x10^6$ units s.c.	Day 1-5 and 15-19 / 28 days	4 mg/m^2 ZOL i.v.	Day 1	1-3	-
Wilhelm M. *et al.* 2014 [40]	AHM	4	pilot	$1.0x10^6$ IU/m^2 s.c.	Day 1-6	4 mg/m^2 ZOL i.v.	Day 0	1	DILI and Hi-Cy/Flu
	Observed effects								
Pressey JG. *et al.* 2016 [39]	- 3- to 10-fold ↑ in circulating γδ T cells after the first week, restoration of the range seen in healthy volunteers - ↑ Treg:, likely due to stimulation from low-dose IL-2								
Wilhelm M. *et al.* 2014 [40]	- *in vivo* expansion of donor γδ T cells (mean 68-fold), with proliferation peak at ± day 8 - donor cells persisted up to 28 days - 3/4 patients achieved a complete remission								

Abbreviations: aBP, aminobisphosphonate; AHM, advanced hematological malignancies; DILI, donor innate lymphocyte infusion (day 0); Hi-Cy/Flu, 'fludarabine 25 mg/m2 day -6 until day -2 and cyclophosphamide day -6 and -5'; Pag, phosphoantigen; Ph, phase; s.c. subcutaneous; ZOL, zoledronate

Table 2. Clinical trials using adoptively transferred γδ T cells

Paper	Disease	n	Ph	Expansion condition		Cycle length (d)	No. of cells	Schedule	Courses	Additional treatments
				IL-2 (IU/mL)	aBP or PAg					
Aoki T. *et al.* 2017 [41]	Pancreatic cancer	56	1	1000	5 µmol/L ZOL	14	>1×10^9	day 4 and 18 / 4 weeks	6	1000 mg/m^2 gemcitabine on day 1,8 and 14
Wada I. *et al.* 2014 [42]	Malignant ascites	7	pilot	1000	5 µmol/L ZOL	14	Median 5.9x10^9 (0.06-6.98×10^9)	weekly	1-4	1 mg ZOL; day 0 i.v., Day 7/14/21 i.p.
Izumi T. *et al.* 2013 [43]	Colorectal cancer	6	pilot	1000	5 µmol/L ZOL	14	Mean 6.8x10^9	weekly	8	-

	Observed effects
Aoki T. *et al.* 2017 [41]	- no apparent additive beneficial effect of γδ T cell therapy on gemcitabine - more γδ T cells were detected in the blood of recurrence-free patients - % γδ T cells in the infused products was linked to clinical outcome (> 80% purity)
Wada I. *et al.* 2014 [42]	- ↓ tumor cells in the ascites, already after the first round of therapy - IFN-γ was detected in the ascites on treatment - i.p. injection → local control of malignant ascites
Izumi T. *et al.* 2013 [43]	- ↑ in circulating γδ T cells, persistence for long period

Abbreviations: aBP, aminobisphosphonate; Pag, phosphoantigen; Ph, phase

Table 3. Effects of cytokines of the common gamma-chain family on γδ T cells

	Conc.	Effect	Condition	Comparison with other cytokines or remarks	Ref
IL-2	100 IU/mL	↑ CD69	PBMC (cynomolgus monkeys)	(-) proliferation, ↑ In combination with phosphoantigens	44
	100 IU/mL	↑ CD25, CD69, CD94, CD152, CD244, ICAM-1, KLRG-1, LFA-1 and NKG2D and ↓ CD27 expression	HMB-PP stimulated PBMC	CD25/CD152/KLRG-1: ↑↑ than IL-4, ↑ than IL-21 CD69: (-) than IL-4, ↓ than IL-21 CD244: ↑ than IL-4, ↓ IL-21 ICAM-1: (-) than IL-4, ↑↑ than IL-21 LFA-1: ↑ than IL-4, (-) IL-21 CD27: ↓↓ than IL-4, ↓ than IL-21	45
	100 IU/mL	↑ CD16	Expanded and isolated γδ T cells	healthy donors and MS patients, ↓ than IL-15	46
	$1x10^9$ IU s.c. Day 1-5	(-) proliferation	cynomolgus macaques	↑ in combination with HMB-PP injection	47
	10 IU/mL	(-) proliferation	PBMC of healthy donors and MM patients	↑ in combination with zoledronate	48
	?	(-) proliferation	Isolated γδ T cells	↑ + IPP, ↑↑ + IPP + (im)mature DCs	47
	100 IU/mL	↑ proliferation	HMB-PP stimulated PBMC	↑↑ than IL-4, ↑ than IL-21	45
	50 ng/mL	IFN- γ, TNF-α	PBMC and isolated γδ T cells	↑ $CD8\alpha\beta^+$, (-) $CD8\alpha\alpha^+$ and ↓ $CD8^-$ γδ T cell subsets	49
	100 IU/mL	(-) IFN-γ, IL-18	PBMC	-	50
	100 IU/mL	(-) IFN- γ, TNF-α	PBMC	↑ In combination with phosphoantigens	51
	100 IU/mL	↑ IFN-γ, TNF-α, GM-CSF, IL-13, CXCL10	HMB-PP stimulated PBMC	CXCL10: ↑↑ than IL-4, (-) than IL-21 Rest: ↑↑ than IL-4 and IL-21	45
	50 IU/mL	↑ IFN-γ	PBMC (influenza infection)	IL-2 restores IFN-γ production in γδ T cells from obese donors	52
	100 IU/mL	(-) CD107a	PBMC	↑ In combination with phosphoantigens	51
	10 ng/mL	↑ cytotoxicity	Expanded γδ TILs	(-) IL-4 and IL-15	53
	100 IU/mL	γδ IL-2 T-APC generation	purified γδ T cells + monocytes + HMB-PP	Limited and no effect of IL-21 and IL-7	54
IL-4	100 ng/mL	↑ transcription factor Eomesodermin	Mice isolated γδ T cells	-	55
	10 ng/mL	↑ CD69, CD62L, CD94, ICAM-1, LFA-1 and NKG2D expression	HMB-PP stimulated PBMC	CD62L: ↑↑ IL-2, ↓ than IL-21 CD69: (-) IL-2, ↓ than IL-21 ICAM-1: (-) IL-2, ↑↑ than IL-21	45

				LFA-1: ↓ than IL-2, ↓ than IL-21	
	4 ng/mL	↓ NGK2D	Vδ1 T cells	Both in activation and proliferative stages	56
	10 ng/mL	↑ proliferation	HMB-PP stimulated PBMC	↓↓ than IL-2, ↓↓ than IL-21	45
	4 ng/mL	↓ proliferation	PBMC + anti-TCR Vδ1 mAb or zol + 100 IU/mL IL-2	But IL-4 promotes the proliferation of activated γδ T cells, ↑ Vδ1/Vδ2 T cell ration	56
	4 ng/mL	↓ IFN-γ, TNF-α, IL-6, IL-17a and ↑ IL-10	PBMC + anti-TCR Vδ1 mAb or zol + 100 IU/mL IL-2	-	56
	10 ng/mL	↑ cytotoxicity	Expanded γδ TILs	(-) IL-2 and IL-15	53
IL-7	10 ng/mL	↑ BTLA expression	Mouse lymphocytes enriched for γδ T cells	-	57
	50 ng/mL	↑ survival	Isolated γδ T cells	-	58
	20 ng/mL	↑ proliferation	purified splenic mouse γδ T cells	↑ IL-17 production only in the presence of IL-1β, TNF-α or TCR stimulation	59
	20 ng/mL *in vitro*	Expansion of IL-17 producing γδ T cells	Mouse *in vitro* and *in vivo* experiments	Similar results *in vitro* as compared to *in vivo* with i.p. injections of 5 µg IL-7 / mouse every 2 days for 1 week ↔ IL-2 (↑ all γδ T cells), IL-15 (↑ Th1 γδ T cells) and IL-21 stimulation	60
IL-9	2 ng/mL	↑ IFN-γ, IL-17	Isolated γδ T cells	In psoriatic arthritis patients, but not in healthy individuals	61
IL-15	10 ng/mL	↑ survival	CBMC (+alendronate)	Decline in survival of γδ T cells with IL-2	62
	2 IU/mL	↑ survival	Expanded γδ T cells	Neither 10 IU/mL IL-2 nor 40 IU/mL IL-7 supports survival and proliferation	43
	10 ng/mL	↑ growth and survival	Expanded γδ T cells	Apoptosis-resistant, but not apoptosis-sensitive γδ T cells show augmented susceptibility to IL-15	63
	10 ng/mL	↑ CD25, CD27, NKG2D	CBMC (+alendronate)	↑ than IL-2	62
	10 ng/mL	↑ CD16	Expanded and isolated γδ T cells	Healthy donors and MS patients ↑ than IL-2	46
	12.5 ng/mL	↑ proliferation, CD69, HLA-DR, CD56	Isolated γδ T cells (+IPP)	IL-2 failed to induce γδ T cell proliferation	64
	20 ng/mL	↑ expansion	TIL (+BrHPP)	IL-2 was inefficient	65
	10 ng/mL	↑ IFN-γ	PBMC (+IPP or +BrHPP)	↑ than IL-2 No IL-10, IL-13 or IL-17	66
	12.5 ng/mL	↑ IFN-γ, TNF-α	isolated γδ T cells (+IPP)	↑ than IL-2 No IL-10, TGF-β	64
	5 IU/mL	↑ IFN-γ, CD107a	Expanded γδ T cells + zol treated Daudi	↑ than IL-2 and IL-7	43

	10 ng/mL	↑ IFN-γ, TNF-α, CD107a	PBMC	IL-15 improves innate immunity after allo-HSCT	67
	10 ng/mL	↑ granzyme, perforin, CD107a, TNF-α	CBMC (+alendronate)	↑ than IL-2	62
	12.5 ng/mL	↑ cytotoxicity	isolated γδ T cells (+IPP)	↑ than IL-2	64
	10 ng/mL	↑ cytotoxicity	Expanded γδ T cells	Against zol pre-treated neuroblastoma	68
	10 ng/mL	↑ cytotoxicity	Expanded γδ TILs	(-) IL-2 and IL-4	53
	20 IU/mL	γδ IL-15 T-APC generation	Isolated γδ T cells + monocytes + HMB-PP	Limited and no effect of IL-21 and IL-7	54
IL-21	10 ng/mL	↑ CD25, CD27, CD62L, CD69, CD94, CD152, CD244, ICAM-1, KLRG-1, LFA-1 and NKG2D expression	HMB-PP stimulated PBMC	CD25/CD152/KLRG-1: ↓ than IL-2, ↑↑ IL-4 CD27/CD244: ↑↑ than IL-2 and IL-4 CD62L: ↑↑ than IL-2, ↑ than IL-4 CD69: ↑ than IL-2, ↑ than IL-4 ICAM-1: ↓↓ IL-2, ↓↓ than IL-4 LFA-1: (-) IL-2, ↑ than IL-4	45
	10 ng/mL	↑ inhibitory receptors ↓ NKG2D	PBMC (+C-HMB-PP)	-	69
	10 ng/mL	(-) proliferation	PBMC (+HMB-PP)	↓↓ than IL-2 No synergism/interference with IL-2	69
	10 ng/mL	↑ proliferation	HMB-PP stimulated PBMC	↑↑ than IL-4, ↓ than IL-21	45
	10 ng/mL	↑ CXCL10	HMB-PP stimulated PBMC	CXCL10: (-) than IL-2, ↑↑ than IL-4,	45
	10 ng/mL	↑ granzyme A/B, perforin, CD56, cytotoxicity	PBMC (+HMB-PP)	Reversible; nullified with the removal of IL-21	69

Note: data are obtained from *in vitro* experiments unless stated otherwise

Abbreviations: (-) same/no effect; BTLA, B and T lymphocyte attenuator; CBMC, cord blood mononuclear cells; Conc., concentration; i.p., intraperitoneal; mAb, monoclonal antibody; MM, multiple myeloma; MS, multiple sclerosis; ref, reference; s.c. subcutaneous administration; zol, zoledronate

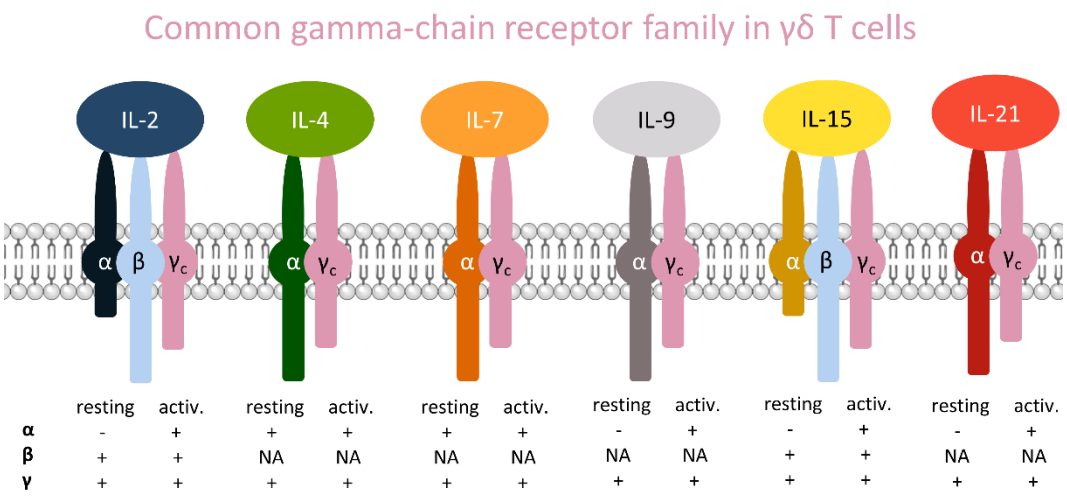

Figure 1. Overview of the expression of common gamma-chain receptors on the γδ T cell surface. Interpretation: -, not expressed; +, expressed; active, activated; NA, not applicable

4.1. INTERLEUKIN-2

IL-2 is one of the earliest cytokines discovered and was the first immunotherapy approved by the FDA to be used as a primary or stand-alone cancer treatment.[70] Originally designated as a T cell growth factor, it is now well-believed that its role in both the innate and adaptive immune system exceeds by far its original description.[71] IL-2 supports among others the cytotoxic capacity of $CD8^+$ T cells and NK cells, promotes naive $CD4^+$ T cell differentiation into Th1 and Th2 cells while inhibiting Th17 and T follicular helper (Tfh) cell differentiation,[72] and regulates plasma cell activity. Moreover, IL-2 induces activation-induced cell death and is indispensable for the generation and preservation of Treg.[73] Although desirable outside the context of cancer immunotherapy, limiting inappropriate immune reactions, the latter features represent a caveat for the use of IL-2 by tumor immunologists. A second disadvantage is the very short serum half-life of IL-2, which is in the range of minutes, due to its rapid renal clearance, necessitating frequent administration of high doses. This leads unfortunately to severe toxicities including hyperpyrexia, hypotension, vascular leak syndrome, pulmonary edema, and heart toxicities.[70]

4.1.1. γδ T cells

Focusing on γδ T cells, they are renowned for their distinct expansion through IL-2 signaling, both paracrine and autocrine.[44,74] Namely, *in vivo* γδ T cells produce high levels of IL-2,

promoting their own survival and proliferation, as a result of (among others) CD28 co-stimulation.[74] Accruing from this, the clinical manipulation of γδ T cells in the field of cancer immunotherapy and beyond largely depends on IL-2 stimulation (in combination with phosphoantigens).[36] For example, IL-2 reverses dysfunctional γδ T cell responses from obese individuals[52] and in non-human primates the combination of IL-2 and phosphoantigens controls *Mycobacterium tuberculosis* infection.[75] However, to date, clinical trials with IL-2 showed only modest efficacy, significant toxicity and even some effects counteracting the anti-tumor benefits, including immunotherapy-induced vascular endothelial growth factor release.[36,76] The question therefore arises whether this approach would generate the best results.

While binding of IL-2 to its receptor results in the activation of all three IL-2 signaling pathways in IL-2 stimulated γδ T cells, direct translation into γδ T cell proliferation or (enhanced) effector functions are not perceived. γδ T cell expansion, and IFN-γ, TNF-α and CD107a expression are only invigorated by IL-2 in addition to phosphoantigen activation.[47,48,51,77] This essential prerequisite of γδ TCR contribution results from the activation of the calcium pathway, leading to the translocation of nuclear factor of activated T cells (NFAT)1 and NFAT4 proteins to the nucleus.[51] Moreover, while clinical trials exploring the combination of IL-2 and phosphoantigens as γδ T cell therapy demonstrate positive effects on tumor growth, this approach is also associated with γδ T cell exhaustion and anergy, conforming to known IL-2 effects.[78-80] Besides, all γδ T cells cannot be tarred with the same brush. Although the majority of γδ T cells are $CD4^-CD8^-$, positive subsets exist, possibly representing functional distinct subsets.[3,81] Indeed, whereas the percentage of $CD8^-$ γδ T cells is decreased in PBMC after IL-2 stimulation, the opposite is seen for $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ γδ T cells.[49] Moreover, IL-2 signaling increases IFN-γ and TNF-α production in the $CD8\alpha\beta^+$ γδ T cells, leaves the cytokine production of the $CD8\alpha\alpha^+$ subset unchanged and obstructs the pro-inflammatory cytokine response of $CD8^-$ γδ T cells.[3] Hence, the use of IL-2 in this study actually had a detrimental effect on the anti-tumor capacity of the majority of γδ T cells.[81] Finally, in line with the immunosuppressive effect of IL-2 on classic αβ T cells, IL-2 supports Th17 γδ T cells, who emerge as an important pro-tumor subset.[24,82-84] The effect is however not unilateral. Even though IL-2 induces Th17 γδ T cell proliferation, it also negatively impacts on their survival via downregulation of the IL-7R.[84] Thus, notwithstanding the undeniable qualities of IL-2, the use of this cytokine warrants careful dosing and implementation.

Turning to the other cytokines of the common gamma-chain family cytokines, interesting properties come to light, considering they have both unique and overlapping effects. Different cytokines could therefore be of interest depending on the context and the aspired

objectives. By way of example, direct comparison between γδ T cells within PBMCs stimulated with HMB-PP and IL-2, IL-4 or IL-21 showed that HMB-PP- and IL-2-stimulated γδ T cells displayed a prototypic proinflammatory phenotype, characterized by IFN-γ and TNF-α production, but surprisingly also the Th2 cytokines IL-5 and IL-13. Conversely, Th2-like γδ T cells obtained by HMB-PP and IL-4 stimulation are found to be deficient in the secretion of IL-5 and IL-13. IL-21 and HMB-PP stimulated γδ T cells had a limited proinflammatory phenotype, although they promoted B cell maturation.[45] This is further evidenced by microarray analysis defining 513 and 328 differentially expressed genes between γδ T cells stimulated with HMB-PP in combination with IL-2 and IL-4 or IL-21, respectively (Table 3). However, these differences between stimulated γδ T cells were far outnumbered by the differences between activated and fresh γδ T cells, reflecting the family ties.[45]

4.2. INTERLEUKIN-4

IL-4 induces differentiation of naive T cells to Th2 and Tfh cells, regulates immunoglobulin class switching, and promotes mast cell survival and proliferation.[85] This results from the binding of IL-4 to IL-4Rα, leading to the recruitment of the common gamma-chain and/or IL-13Rα1, and ultimately the formation of three different types of IL-4R complexes; (I) IL-4Rα in combination with the IL-2Rγ, (II) IL-4Rα and IL-13Rα1 and (III) all three chains together, namely IL-4Rα, IL-2Rγ and IL-13Rα1.[86] In cancer, IL-4 has been ascribed both an anti-tumor and a tumor-promoting role. IL-4 was first used as an immunotherapeutic agent against malignancy.[87] However, more and more evidence is gathered of (direct) tumor-promoting effects of IL-4. Namely, increased IL-4 levels are seen in cancer patients, associated with tumorigenesis, Th2-polarized immune responses, reduced CTL activity and the differentiation of macrophages into an “M2” phenotype, exhibiting poor antigen-presenting capacity and local anti-inflammatory effects.[88-92] Owing to these knowledge gaps, more research is warranted to unravel the complete extent of the IL-4 signaling, also keeping in mind that not all of the effects are mediated via a common gamma-chain containing receptor.

4.2.1. γδ T cells

To date, the effect of IL-4 on γδ T cells is poorly studied. Potentially, IL-4 assists in the differentiation of Th1-like effector γδ T cells in the thymus and periphery, as evinced by the induction of eomesodermin, a T-box transcription factor.[55] Furthermore, although no direct effect of IL-4 on γδ T cells was reported, patients with low-grade non-Hodgkin lymphomas had a better prognosis with increased circulating Vδ1 T cells and high levels of serum IL-4.[93] Conversely, IL-4 confers an inhibitory effect on the activation of naive γδ T cells, namely

reduced γδ T cell proliferation and seriously suppressed IFN-γ and TNF-α production, but induced IL-10 production.[56] This IL-4-mediated inhibition of γδ T cell activation, in the presence of TCR stimulation, was attributed to the suppression of among others the Ca^{2+} release.[93] Unexpectedly, IL-4 does promote the proliferation of already activated γδ T cells, with a predisposition of the Vδ1 subset.[93] Therefore, IL-4 signaling seems to induce a seemingly conflicted dual effect; inhibition of TCR signaling and promotion of proliferation. Altogether, in view of the immunoregulatory profile of the expanded Vδ1 T cell subset by IL-4, it can be speculated that IL-4 will weaken the γδ T cell-mediated anti-tumor immune response.

4.3. INTERLEUKIN-7

IL-7 is indispensable for the adaptive immune system, being required for optimal T cell DC interaction and promoting proliferation and cell survival of both naive and memory T cells.[94] Furthermore, IL-7 signaling is essential in early thymocyte development, it enhances the function of immune effector cells and is able to antagonize the immunosuppressive milieu created by malignancies.[95] Consequentially, IL-7 raises interest as an immunotherapeutic in view of its potential to reconstitute the immune system.[96] This also includes the use of IL-7 as stimulus for immune cells for adoptive transfer. For example, CAR-modified T-cell therapy is an emerging strategy that has already shown unprecedented results in CD19-expressing hematological malignancies.[97] Whereas IL-2 stimulation for T cell expansion is standard, it has been shown that IL-7 and IL-15 are superior for preserving T memory stem cells in the product, as well as improve anti-tumor activity and survival of CAR-modified T cells after consecutive antigen exposure.[98] Moreover, clinical trials with recombinant IL-7 have shown both its safety and immunostimulatory capacity, manifested by elevated numbers of circulating $CD4^+$ and $CD8^+$ T cells, enhanced diversity in TCR repertoire and decreased figures of Treg cells.[99-102]

4.3.1. γδ T cells

It seems that γδ T cell propagation and IL-7 signaling are linked as well. For example, in humans a correlation was reported between γδ T cell numbers and IL-7 levels in the gastric mucosa in the context of gastritis.[103] Mice with transgenic overexpression of IL-7 showcase a substantial increase in dermal γδ T cells.[104] On the other hand, thymi of $IL\text{-}7^{-/-}$ mice are completely devoid of γδ T cells and lack $TCR\gamma\delta^+$ dendritic epidermal T cells.[104,105] Additionally, intraepithelial γδ T cells are considerably decreased in the small intestines of $IL\text{-}7^{f/f}$ FoxN1-Cre mice.[105] Therefore, the question arises as to which γδ T cell subset is promoted by IL-7. It has been shown that IL-7 preferentially enriches IL-17-competent γδ T

cells, rendering Th17 γδ T cells the main subset in the thymus at the expense of IFN-γ-competent γδ T cells.[60,106] These data are opposed to IL-2 stimulation, supporting all γδ T cells (with a predisposition of the IFN-γ producing subset) and IL-15 stimulation which only supported the Th1 γδ T cell subset. The induction of lymph node and skin resident Th17 γδ T cells seems, however, self-limiting, considering IL-7 signaling induces the expression of the inhibitory receptor B and T lymphocyte attenuator (BTLA), which in turn limits IL-7-dependent responses.[57] In mice, the induction of Th17 γδ T cells has been therapeutically investigated with a recombinant *Mycobacterium bovis* bacillus Calmette–Guérin expressing antigen 85B-IL-7 fusion protein.[107] Interestingly, here the increase in Th17 γδ T cells led to an elevated Th1 response as a reaction to mycobacterial infection.[107] The latter might explain why in a mouse model of disseminated neuroblastoma, the addition of IL-7 as a combination therapy with γδ T cells and anti-GD2 antibodies offered a supplementary survival benefit on top of a protective effect on γδ T cell survival.[58]

4.4. INTERLEUKIN-9

Similar to the other members of common gamma-chain family cytokines, IL-9 has pleiotropic functions, inducing the proliferation, differentiation and effector functions of numerous immune cell subsets, among others T and B cells, eosinophils, neutrophils and mast cells.[108] Herewith, IL-9 is suggested to be involved in the anti-tumor immune response of which most evidence exists in the melanoma setting.[109-111] The general research interest in the characteristics of IL-9 is, nevertheless, fairly recent, making the available knowledge on the effects of IL-9 on γδ T cells scarce. The only report of Guggino *et al.* investigated IL-9 signaling in γδ T cells of psoriatic arthritis patients, showing an increase in IFN-γ^+ IL-17^+ γδ T cells of patients after stimulation with IL-9, but not in healthy controls in accordance with their reduced expression of IL-9R.[61]

4.5. INTERLEUKIN-15

The IL-15R is a heterodimeric receptor consisting of the common cytokine receptor gamma-chain and the IL-2/IL-15Rβ-chain, also known as CD122. Similar activities as IL-2 are therefore expected of IL-15. However, IL-15 also binds to a specific receptor part, namely IL-15Rα, forming a heterotrimeric receptor complex with IL-2/IL-15Rβ and γ_c on neighboring cells (trans-presentation) or the same cell (cis-presentation, using IL-15 derived from autocrine or paracrine sources). Although binding with IL-15Rα increases the bioavailability of IL-15, signaling by membrane-anchored IL-15 and (high concentrations of) soluble IL-15 as such, is possible as well.[112]

IL-15 plays a central part in stimulation of both innate and adaptive immune cells. This includes the development, proliferation and activation of classic αβ T cells, γδ T cells, NK cells, NKT cells and B cells, the reactivation of memory T cells and the induction of DC maturation.[113-115] Furthermore, IL-15 endorses the survival of immune cells by averting apoptosis through the upregulation of anti-apoptotic and downregulation of pro-apoptotic factors. An absolute plus for cancer immunotherapy is the fact that IL-15, in stark contrast to IL-2, does not provoke activation-induced cell death and Treg induction, and it can actually protect effector immune cells from the immunosuppressive activities of Treg cells.[116,117] Because of the drawbacks of IL-2 therapy and the additional advantageous properties of IL-15, interest has grown in IL-15 as stimulator of the immune system over IL-2. The latter observation is reinforced by the prognostic value of IL-15 as a biomarker in cancer.[118] It may therefore come as no surprise that IL-15 was ranked third on the CITN priority list of immunotherapy agents after anti-PD-1 and anti-CD40.[119]

The first-in-human clinical trial was, moreover, very promising, demonstrating the safety of IL-15 administration in metastatic cancer patients as well as NK cell, monocyte, γδ T cell and memory $CD8^+$ T cell activation.[120] In analogy with this, the first clinical trial with the IL-15 superagonist ALT-803 showed that the therapy was well-tolerated and even resulted in clinical responses in AML and myelodysplastic syndrome patients who relapsed following HSCT.[121] Additionally ALT-803 stimulated NK cell, $CD8^+$ T cell and γδ T cell expansion.[121,122] Proceeding to immune cell therapy for adoptive transfer, CAR T cells stably co-expressing IL-15/IL-15Rα countered leukemia engraftment and exhibited the promise of sustained resistance by displaying a memory stem cell phenotype.[123]

4.5.1. γδ T cells

From the above, it stands to reason to have high expectations of IL-15-mediated γδ T cell activation. In celiac disease intestine, the presence of IL-15 induces γδ T cell recruitment into the intraepithelial compartment, which is not seen with IL-2 and IL-4, and only to a lesser extent with IL-7, suggesting that IL-15 may be a potent chemotactic for γδ T cells.[124,125] In addition, IL-15 controls the generation of the restricted TCR repertoire of γδ intestinal intraepithelial lymphocytes and is critical for their effector functions.[126,127] Next, the redundant role of IL-15 regarding γδ T cell homeostatic expansion has been shown in murine models, demonstrating the dependency of both classic αβ T cells and γδ T cells on IL-15, and to a lesser degree on IL-7, for their proliferation. This to such an extent that competition for IL-15, trans-presented by DCs, dictates the interplay between γδ T cells and αβ T cells.[128-130] Furthermore, in line with the characteristics typical of a survival factor, apoptosis-resistant γδ T cells, but not their susceptible counterparts, exhibit enhanced sensitivity to IL-15, through enhanced expression of the IL-15Rα.[63] The same is seen with

ex vivo cultures of γδ T cells, showing a prolonged survival with IL-15, as compared to IL-2, associated with improved effector functions.[43,62] Herewith, isolated γδ T cells are able to proliferate in response to IL-15 + IPP, but not to IL-2 + IPP,[64] and the combination of IL-15 and bromohydrin pyrophosphate (BrHPP) was able to expand γδ tumor-infiltrating lymphocytes (TILs) from renal cell carcinoma patients, whereas BrHPP and IL-2 did not.[65] In the context of adoptive cell transfer, IL-15 boosts IL-2/zoledronate-mediated expansion and activation of γδ T cells of healthy donors and of AML patients.[64] The latter observation alludes to cellular immunotherapy benefiting of the unique characteristics of IL-15 stimulation.

It has been shown that IL-2/IL-15 signaling is required for the differentiation of immature γδ thymocytes into Th1 cytotoxic γδ T cells.[131] In general, opportune γδ T cell subsets are supported by IL-15, namely $CD122^+$ Th1 γδ T cells are maintained by IL-15 whereas $CD25^+$ Th17 γδ T cells are sustained by IL-2.[64,132-134] With regard to the influence of IL-15 on γδ T cell cytotoxicity, γδ T cells expanded from cord blood mononuclear cells using IL-15 and alendronate produce the cytotoxic mediators perforin, granzyme B and TNF-α.[62] Interestingly, this production was associated with CD56 expression, which defines γδ T cells with increased anti-tumor activity.[62,135] The same CD56 upregulation was seen with isolated γδ T cells stimulated with IPP and IL-15, concomitant with improved tumor cell killing.[64] Expanded γδ TILs from rectal tumors have also been shown to demonstrate improved killing capacity against the rectal carcinoma cell line HR-8348 and the Burkitt's lymphoma cell line Daudi.[53] The low affinity type III Fcγ receptor CD16, involved in, among others, phagocytosis, ADCC, and the acquisition of potent APC function, is as well upregulated by IL-15.[46,136] The combination of TCR triggering and IL-15 generates γδ T APCs that are able to process different antigens for presentation and to stimulate other immune cells.[54,137] In turn, the IL-15 DC vaccine has been shown to induce γδ T cell activation through *in situ* IL-15 secretion.[138] From this we can conclude that, thus far, IL-15 seems the most preferable cytokine from the common gamma-chain cytokine family to harness γδ T cells in the anti-tumor immune response, improving clinical responses of human γδ T cell-based immunotherapy.

4.6. INTERLEUKIN-21

IL-21, mainly produced by $CD4^+$ T cells and NKT cells, is the most recently described cytokine belonging to the common gamma-chain cytokine receptor family. Also, this cytokine has been ascribed pleiotropic properties, including, but not limited to, enhancing NK cell and $CD8^+$ T cell cytotoxicity, modulating plasma cell differentiation and inhibiting Treg cells. On the other hand, IL-21 seems to sustain Th17 development, induces IL-10 secretion from T

cells and NK cells, and prevents GM-CSF-mediated activation and maturation of DCs.[139,140] Although careful evaluation of the breadth of actions of IL-21 is necessary to optimize its favorable anti-cancer effect, the results of the first clinical trial with IL-21 are promising.[62]

4.6.1. γδ T cells

IL-21 induces γδ T cell division to some extent, but is unable to sustain long-term antigen-induced proliferation of *ex vivo* γδ T cells as opposed to for example IL-2 and IL-15. Therefore, it will not be likely to design γδ T cell adoptive transfer protocols solely based on IL-21. Moreover, there is no contribution of IL-21 to IL-2-mediated γδ T cell expansion.[69] Advantage could, however, be attained by IL-21 as an enhancer of the expression of lytic effector molecules, including granzyme A/B, perforin and CD56,[69] and the suppression of IL-17 producing γδ T cells through induction of apoptosis.[141,142] On the flip side, increased expression levels of inhibitory NK receptors and lower levels of natural killer group 2D (NKG2D) receptor were seen as well with the addition of IL-21.[69] Yet, the net effect of IL-21 stimulation on γδ T cell killing capacity seems positive, albeit reversible, fading away rapidly upon IL-21 removal.[69]

5. CONCLUSON

As discussed in this review, the use of γδ T cells for (cellular) immunotherapy is highly promising. Today γδ T cell-based immunotherapies are still early stage and have likely not yet reached their full potential. More insight in γδ T cell biology is needed as to why only certain γδ T cell subsets hold robust anti-tumor effects, whereas others possess immunoregulatory properties. The use and/or activation of the appropriate γδ T cell subset, with preservation of their functionality during treatment, is therefore of key importance. This is the first comprehensive review analyzing our current understanding of the effects that cytokines pertaining to the common gamma-chain receptor family exert on γδ T cells. From the presented data the pivotal role played by these cytokines concerning γδ T cell functionality emerges. In particular, the role and effect of IL-15 seems to outcompete the other members of this family (Figure 2). This cytokine provides the necessary stimulatory signals for γδ T cell expansion, survival, unbiased Th1 promotion and cytotoxicity, with little evidence of potential downsides so far, holding potential as a protective agent against immunosuppressive cells and milieus. Hence, future advances in the field are expected with the implementation of IL-15 in adoptive transfer protocols or its use *in vivo* as general immune cell activator. Therefore, the importance of potential combinatory treatments is warranted; not only to increase the anti-tumor activity of effector killer cells such as γδ T cells, but also to overcome the myriad of immune escape

mechanisms used by cancer cells. From this review, we conclude that IL-15-activated γδ T cells will arise as strong candidates for future cancer immunotherapies, holding the key to unlock cancer immune escape and resistance in both active and passive strategies.

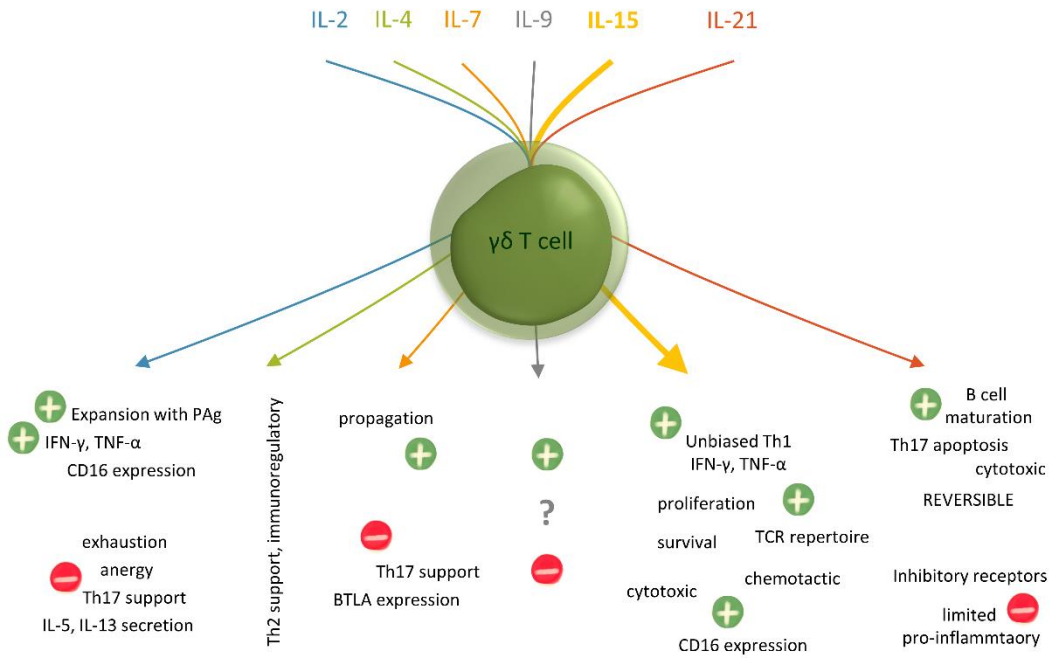

Figure 2. Graphical summary. Illustration of the effects of the common gamma-chain receptor family cytokines on γδ T cells. Pro-tumor responses are grouped around red minus signs and anti-tumor effects are grouped around green plus signs.

Abbreviations: IFN-γ, interferon-γ; IL, interleukin; PAg, phosphoantigen; TCR, T cell receptor; Th, T helper, TNF-α, tumor necrosis factor alpha

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CHAPTER 2:

INTERLEUKIN-15 ENHANCES THE PROLIFERATION, STIMULATORY PHENOTYPE AND ANTI-TUMOR EFFECTOR FUNCTIONS OF HUMAN GAMMA DELTA T CELLS

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ABSTRACT

Background. Adoptive immunotherapy is gaining momentum to fight malignancies, whereby γδ T cells have received recent attention as an alternative cell source as to natural killer cells and αβ T cells. The advent of γδ T cells is largely due to their ability to recognize and target tumor cells using both innate-characteristic and TCR-mediated mechanisms, their capacity to enhance the generation of antigen-specific T cell responses and their potential to be used in an autologous or allogeneic setting.

Methods. In this study, we explored the beneficial effect of the immunostimulatory cytokine IL-15 on purified γδ T cells and its use as stimulatory signal in the *ex vivo* expansion of γδ T cells for adoptive transfer. The expansion protocol was validated both with immune cells of healthy individuals and AML patients.

Results. We report that the addition of IL-15 to γδ T cell cultures results in a more activated phenotype, a higher proliferative capacity, a more pronounced T helper 1 polarization and an increased cytotoxic capacity of γδ T cells. Moreover γδ T cell expansion starting with peripheral blood mononuclear cells from healthy individuals and AML patients is boosted in the presence of IL-15, whereby the anti-tumor properties of the γδ T cells are strengthened as well.

Conclusion. Our results support the rationale to explore the use of IL-15 in clinical adoptive therapy protocols exploiting γδ T cells.

1. INTRODUCTION

Cellular immunotherapy for cancer is a rapidly evolving field, endeavoring to deal with common relapse or resistance to conventional treatments. Herewith, most interesting are the γδ T cells, a T cell subset possessing a combination of innate and adaptive immune cell traits.[1] Activated γδ T cells have strong cytotoxic effector functions, by means of both death receptor/ligand and cytolytic granule pathways. Moreover, they have an immunoregulatory function, producing various cytokines, including Th1-associated cytokines TNF-α and IFN-γ.[1] γδ T cells are considered to be important players in cancer immune surveillance evidenced by; *(a)* the increased incidence of tumors in γδ T cell deficient mice,[2,3] *(b)* the overrepresentation of γδ T cells in reactive lymphatic regions associated with neoplasia, including AML,[4] *(c)* their infiltration into solid tumors[5] and *(d)* their potential to kill a variety of tumor cells.[1] Pointedly, tumor-infiltrating γδ T cells recently emerged as the most significant favorable prognostic immune population across 39 malignancies.[6] As γδ T cells can kill tumor cells without previous contact and do not induce graft-versus-host disease, the adoptive transfer of γδ T cells could be a promising alternative for HSCT and adoptive transfer of αβ T cells.[7]

Among the various tumor targets of γδ T cells[8] we focused on AML, a heterogeneous hematological malignancy involving the clonal expansion of myeloid blasts in the bone marrow and peripheral blood. Although significant improvement in treatment of AML has been made, the unfortunate reality is that currently available treatments are largely ineffective for most AML patients.[9,10] In pursuit of new treatment options, several lines of evidence suggest that immunotherapy is an active modality in AML.[11] This includes the graft-versus-leukemia effect associated with allogeneic HSCT, where it has been demonstrated that elevated γδ T cell immune recovery after HSCT is associated with a better outcome in terms of infections, graft-versus-host disease and overall survival.[12-14] Moreover, while the use of HSCT is restricted to a minority of patients due to, among other, its high transplant-related mortality and morbidity,[10,15] γδ T cell immune therapy is well-tolerated and safe.[7,16] Adoptive transfer of γδ T cells is therefore an interesting alternative to tackle minimal residual disease and the high relapse rate in AML patients, optionally in combination with HSCT[17] or other (new) therapeutic agents.[18-20] Overall, while γδ T cell therapy holds great promise, clinical results are thus far modest, underscoring the need to further enhance the immunogenicity of the γδ T cell product.[21]

γδ T cells can be expanded, using combinations of cytokines and phosphoantigens (e.g. IPP) or aminobisphosphonates (e.g. zoledronate).[22] The inclusion of aminobisphosphonates relies on their inhibition of farnesyl pyrophosphate (FPP) synthase, a key enzyme of the

mevalonate pathway, leading to accumulation of mevalonate metabolites such as IPP.[23] When PBMCs are treated with zoledronate, IPP will selectively accumulate in monocytes, due to the efficient drug uptake by these cells.[24] Therefore in the absence of monocytes, addition of aminobisphosphonates is inefficient for induction of γδ T cell expansion, and the use of IPP or related phosphoantigen is required. To date, the standard protocol for the expansion of γδ T cells out of PBMC relies on the combination of zoledronate and IL-2.[25]

Limitations on the use of IL-2, relating to among other its toxicity, invigorate the investment in exploring other cytokines of the IL-2 family (also called the common γ chain cytokine family; IL-4, IL-7, IL-9, IL-15, and IL-21).[26] IL-15 is of interest as it is closely related to IL-2 and has got the top position in the US National Cancer Institute's ranking of 20 immunotherapeutic drugs with the greatest potential for broad usage in cancer therapy.[27] Moreover, both cytokines mediate their effects through a heterotrimeric receptor complex consisting of a cytokine specific α-chain (IL-2Rα or IL-15Rα), the IL-2/15R β-chain and the common γ-chain.[28] IL-2 and IL-15 share many functions in regulating both adaptive (stimulation of T cell proliferation and induction of CTLs) and innate (activating NK cells) immune responses.[29] In spite of the resemblances between both cytokines, they also have different functions *in vivo*. IL-15 is specifically known for its role in the maintenance of long-lasting, high-avidity T-cell responses, whereas IL-2 may induce activation-induced cell death and provoke maintenance of regulatory T cells.[26,30] Therewithal, IL-15 has shown efficacy in murine models of malignancy, even when IL-2 failed.[31,32] Clinical trials with IL-15 as monotherapy have recently been initiated, investigating its therapeutic potential. When looking at the lymphocyte subsets in blood, both γδ T cell proliferation and activation were observed after IL-15 administration.

These results stressed the need for a detailed characterization of the sheer effect of IL-15 on untouched and isolated γδ T cells. The latter will be discussed in the first part of this paper, followed by the translation of the results into a γδ T cell expansion protocol for adoptive transfer.

2. MATERIALS AND METHODS

2.1. ETHICS STATEMENT AND CELL MATERIAL

This study was approved by the Ethics Committee of the Antwerp University Hospital (UZA; Edegem, Belgium) under the reference number B300201419756. Experiments were performed using buffy coats derived from healthy volunteer whole blood donations (supplied by the blood bank of the Red Cross, Mechelen, Belgium) and blood samples from

patients with AML obtained from the hematological division of the UZA (Table 1). PBMC were isolated by Ficoll density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare, Diegem, Belgium). Untouched γδ T cells were isolated from PBMC using the EasySep™ Human Gamma/Delta T Cell Isolation Kit (Grenoble, France), according to the manufacturer's instructions. The purity of the γδ T cells was on average 94% [min-max; 89-98%]. Isolated γδ T cells ($1x10^6$ cells/mL) were cultured for 5 days in Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Merelbeke, Belgium), supplemented with 10% fetal bovine serum (FBS, Life Technologies), IPP (30 µg/mL; tebu-bio, Le-Perray-en-Yvelines, France) and IL-2 (100 IU/mL; Immunotools, Friesoythe, Germany) or IL-15 (12.5 ng/mL; Immunotools). The Burkitt's lymphoma tumor cell line Daudi was kindly provided to us by the laboratory of Prof. Kris Thielemans (Free University of Brussels, Brussels, Belgium) and the multiple myeloma cell (MM) line U266 was a gift from Prof. Wilfred Germeraad (Maastricht University Medical Center, Maastricht, the Netherlands).

Table 1. AML patient characteristics

P	Sex	Age (yr)	WHO type	Disease stage	PB blast %	BM blast %	Molecular	Cytogenetics
A	f	51	AML-nos	R1	6.5	16.2	$WT1^+$	Nl
B	m	51	AML-rga	Dx	50	64.4	$NPM1^+$, $WT1^+$	nl
C	m	62	AML-nos	Dx	2	72	$ASXL1^+$	trisomy 8
D	m	61	AML-nos	Dx CR1	47.8 0	79.8 0.6	negative	deletion 17p
E	f	76	AML-mds	evolution from MDS	18	ND	ND	ND
F	m	52	AML-rga	Dx	92.5	94.5	$WT1^+$, $NPM1^+$, $FLT3\text{-}ITD^+$	inv(3)(q21q26)
G	f	62	AML-nos	CR1	0	0.8	$ASXL1^+$	trisomy 8

Abbreviations: AML, acute myeloid leukemia; P, Patient; f, female; m, male; WHO, World Health Organization (WHO) 2008 classification for AML; AML-nos, AML, not otherwise specified; AML-rga, AML with recurrent genetic abnormalities; AML-mds, AML with myelodysplasia (MDS)-related changes; R1, first relapse of AML; Dx, diagnosis stage; CR1, first complete hematological remission of AML; PB blast %, percentage of AML blasts in peripheral blood; BM blast %, percentage of AML blasts in bone marrow; ND, no data; WT1, overexpression of Wilms' tumor 1 (WT1) gene transcript; NPM1, presence of mutated nucleophosmin 1 (NPM1); ASXL1, presence of mutation in additional sex combs 1 (ASXL1) gene; FLT3-ITD, presence of internal tandem duplication of fms-like tyrosine kinase 3 (FLT3); nl, normal karyotype.

2.2. PROLIFERATION ASSAY

To test the ability of IL-2 and IL-15, in combination with IPP, to induce γδ T cell proliferation, a 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Merelbeke, Belgium) flow cytometry-based proliferation assay was performed with isolated γδ T cells.

Unstimulated CFSE-labeled γδ T cells served as negative control. After 5 days, cells were stained with LIVE/DEAD® Fixable Aqua Stain (Life Technologies), CD56-PE (BD; Erembodegem, Belgium), CD3-PerCP-Cy5.5 (BD) and γδ TCR-APC (Miltenyi) and analyzed using a FACSAria II cytometer (BD). γδ T cell proliferation was assessed by quantifying the percentage of proliferating (CFSE-diluted) cells within the viable (LIVE/DEAD$^-$) CD3$^+$γδTCR$^+$ gate.

2.3. EXPANSION PROTOCOL OF γδ T CELLS (FOR ADOPTIVE TRANSFER)

PBMC were resuspended in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated human AB serum (hAB; Invitrogen, Merelbeke, Belgium), zoledronate (5 µM; Sigma-Aldrich, Diegem, Belgium), IL-2 (100 IU/mL) and/or IL-15 (100 IU/mL) at a final concentration of 1x10^6 cells/mL. Cell cultures were maintained at a cell density of 0.5-2x10^6 cells/mL and were replenished every 2 to 3 days by adding IL-2/IL-15-supplemented medium. Phenotypic and functional assays were performed on cells harvested at least 14 days after first stimulations.

2.4. IMMUNOPHENOTYPING

Freshly isolated and 5-day proliferated γδ T cells were membrane-stained with the following monoclonal antibodies; γδ TCR-FITC (Miltenyi), CD56-PE (BD), CD69-PE (BD) and HLA-DR-PE (BD). Propidium iodide (PI; Life Technologies) was added to exclude dead cells from phenotypic analysis. Data acquisition was performed on a FACScan multiparametric flow cytometer (BD). Phenotypic characterization of γδ T cells was examined pre- and post-expansion, using CD27-FITC (BD), CD69-FITC (BD), CD56-PE (BD), CD80-PE (BD), CD45RA-PE-Cy7 (BD), CD28-PerCP-Cy5.5 (BD), CD16-PB (BD), CD86-V450 (BD), γδ TCR-APC (Miltenyi), HLA-DR-APC-H7 (BD). Live/Dead® Fixable Aqua Stain was used to distinguish viable from non-viable cells. Data were acquired on a FACSAria II flow cytometer (BD). Corresponding species- and isotype-matched antibodies were used as controls.

2.5. CYTOKINE PRODUCTION

γδ T cell cultures were set up as described above. After five days of proliferation, cell-free supernatants were harvested and stored at -20°C before analysis. Samples were assessed by using enzyme-linked immunosorbent assay (ELISA) for the presence of Transforming growth factor (TGF)-β (eBioscience, Vienna, Austria) and by using electrochemiluminescence immunoassay (ECLIA; Meso Scale Discovery [MSD], Rockville,

MD, USA) for the presence of IFN-γ, TNF-α, IL-5, IL-10 and IL-17. Cytokine measurements were also performed on supernatant of γδ T cell cultures stimulated for an additional 4 hours with the tumor cell lines Daudi and U266 at an effector-to-target (E:T) ratio of 5:1.

2.6. INTRACELLULAR STAINING

After 14 days of γδ T cell expansion, IFN-γ and TNF-α production was measured using a flow cytometric-based intracellular staining assay. Measurements were also performed after an additional hour of stimulation with Daudi or U266 cells (E:T ratio = 5:1). Brefeldin A (Golgi-Plug 1 µL/mL; BD) was added to the different conditions ($1x10^6$ cells/mL) and incubated for 3 hours at 37°C/5% CO2. γδ T cells were then washed and incubated with Live/Dead® Fixable Aqua Stain, CD3-PerCP-Cy5.5 (BD) and γδ TCR-APC (Miltenyi) for 30 minutes at 4°C. Subsequently, cells were fixed and permeabilized, using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), according to the manufacturer's instructions. Intracellular staining antibodies (IFN-γ-FITC and TNF-α-PE-Cy7, BD) or the corresponding isotype control were added and allowed to bind for 1 hour at 4°C.

2.7. CYTOTOXICITY ASSAY

A flow cytometry-based lysis assay was performed in order to determine the killing activity of γδ T cells against the tumor cell lines Daudi and U266. Tumor cells were labeled prior to co-culture with PKH67 Green Fluorescent Cell Linker dye (Sigma-Aldrich), according to the manufacturer's protocol, and subsequently co-cultured with γδ T cells at different E:T ratios (1:10, 1:5, 1:1, 5:1 and 10:1). After 4 hours, cells were acquired on a FACSAria II flow cytometer following staining with annexin V-APC (BD) and PI. Killing was calculated based on the percentages of viable (annexin V^-/PI^-) cells within the $PKH67^+$ tumor cell population using the following equation: % killing = 100 − [(% viable tumor cells with γδ T cells / % viable tumor cells without γδ T cells) x 100].

2.8. STATISTICS

Flow cytometry data were analyzed using FlowJo (v10; Treestar, Ashland, OR, USA). GraphPad Prism software (v5.0; San Diego, CA, USA) was used for statistical calculations and artwork. Shapiro-Wilk normality test was performed to ascertain the distribution of the data. *P*-values < 0.05 were considered statistically significant. All data are depicted as means ± standard error of the mean.

3. RESULTS

3.1. ENHANCED γδ T CELL PROLIFERATION IN RESPONSE TO IL-15, CONCOMITANT WITH AN ACTIVATED PHENOTYPE

Stimulation of αβ T cell proliferation is a characteristic of both IL-2 and IL-15. To assess their effect on γδ T cells, in combination with IPP, a 5-day proliferation assay was performed using isolated γδ T cells (Figure 1). Unstimulated γδ T cells did not proliferate and were used as negative control. Although stimulatory effects were seen to some extent with the combination of IL-2+IPP, as evidenced by CFSE-dilution, no statistical significance was reached relative to unstimulated γδ T cells. In contrast, IL-15+IPP stimulation induced distinct γδ T cell proliferation (Figure 1). This finding was confirmed by a higher absolute number of γδ T cells after 5-day culture with IL-15+IPP. Calculating mean fold expansion, being the number of γδ T cells after stimulation divided by the number of γδ T cells at the start, IL-2+IPP and IL-15+IPP stimulation resulted in a mean fold expansion of 1.35 ± 0.15 $x10^6$ and 2.18 ± 0.31 $x10^6$ γδ T cells, respectively.

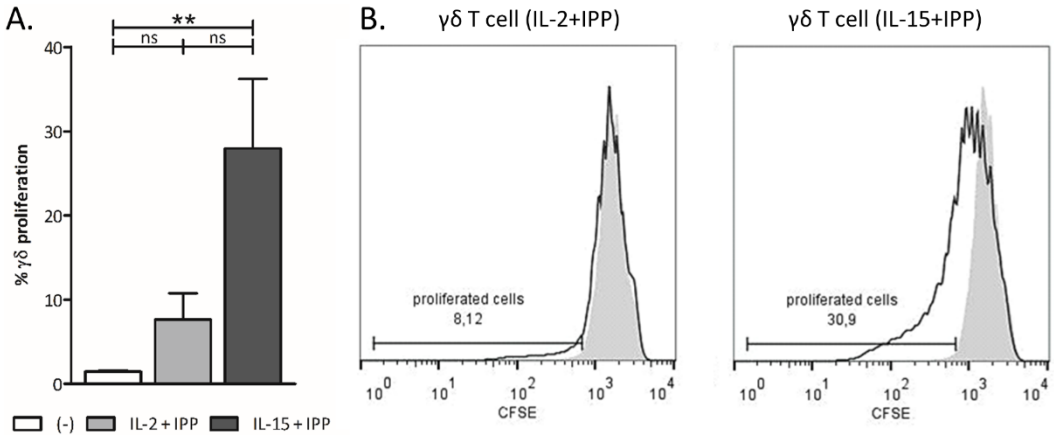


Figure 1. Purified γδ T cell stimulation with IL-15 and IPP for 5 days induces their proliferation. A. Isolated γδ T cells were stimulated with IL-2+IPP (grey bar) or IL-15+IPP (dark bar) for 5 days. Unstimulated γδ T cells (white bar) were used as negative control. The percentage of proliferated (CFSE-diluted) cells within the viable γδ T cell population was determined by flow cytometry (n = 5) **B.** Histogram overlays show CFSE dilution and gating of unstimulated γδ T cells (grey filled area) and γδ T cells exposed to IL-2+IPP or IL-15+IPP (black line) for one representative donor. Friedman test with Dunn's Multiple Comparison Test. **, $p < 0.01$; ns, not significant

Furthermore, exposure of human peripheral blood γδ T cells to IL-15+IPP resulted in an improved activated phenotype. Surface expression of CD69 augmented from 1.63 ± 0.52% (basal level) to 15.85 ± 3.80% (IL-2+IPP) and 26.55 ± 3.83% (IL-15+IPP), and HLA-DR expression went up from 12.16 ± 2.36% (basal level) to 64.58 ± 5.63% (IL-2+IPP) and 63.93 ± 7.63% (IL-15+IPP). Notably, CD56 expression was significantly higher on IL-15+IPP stimulated γδ T cells (38.34 ± 4.83%) than on unstimulated (19.20 ± 4.49%) and on IL-2+IPP stimulated γδ T cells (28.14 ± 4.73%).

3.2. IL-15+IPP STIMULATED γδ T CELLS DISPLAY STRONG TH1 CYTOKINE RESPONSES AND ARE MORE EFFECTIVE KILLERS THAN IL-2+IPP γδ T CELLS

An important question in the context of cancer immunotherapy is whether IL-15, as compared to IL-2, can boost γδ T cell effector functions, in terms of cytokine secretion and cytotoxic capacity. γδ T cells stimulated with either IL-2+IPP or IL-15+IPP are characterized by a largely pro-inflammatory cytokine profile comprising IFN-γ and TNF-α. IFN-γ levels were typically >68ng/mL/1x10^6 γδ T cells. On average 3512 ± 2140 and 4506 ± 2527 pg/mL/1x10^6 γδ T cells of TNF-α was detected in supernatant of 5-day cultures of IL-2+IPP and IL-15+IPP activated γδ T cells, respectively. With the exception of the IL-5 concentration, for which a 4.4-fold higher concentration was produced by IL-2+IPP γδ T cells, no significant difference was found in the non Th1 cytokine secretion (IL-10, IL-17 and TGF-β tested) between both γδ T cell types. After 5-days, γδ T cell cultures were harvested and exposed to Daudi or U266 tumor cells, to verify their anti-tumor activity, including cytokine secretion (Figure 2A) and cytolytic capacity (Figure 2B). The same trends were observed as prior to tumor cell exposure, i.e. a clear Th1 response for both IL-2 and IL-15-cultured γδ T cells. However, IL-15+IPP γδ T cells gave rise to significantly higher concentrations of IFN-γ compared to IL-2+IPP γδ T cells, whereas the latter were more prone to a Th2 and Th17 response. As direct anti-tumor activity is a hallmark of γδ T cells, and an important asset for immunotherapy, γδ T cell-mediated cytotoxicity was tested next against Daudi and U266 cells. γδ T cell cytotoxicity augmented with increasing E:T ratios and γδ T cells from all donors were able to kill both tumor cell lines. In addition, γδ T cell-mediated lysis was unambiguously stronger for the IL-15+IPP stimulated γδ T cells in comparison with the IL-2+IPP stimulated ones against both Daudi and U266 (Figure 2B).

3.3. IL-15 BOOSTS IL-2/ZOLEDRONATE-MEDIATED EXPANSION OF γδ T CELLS

As a first step in the clinical translation of the preceding results towards an improved protocol for the expansion of γδ T cells for adoptive transfer, expansion based on stimulation of PBMC with IL-2 and zoledronate was compared with responsiveness to IL-15

and zoledronate. Of the four healthy donors tested, only half of the cultures stimulated with IL-15 and zoledronate succeeded.

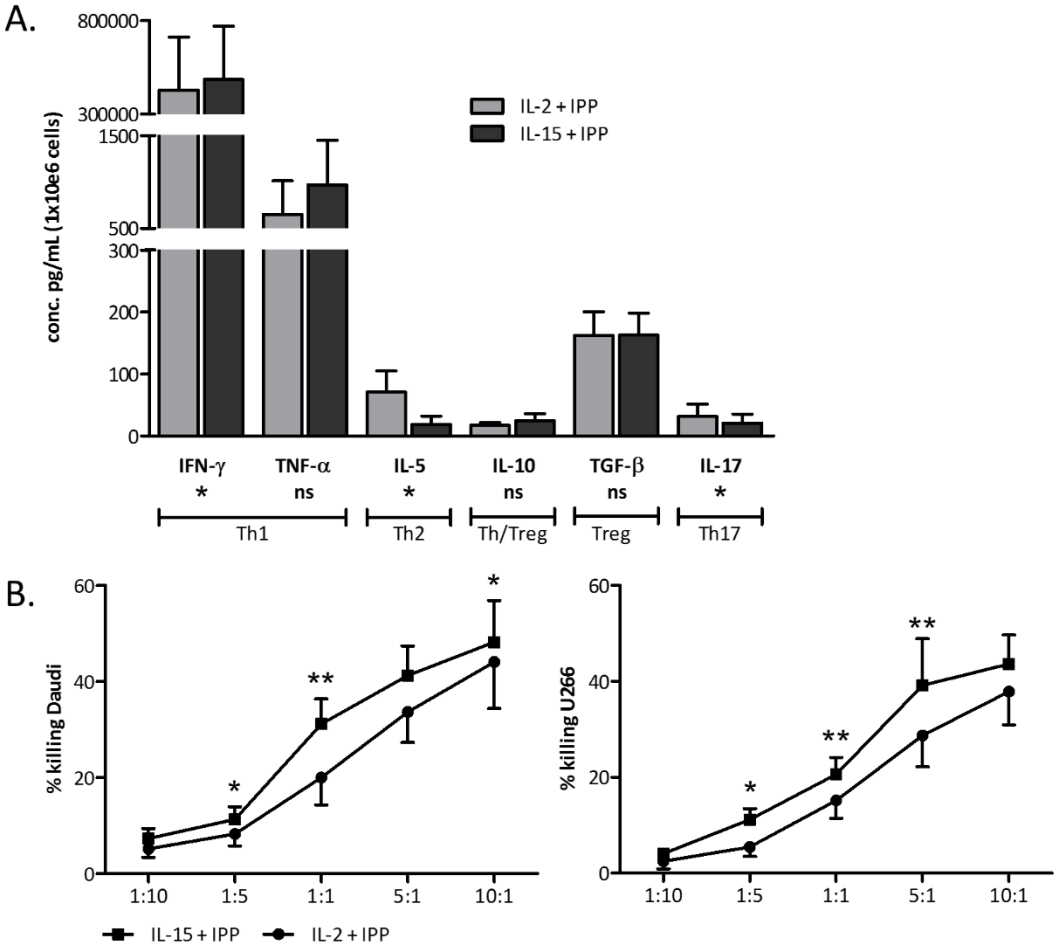


Figure 2. Distinct Th1 cytokine release and marked tumor cell killing by IL-15+IPP activated γδ T cells. A. γδ T cell invigorated for 5 days with IL-15 and IPP (dark bar) or IL-2 and IPP (grey bar), were stimulated for 4 hours with U266 cells (E:T = 5:1). Bar graphs represent the cytokine secretion level of 1 million γδ T cells (pg/mL), as determined by ECLIA (IFN-γ, TNF-α, IL-5, IL-10 and IL-17) and ELISA (TGF-β). Data are depicted as mean of duplicate measurements of six independent donors. Wilcoxon matched-pairs signed rank test. **B.** γδ T cells stimulated for 5 days with IL-15+IPP (■) or IL-2+IPP (●) were analyzed by flow cytometry for cytotoxicity against the tumor cell lines Daudi and U266. Target cell killing was determined by annexin-V/PI staining after 4 hour incubation at different E:T ratios. Results are expressed as mean percentage killing, calculated using the formula specified in "Methods". Data are from six different experiments involving 6-12 different donors. Paired t-test. **, $p < 0.01$; *, $p < 0.05$

One culture was considered to have failed due to a culture viability of less than 50% and another one due to the absence of γδ T cell expansion. Yet, IL-2+zoledronate-mediated γδ T cell expansion occurred in all 4 donors. On the other hand, when IL-15 was added as a third stimulus to the standard expansion protocol (IL-2+zoledronate), a clear effect was detected (Figure 3). Despite the fact that the percentage (Figure 3B) and viability of γδ T cells after 14 days were similar between cultures stimulated with IL-2+zoledronate or IL-2+IL-15+zoledronate, a significant increase in total γδ T cell yield was observed when IL-15 was added to the cultures (Figure 3A). On average, IL-2+zoledronate cultures showed a 590-fold increase in γδ T cells after 14 days, whereas addition of IL-15 almost led to a 1000-fold increase. To ascertain the strength of the immune proliferative and stimulatory effect of IL-15, immune cells of AML patients (Table 1) were included. First, AML patients had a significantly lower proportion of γδ T cells in PBMC in comparison with healthy donors (0.30 ± 0.13% versus 1.91 ± 0.37%, respectively; p = 0.0234). Next, it should be noted that in contrast to the 100% success rate of γδ T cell expansion (defined as ≥ 70% γδ T cells after culture)[33,34] starting from PBMC of healthy donors, less than half of the γδ T cell expansion cultures from AML patients succeeded (Table 2).

Table 2. Outcome of *in vitro* expansion of γδ T cells from PBMC of AML patients

P	% γδ T cells IL-2+zoledronate	% γδ T cells IL-2 + IL-15+zoledronate	success
A	75.9	91.9	yes
B	16.8	12.4	no
C	84.4	94.0	yes
D	17.2	46.6	no
Dr	76.6	89.4	yes
E	5.65	1.80	no
F	4.31	1.84	no
Gr	27.1	50.8	no

Abbreviations: P, Patient; Pr, Patient in remission

However, no direct correlation could be made between γδ T cell percentages in PBMC at start and the success of the expansion culture. Cultures that expanded relatively poorly, but in which nonetheless sufficient (viable) γδ T cells were induced for (functional) testing, were also included. Importantly, similar to the healthy donors, γδ T cells expansion was convincingly stronger for all AML patients when IL-15 was added to the cultures (Figure 3C).

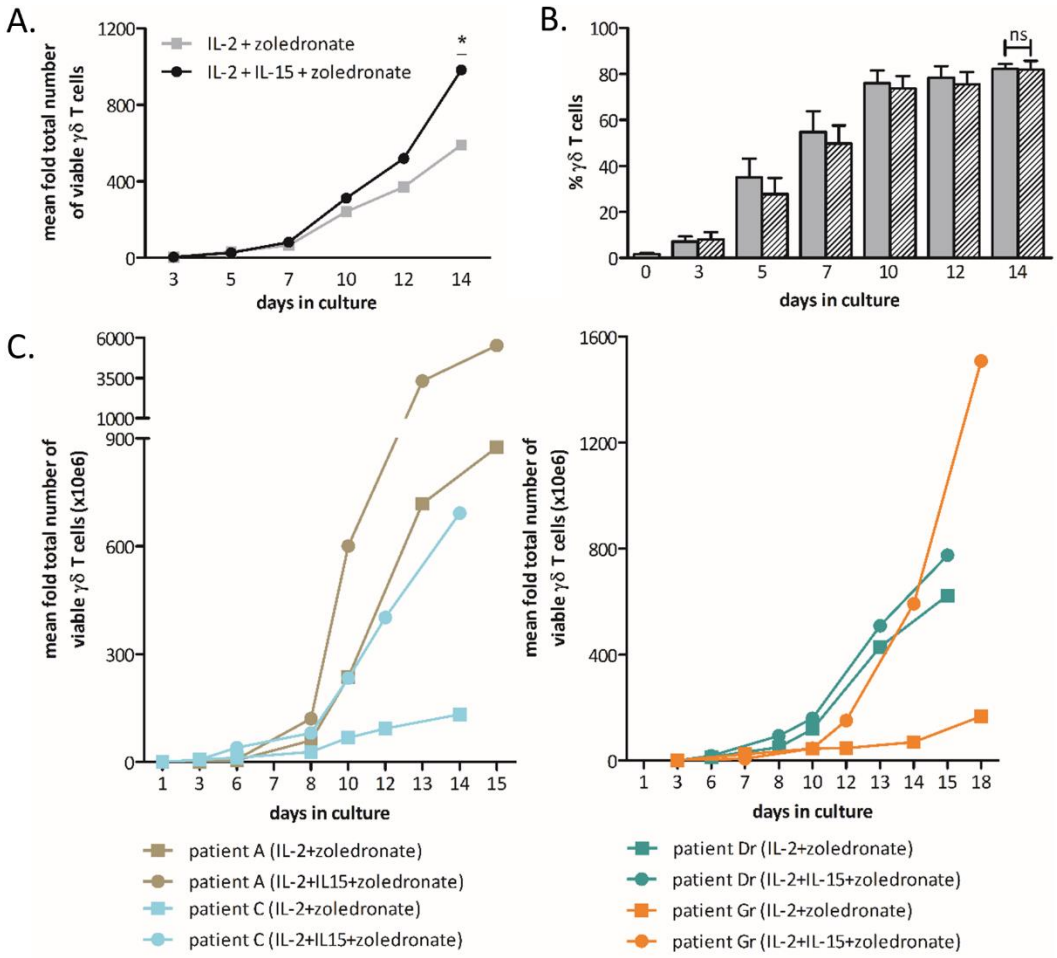
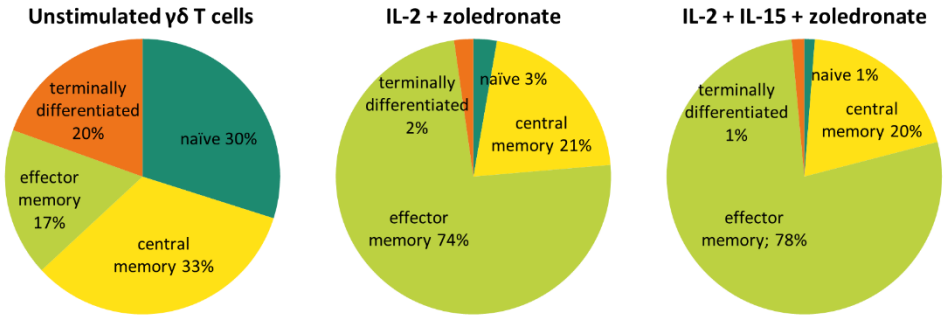


Figure 3. Kinetics of γδ T cell expansion. A. The graph represents the average (n = 6, healthy donors) mean fold total number of γδ T cells, calculated as the absolute number of γδ T cells at the respective time point divided by the absolute number of γδ T cells at start of the expansion culture. **B.** Bar graphs showing the proportion of γδ T cells at various times during the culture, starting from day 0. Cultures expanded with zoledronate and IL-2 are displayed as grey bars, and cultures supplemented with zoledronate, IL-2 and IL-15 are shown as hatched bars. Data are expressed as mean percentages of 6 healthy donors from 3 experiments. **C.** The increase in number of 'successfully' cultured γδ T cells from different AML patients is presented as mean fold total number of viable γδ T cells. Patients in remission are indicated by a lowercase r after their letter of identification (right panel). Wilcoxon matched-pairs signed rank test. P, patient; *, $p < 0.05$; ns, not significant

3.4. 14-DAY EXPANSION USING IL-2+IL-15+ZOLEDRONATE GIVES RISE TO γδ T CELLS WITH AN ACTIVATED MEMORY PHENOTYPE

At culture initiation (day 0) and termination (day 14-15), we subjected the γδ T cell cultures to immunophenotyping. Whereas the four distinct effector/memory states were nearly evenly represented in terms of percentages by circulating γδ T cells of healthy donors at day 0, with a small preponderance for the naïve and central memory phenotype, the vast majority of expanded cells were effector memory γδ T cells and to a lesser extent central memory γδ T cells (Figure 4). γδ T cells of AML patients, however, deviated from this pattern (Figure 4). Likely, the stage of the disease, i.e. diagnosis, relapse or remission, plays a role in this phenomenon. For example, notwithstanding the rather small patient group, patients at diagnosis or in relapse displayed at day 0 a higher amount of central memory γδ T cells as compared to healthy donors, whereas remission patients strikingly had less central memory γδ T cells. Undoubtedly, patient-dependent factors also play a role, for instance when looking at the distribution of patient G (Figure 4). Still, in analogy with healthy donors, expanded γδ T cells of AML patients show a clear predominance for the memory stages (Figure 4).



	naive CD27⁺ CD45RA⁺			effector memory CD27⁻ CD45RA⁻			central memory CD27⁺ CD45RA⁻			terminally differentiated CD27⁻ CD45RA⁺		
	unst.	IL-2 +zol	IL-2+IL-15 +zol	unst.	IL-2 +zol	IL-2+IL-15 +zol	unst.	IL-2 +zol	IL-2+IL-15 +zol	unst.	IL-2 +zol	IL-2+IL-15 +zol
A	26.30	2.17	0.96	2.76	78.10	86.70	49.30	19.30	11.90	21.60	0.43	0,50
C	25.60	5.67	6.90	3.32	85.40	78.60	52.70	0.89	1.81	18.40	8.02	12.70
D	17.10	-	0.16	3.50	-	81.60	42.70	-	18.10	36.70	-	0.13
Dr	31.5	7.26	6.05	7.95	26.9	50.5	16	64.9	41.7	44.6	0.91	1.8
Gr	0	2.15	0.62	74.6	57.8	83.5	8.88	36.2	13.7	16.6	3.83	2.15

Figure 4. Effector memory phenotype of γδ T cells pre and post expansion. Average percentage of naïve ($CD45RA^+CD27^+$), effector memory ($CD45RA^-CD27^-$), central memory ($CD45RA^-CD27^+$) and terminally differentiated ($CD45RA^+CD27^-$) γδ T cells, before (unstimulated) and after expansion, represented as pie charts for six healthy donors. Values of γδ T cells of patients (A, C, D, G) are listed in the table below. -, no value due to low cell number; Xr, patient in remission; unst., unstimulated; zol, zoledronate

Further phenotypic characterization revealed a marked increase in expression level of the activation associated markers CD56, CD69 and HLA-DR on expanded γδ T cells of healthy donors, in combination with an increase in the co-stimulatory molecules CD80 and CD86 (Figure 5). Expansion, however, had no effect on the expression of CD16 and CD28. If we then looked at the phenotype of patient-derived γδ T cells after expansion, we perceive an increase in the early and late activation markers CD69 and HLA-DR as well (Figure 5). Interestingly, γδ T cells of patients in remission express less HLA-DR (and CD69) after expansion in comparison to healthy controls and patients with acute disease. The activated phenotype of γδ T cells from remission patients is nonetheless clearly improved upon addition of IL-15. With regard to CD56 expression, all patient γδ T cells exhibited already elevated levels at start, similar to those of healthy donors after expansion. After expansion, a down-regulation of CD56 is seen, which is more pronounced in patients in remission and γδ T cells expanded with IL-2+zoledronate. The same phenomenon holds true for CD16 upon expansion. Concerning the expression of CD80, a marked upregulation was detected after expansion, even more pronounced as compared to healthy donors. Whereas the expression of CD86 is similar for patient A, patient C and healthy donors, γδ T cells of patients in remission expressed less of this co-stimulator after expansion (Figure 5). In summary, expanded γδ T cells display an activated memory phenotype, based on the enhanced expression of CD69, HLA-DR, CD56, CD80 and CD86.

3.5. THE FREQUENCY OF IFN-γ-PRODUCING γδ T CELLS IS CONSIDERABLY HIGHER AFTER IL-2+IL-15+ZOLEDRONATE EXPANSION COMPARED TO IL-15-FREE EXPANSION

To study the functionality of the expanded γδ T cells, we first determined their Th1 polarization. In the absence of additional stimuli, the mean frequency of IFN-γ-producing γδ T cells after expansion was significantly higher when stimulated with IL-2+IL-15+zoledronate (3.28 ± 0.81%), as compared to IL-2+zoledronate (1.49 ± 0.43%) (Figure 6A). This more pronounced Th1 profile of γδ T cells, seen when IL-15 is present during expansion, also holds true upon addition of tumor cells to the cultures. When Daudi cells were added, we even observed an amplified IFN-γ production as compared to the control (no tumor cells) condition (Figure 6A). Despite having low numbers of TNF-α-producing γδ T cells, generally below 1%, the same trends were seen as with IFN-γ. Proceeding with immune cells of AML patients, different observations were seen as compared to expanded γδ T cells of healthy donors (Figure 6B). Whereas the fraction of γδ T cells positive for IFN-γ after stimulation with IL-2+IL-15+zoledronate was comparable between healthy donors and AML patients, IL-2+zoledronate stimulation gave rise to more IFN-γ positive γδ T cells when derived from AML patients relative to healthy donors.

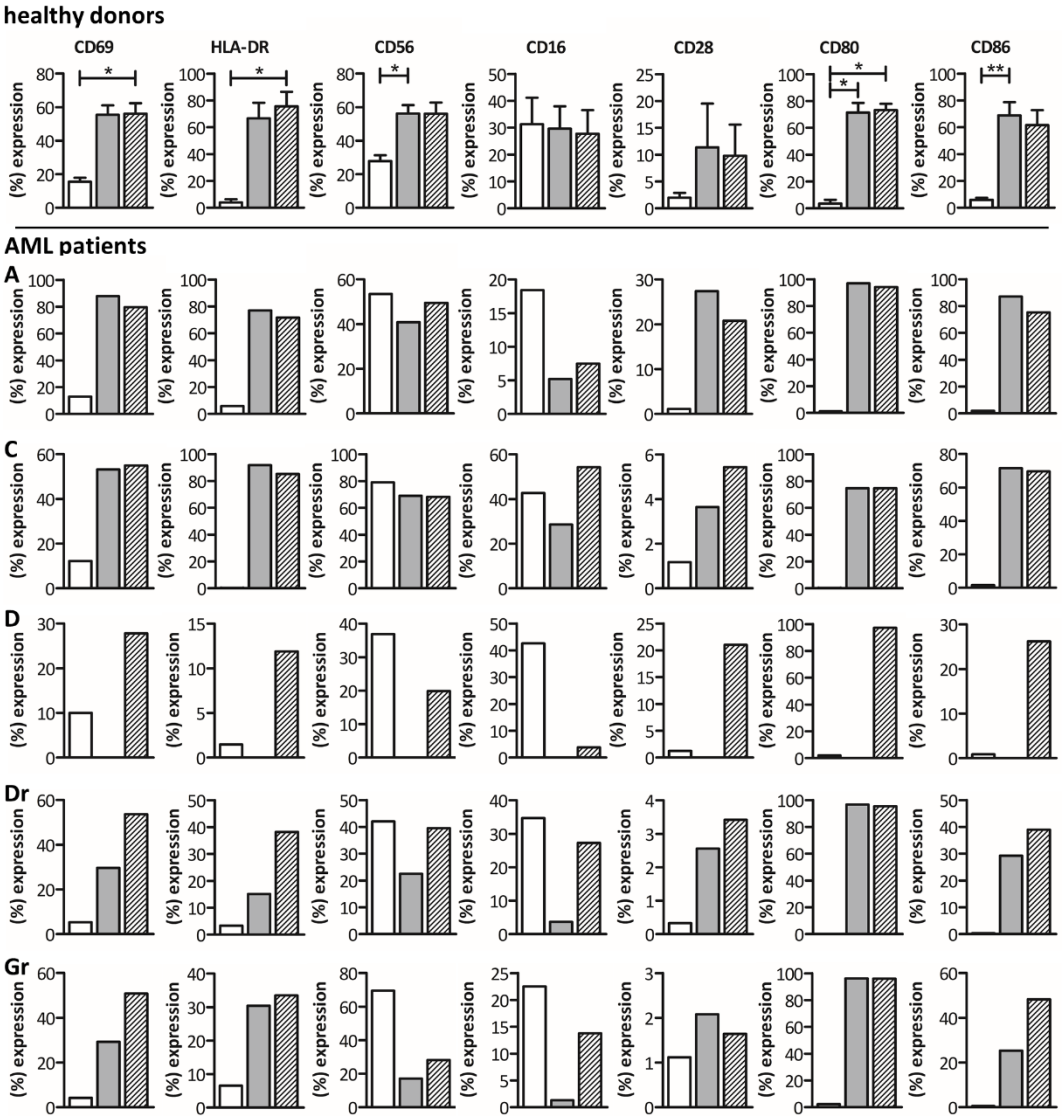
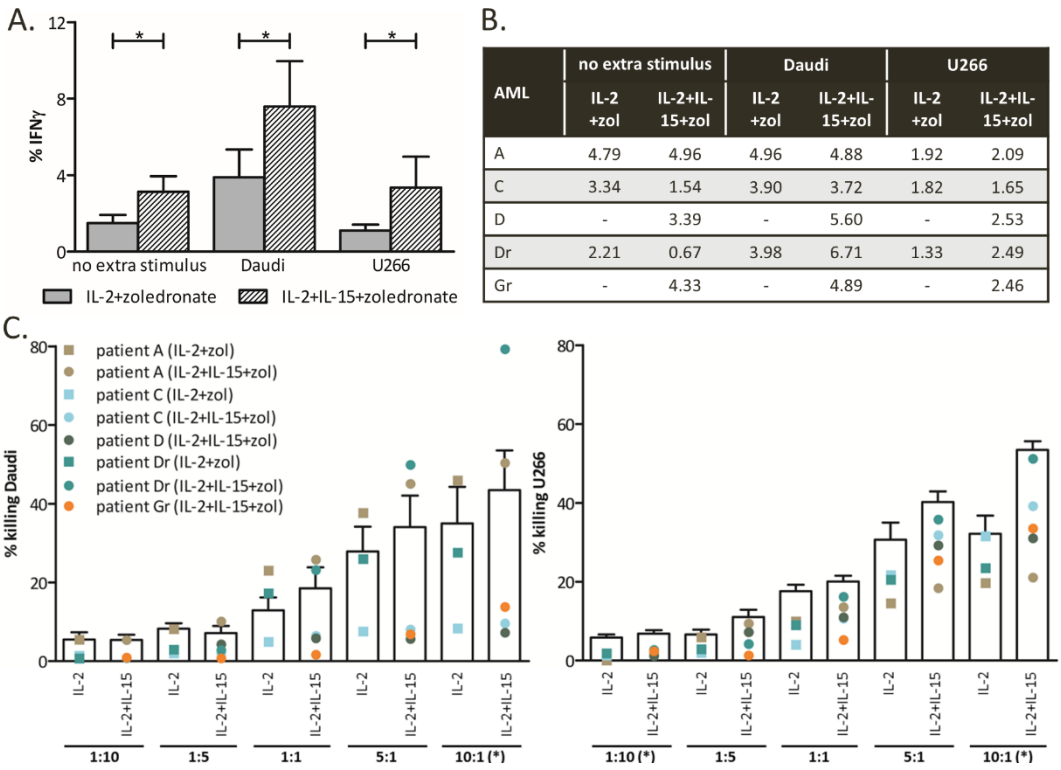


Figure 5. γδ T cell phenotype prior and subsequent to expansion. Comparison of the expression of CD69, HLA-DR, CD56, CD16, CD28, CD80 and CD86 on unstimulated (white bars), IL-2+zoledronate expanded (grey bars) and IL2+IL-15+zoledronate expanded (hatched bars) γδ T cells. Results are shown for six healthy donors on the uppermost row as mean percentage. Expression levels of the respective markers of patient γδ T cells are showed below. Due to the low number of γδ T cells after expansion with IL-2+zoledronaat of patient D, no data are available. Friedman test with Dunn's Multiple Comparison Test. **, $p < 0.01$; *, $p < 0.05$

Moreover, the IL-2+zoledronate cultures of AML patients performed equally well in terms of IFN-γ production, if not better, than the IL-2+IL-15+zoledronate condition. Next, when

tumor cells were added to expanded γδ T cells from patients, blunting of the γδ T cell helper function could be observed to a certain degree pertaining to healthy donor expanded γδ T cells. Here, γδ T cells of patients with active disease did similar in terms of percentage IFN-γ positive cells after expansion with or without IL-15. On the other hand, γδ T cells of remission patient Dr, of which both protocols had yielded sufficient γδ T cells for functional testing, exhibited an almost doubling in the number of IFN-γ positive cells when expanded in the presence of IL-15 and challenged with tumor cells



AML	no extra stimulus		Daudi		U266	
	IL-2 +zol	IL-2+IL-15+zol	IL-2 +zol	IL-2+IL-15+zol	IL-2 +zol	IL-2+IL-15+zol
A	4.79	4.96	4.96	4.88	1.92	2.09
C	3.34	1.54	3.90	3.72	1.82	1.65
D	-	3.39	-	5.60	-	2.53
Dr	2.21	0.67	3.98	6.71	1.33	2.49
Gr	-	4.33	-	4.89	-	2.46

Figure 6. Pro-inflammatory and cytolytic γδ T cell capacity after expansion. Percentage IFN-γ-positive γδ T cells after expansion (no extra stimulus) and after an additional 4 hour stimulation with Daudi or U266 cells (E:T ratio = 5:1) measured by flow cytometry for **A.** six healthy donors **B.** five AML patients. **C.** Expanded γδ T cells, with IL-2+zoledronate (IL-2+zol) or IL-2+IL-15+zoledronate (IL-2+IL-15+zol), were analyzed by flow cytometry for cytotoxicity against Daudi and U266. Target cell killing was determined by annexin-V/PI staining after 4 hours incubation at different E:T ratios. Bar graphs (white) represent the mean percentage killing of 6 healthy donors in three independent experiments. The dots display the proportion of tumor cell killing by γδ T cells of the different patients. Wilcoxon matched-pairs signed rank test. -, no value due to low cell number; *, $p < 0.05$

3.6. ADDITION OF IL-15 TO THE EXPANSION MEDIUM RESULTS IN A SIGNIFICANT INCREASE IN THE γδ T CELL-MEDIATED CYTOTOXICITY AGAINST TUMOR CELLS

Finally, we determined the cytotoxic potential of the expanded γδ T cells against the Daudi and U266 tumor cell lines (Figure 6C). γδ T cells of healthy donors displayed an increase in tumor cell killing upon increasing E:T ratios. Notably, addition of IL-15 in the expansion cultures evoked a superior cytotoxic capacity of expanded γδ T cells at the 10:1 ratio for both tumor cell lines. Similarly, the cytotoxic capacity of γδ T cells originating from AML patients was improved upon expansion in IL-15. However, in general, killing values of AML-derived γδ T cells were lower than those of healthy donor γδ T cells. At the 10:1 ratio, average killing of Daudi by expanded γδ T cells was 27.26 ± 10.86% and 32.03 ± 14.17%, with respectively the IL-2+zoledronate (n=3) and IL-2+15+zoledronate (n=5) expansion protocols. For U266 killing, percentages of 24.89 ± 3.51% and 35.19 ± 4.96% were true. Finally, for some AML patients e.g. patient A, cytotoxicity capacity is highly dependent on the target cell line (Figure 6C).

4. DISCUSSION

To date, adoptive immunotherapy using T lymphocytes has been applied for nearly 30 years.[35] Since then, vast improvements, including the use of CAR or TCR gene-modified killer T cells, have been made. This resulted in proven clinical benefit for end-stage cancer patients for which standard therapy failed and the provision of long-term protection in some cases.[36-38] Notwithstanding this progress in the field, one of the major problems encountered with the adoptive transfer of classical αβ T cells, gene-modified or not, is the occurrence of off- and on-target toxicity.[37] Here, γδ T cells have received recent attention as an alternative cell source for T-cell mediated anti-cancer therapy.[7] Namely, both adoptive transfer and *in vivo* activation/expansion of γδ T cells are safe therapeutic modalities that can result in objective clinical responses in the treatment of cancer.[7,16] A major advantage with using γδ T cells is that they are unlikely to cause graft-versus-host disease, allowing them to be generated from healthy donors and given in an allogeneic setting as an “off-the-shelf” therapeutic.[7,14] In this study, we explored the beneficial effect of IL-15 on γδ T cells and its use as stimulatory signal in the *ex vivo* expansion of γδ T cells for adoptive transfer. The strength of IL-15-mediated activation was further validated in γδ T cells originating from AML patients.

A recent study in rhesus macaques showed that continuous administration of IL-15 is, among other, associated with increased numbers of circulating γδ T cells.[39] This has now

been confirmed in the first-in-human trial of recombinant IL-15, whereby IL-15 administration in cancer patients induced both γδ T cell proliferation and activation.[26] These *in vivo* findings corroborate with our *in vitro* results, showing substantial proliferation of isolated γδ T cells upon stimulation with IL-15+IPP, but not with IL-2+IPP. In line herewith, Viey *et al.* demonstrated γδ T cell expansion out of tumor-infiltrating lymphocytes of renal cell carcinoma patients with BrHPP, a synthetic phosphoantigen, in combination with IL-15, while the combination with IL-2 was inefficient.[5]

However, when we sought to extrapolate these results into a clinical two-week expansion protocol based on IL-15 and zoledronate, inconsistencies in expansion successes were detected. In our hands, the best results were obtained when IL-15 and IL-2 were combined, suggesting that these cytokines have distinct roles in γδ T-cell biology. An advantage of IL-15 during expansion could be that IL-15 mediates homeostatic competition between αβ T cells, NK cells and γδ T cells by a yet unknown indirect mechanism.[40,41] While we detect no difference in the final percentage of γδ T cells in expansion cultures of healthy donors with or without IL-15, this is certainly the case with the cultures of AML patients. In the IL-2+zoledronate cultures, we observed a higher proportion of other blood mononuclear cells, of which the vast majority were αβ T cells. Addition of IL-15 may therefore have counteracted the growth of other cell types such as αβ T cells, constraining the growth of the γδ T cells, preventing cluster formation and subsequently successful γδ T cell expansion.[40,41] It is possible that this process is more important when there is only a low percentage of γδ T cells in the starting material or when γδ T cells are less responsive, which is often the case in a malignant setting.[42] Moreover, γδ T cells of AML patients generally exhibited a higher viability after expansion with IL-15, which was not observed with healthy donors. The question remains whether this improved viability is a result or the cause of the enhanced degree of γδ T cell expansion. Nevertheless, it has been shown that IL-15 is very competent in supporting survival of activated γδ T cells, superior to IL-2,[43] and T cell survival in general.[44] This does not mean that IL-2 is by definition redundant, as evidenced by our expansion results. The synergism between IL-2 and IL-15 could lie in the fact that γδ T cells, in response to zoledronate and IL-2, upregulate their expression of *inter alia* IL-2Rβ and γ_c.[43]

With regard to the effector/memory state of the γδ T cells after expansion, our results correspond with the available literature concerning γδ T cells expanded with IL-2 and zoledronate.[25,45] In particular, the majority of expanded γδ T cells from healthy donors and patients, showed for both expansion protocols a predominant effector memory type and, to a lesser extent, a central memory phenotype. Hence, the addition of IL-15 had no apparent influence on the effector/memory state. This is encouraging, since increased

effector memory γδ T cells are reported to correlate with objective clinical outcomes in patients treated with zoledronate and IL-2.[46] On the other hand, adoptive transfer with αβ T cells has shown that it may also be important to preserve some less differentiated T cell subsets within the infused T cell product, to ensure T cell expansion and potentially long-term T cell persistence.[47-49] Given the presence of γδ T cells with a central memory phenotype upon expansion, our culture protocol fulfills the above-mentioned necessity. Moreover, contributing to the rationale of implementing IL-15 in expansion protocols, it has recently been shown that IL-15 instructs the generation of human memory stem T cells, a T cell subset with superior anti-tumor responses,[50] from naive precursors.[49,51]

When looking in detail at the phenotype of cultured γδ T cells, two findings stand out from our results. First, incubation with IL-15+IPP led to a significant upregulation of CD56 relative to IL-2+IPP stimulated and unstimulated γδ T cells. In addition to the stringent association of CD56 with NK cells, CD56 has also been detected on other lymphoid cells, including γδ T cells and activated $CD8^+$ T cells.[52-55] Moreover, CD56 in the human hematopoietic system is not restricted to lymphoid cells. Both $CD56^+$ plasmacytoid DCs and myeloid DCs, including our IL-15 DCs, feature cytotoxic activity, in addition to serving classical DC functions.[56] In this context, it has been presumed that CD56 is associated with activated/cytotoxic effector immune cells.[52,54,57-59] This would implicate that IL-15 stimulation raises stronger cytotoxic γδ T cells, as effectively confirmed by the enhanced killing of tumor cells in our experiments. Moreover, CD56 expression on γδ T cells following expansion was enhanced as well. Secondly, upon expansion, an increased expression of the co-stimulatory molecules CD80 and CD86 was observed. It has been shown that activated γδ T cells are able to acquire a professional APC function, expressing high levels of co-stimulatory molecules.[60] This function may further boost the generation of a potent and long-lasting immune response.

In view of the functionality of γδ T cells, our experiments clearly show that IL-15, in comparison with IL-2, has a superior Th1 polarizing effect on γδ T cells and markedly strengthens the γδ T cell cytotoxic capacity. These results are supported by *in vivo* data where intestinal γδ T cells of $IL\text{-}15^{-/-}$ knockout mice only produced small amounts of IFN-γ upon stimulation and showed significantly lower cytotoxicity against target cells as compared to wild type mice.[61] This indicates that IL-15-mediated signals are indeed indispensable for the development of potent anti-tumor functions. Furthermore, IL-15 has proved itself superior at maintaining effector functions of already expanded and activated γδ T cells, evidenced by a higher IFN-γ production and CD107a expression after stimulation with zoledronate pre-treated Daudi cells.[43] In addition, within the context of neonatal immunity, it has been shown that expansion of cord blood γδ T cells with alendronate and

IL-15 gave rise to γδ T cells capable of strong protective immune responses.[62] Although the differences between the effects of IL-2 and IL-15 were subtle, higher expression of among other granzyme B and perforin was detected after activation with IL-15.[62] All these data therefore point towards the fact that IL-15 is a pivotal signal to generate more powerful effector γδ T cells. This has recently been substantiated by Ribot *et al.*, identifying the MAPK/ERK-mediated IL-2/IL-15 signaling as the major functional differentiation pathway of human γδ T cells towards anti-tumor (cytotoxic type 1) effector cells.[63]

5. CONCLUSION

Taken together, we have demonstrated that the addition of immunostimulatory cytokine IL-15 to *in vitro* γδ T cell cultures, derived from both AML patients and healthy blood donors, resulted in a more activated phenotype and significantly increased the anti-tumor functions of γδ T cells. This was manifested in a higher yield of expanded γδ T cells, a more pronounced Th1 polarization and an increased cytotoxic capacity, all desirable characteristics for their further use in adoptive immunotherapy. Therefore, our results strengthen the rationale to explore the use of IL-15 in clinical adoptive therapy protocols.

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CHAPTER 3:

EMPOWERING GAMMA DELTA T CELL WITH ANTI-TUMOR IMMUNITY BY DENDRITIC CELL-BASED IMMUNOTHERAPY

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ABSTRACT

γδ T cells are the all-rounders of our immune-system with their major histocompatibility complex-unrestricted cytotoxicity, capacity to secrete immunostimulatory cytokines and ability to promote the generation of tumor antigen-specific $CD8^+$ and $CD4^+$ T cell responses. DC-based vaccine therapy has the prospective to harness these unique features of the γδ T cells in the fight against cancer. In this review, we will discuss our current knowledge on DC-mediated γδ T cell activation and related opportunities for tumor immunologists.

1. INTRODUCTION

Since the discovery of γδ T cells in the late 1980s, insights have been gathered of this enigmatic and versatile T cell subtype, bridging innate and adaptive immunity.[1,2] Over the last decade this component of cellular immunity received a growing body of interest from tumor immunologists, for the sake of their immunostimulatory properties and their ability to recognize and eradicate tumor cells. In particular their ability to identify antigens out of the context of classical MHC molecules, has raised expectations for their use in therapeutic anti-cancer applications.[3] Translational research efforts in the field of γδ T cell-based immunotherapy are currently focusing on two main approaches: (a) adoptive transfer of *ex vivo* activated and expanded γδ T cells, and (b) *in vivo* targeting of γδ T cells using therapeutic agents, such as IL-2, bisphosphonates or tumor-directed monoclonal antibodies (mAbs; e.g. rituximab and trastuzumab) specifically aimed at the recruitment and expansion of γδ T cells (excellently reviewed elsewhere).[4,5] Another path that could be explored in view of γδ T cell activation is DC vaccination. DCs are the orchestrators of our immune system. As sentinels, they recognize foreign invaders as well as stressed cells, after which they initiate an immune response appropriate to the hazard. Hence, DCs have caught the attention for their use as therapeutic agents for immunotherapy of cancer[6] and infectious diseases, such as human immunodeficiency virus (HIV).[7,8] Research on DC-based immunotherapy is currently focusing on the vaccine-mediating effects on the adaptive immune system, aiming at inducing (tumor)antigen-specific CTLs. Less extensively studied is the effect of DC-based immunotherapy on γδ T cells. Within this context, recent evidence has emerged that DCs can induce the activation and proliferation of γδ T cells, enhancing their cytotoxic and immunoregulatory functions.[9,10] γδ T cells, in turn, promote DC maturation and improve their capacity to stimulate adaptive αβ T-cell responses.[1] This review summarizes for the first time the current knowledge on DC-mediated γδ T cell activation, mechanisms behind the cell-to-cell interactions and its therapeutic potential for implementation in DC-based cancer immunotherapy (Figure 1).

2. γδ T CELLS AND CANCER IMMUNITY

Human γδ T cells are a group of unconventional T cells that can be subdivided based on their δ TCR chain into Vδ1 and Vδ2 T cells. The majority of the tissue-associated γδ T cells bear the Vδ1 TCR, whereas the δ2 T cells represent the largest group in the blood, reaching up to 95%.[11] γδ T cells in blood represent 1-10% of the entire T-cell population,[11] but an impressive increase in their relative share is observed upon infection, detecting increments

from as low as 1% to over 50% in a few days. The presence of activated γδ T cells is associated with resistance against infectious pathogens, such as *Listeria monocytogenes* [12] and the influenza virus,[13] underscoring their role in protecting the host against infectious disease.[14,15]

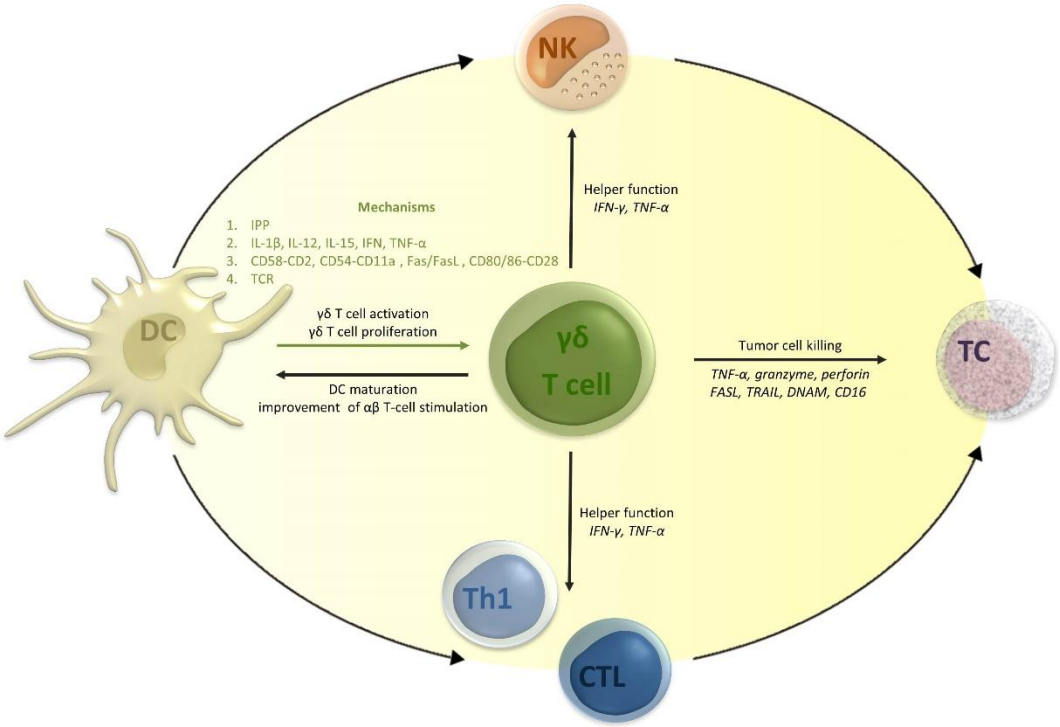

Figure 1. How γδ T cells can contribute to the anti-tumor efficacy of DC-based vaccination. It can be postulated that DC vaccination has the ability to activate γδ T cells and initiate their expansion. In turn, activated γδ T cells can *(I)* further stimulate vaccine and host DCs indirectly supporting sustained anti-tumor T-cell immunity and NK-DC crosstalk, *(II)* fulfill their immunomodulatory function through the secretion of pro-inflammatory cytokines regulating innate (NK cells) and adaptive (T cells) cellular immunity, and *(III)* directly kill tumor cells.

Abbreviations: CTL, cytotoxic T lymphocyte; DC, dendritic cell; DNAM, DNAX accessory molecule; FASL, Fas ligand; IFN, interferon; IL, interleukin; IPP, isopentenyl pyrophosphate; NK, natural killer cells; TC, tumor cell; TCR, tumor cell; Th1, T-helper 1 cell; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand

Furthermore, γδ T cells are considered to be important players in cancer immune surveillance.[2] This is, among other things, substantiated by *(I)* their overrepresentation in reactive lymphatic regions associated with neoplasia,[16] *(II)* their potential to kill a variety of tumor cells including bladder cancer,[17] colon cancer,[18] glioblastoma multiforme,[19]

hematological malignancies[20] and MM,[21] and *(III)* the observed trend between the presence of activated γδ T cells in cancer patients and a beneficial outcome.[22-24]

γδ T cells dispose of a sensitive ability to distinguish "foreign" or transformed cells from healthy self-cells, using activating receptors and inhibitory killer Ig-like receptors (Table 1). γδ T cells are therefore not restricted to MHC priming, in contrast to the classic αβ T cells, which is a major asset in cancer cell recognition.[25,26] γδ T cells exert their cytotoxicity by releasing TNF-α and granzymes in conjunction with perforins, and by binding Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL) and DNAX accessory molecule-1. Membrane-expression of CD16 leaves the possibility of ADCC.[4,27,28] Another feature of γδ T cells is their immunoregulatory function. As being an important early source of pro-inflammatory cytokines such as IFN-γ, TNF and IL-17, γδ T cells support DC, T cell, B cell and stromal cell functions.[1] γδ T cells can also develop properties of APCs, reminiscent of mature DCs, supporting the development of Th1 cells and cytotoxic T cells.[29] Both classic MHC I and MHC II loading pathways are therefore present in γδ T cells.[30] Even though γδ T cells predominantly exert innate-like responses, they are able to transform into memory cells. Thus naïve ($CD45RA^+CD27^+$) and central memory ($CD45RA^-CD27^+$) γδ T cells will home to secondary lymphoid organs, while effector memory ($CD45RA^-CD27^-$) and terminally differentiated ($CD45RA^+CD27^-$) γδ T cells show immediate effector functions on inflammatory sites.[28]

Tumor cells, however, exploit different mechanisms to escape γδ T cell-mediated anti-tumor immunity, reviewed by others.[31,32] For example, tumor cells promote γδ T cells to adopt a Treg phenotype. Such γδ Tregs damp anti-tumor immunity[33] and contribute to the immunosuppressive micro-environment that is characteristic of most tumor cells.[34] Deficient γδ T cell functions have already been observed in various malignancies, including hematological,[20] liver,[35] breast[35] and gastric cancers.[36] Reestablishment of γδ T cell anti-tumor activity is therefore valuable.

3. DC-MEDIATED γδ T CELL ACTIVATION

Since the discovery by Ismaili *et al.* (2002) that phosphoantigen-activated γδ T cells are capable of inducing maturation of monocyte-derived DCs, a plethora of research papers focused on the activation of DCs by γδ T cells.[37-40] It is only recently that γδ T cell activation by DCs and their crosstalk got its rightful attention. Table 2 and Figure 1 summarize our current understanding of DC-mediated γδ T cell activation.

3.1. DC-MEDIATED γδ T CELL PROLIFERATION

Seminal work by Takamizawa *et al.*[41] in the mid-1990s provided the first evidence that human DCs can mediate γδ T cell activation by inducing their proliferation, a finding that was later confirmed by Ye et al. in the early 2000s.[42] Although the nomenclature of the DC system is still expanding, two main *in vivo* DC subsets can be distinguished: plasmacytoid (pDCs) and myeloid DCs (mDCs). However, most DC studies, including those on the interaction between DCs and γδ T cells (Table 2), have relied on the use of ex vivo generated monocyte-derived DCs (mo-DCs). To obtain mo-DCs, peripheral blood monocytes are cultured *in vitro* into immature (i)DCs in the presence of differentiation stimuli such as GM-CSF and IL-4.

Table 1. Main inhibitory and activating γδ T cell receptors and their ligands

	receptor on γδ T cell		ligand(s)
activating receptors	TCR	- -	both MHC-related and non-related molecules
	CD28	-	CD80/CD86
	CD27	-	CD70
	LFA-1	-	ICAM-1
	CD2	-	LFA-3
	CD6	-	CD166
	CD30	-	CD30L
	NKG2C	-	HLA-E
	NKG2D	- -	MICA/B ULBP1-6
	CD16	-	Fc portions IgG1, IgG3
	DNAM-1	-	Nectin-like-5, Nectin-2
	NKp30	-	B7-H6, BAT3
	NKp44	-	?
	NKp46	-	?
inhibitory receptors	PD-1	-	PDL1
	ILT2	-	HLA-A
	KIR2D	-	HLA-C
	NKG2A	-	HLA-E
	KLRG1	-	classical cadherins (E-,N-, R-)
	BTLA	-	HVEM, B7H4

Abbreviations: BAT3, HLA-B associated transcript 3; BTLA, B- and T-lymphocyte attenuator; CD, cluster of differentiation; DNAM-1, DNAX accessory molecule-1; HLA, human leukocyte antigen; Ig, Immunoglobulin; HVEM, herpesvirus entry mediator; ICAM-1, intercellular adhesion molecule 1; ILT, immunoglobulin-like transcript; KIR, killer-cell immunoglobulin-like receptor; KLRG1, Killer cell lectin-like receptor subfamily G member 1; LFA, lymphocyte function-associated antigen; MHC, major histocompatibility complex; MIC, major histocompatibility complex class I-related chain; NKG, natural killer group; NKp, natural killer cell px-related protein; PD-1, programmed cell death protein 1; TCR, T cell receptor; ULBP, UL16-binding protein

iDCs can in turn be converted into mature DCs by exposure to maturation stimuli such as cytokines and TLR ligands.[46] Neither immature mo-DCs or their mature counterparts are capable of inducing γδ T cell proliferation,[44] but they can acquire this capacity when stimulated by the bisphosphonate zoledronate.[44] Such zoledronate-treated mo-DCs were found to preferentially stimulate the proliferation of central memory and effector memory γδ T cells.[44] Recently, the ability to induce γδ T cell proliferation was also demonstrated for zoledronate-exposed $CD56^+$ mDCs isolated from the blood of healthy volunteers.[10] The mechanism by which zoledronate-treated DCs mediate γδ T cell activation (Vγ9Vδ2 subset) relies on inhibition of the mevalonate pathway in DCs, leading to accumulation of mevalonate metabolites such as IPP.[54,55] Healthy somatic mammalian cells constitutively express the latter, but in a too low concentration for γδ T cell activation.[54] IPP and other phosphoantigens, such as HM-BPP, have been shown to promote Vγ9δ2 T cell proliferation, provided that there are MHC class II-positive cells (e.g. DCs) present.[43] However, this is not a property of the entire group of the phosphoantigens since BrHPP, did not show any stimulatory effect on proliferation.[46]

Similar to bisphosphonate and phosphoantigen stimulation, infection-related signals can also provide the necessary stimulus for DC-mediated γδ T cell proliferation (Table 2). For example, *Bacillus Calmette-Guérin* (BCG)- and *Mycobacterium tuberculosis*-infected DCs have been shown to induce proliferation of Vγ9δ2 T cells with a central memory phenotype, both in autologous and allogeneic settings.[45,51] In contrast, DCs infected with HIV are not able to induce Vγ9δ2 T cell proliferation,[52] indicating that DC-mediated γδ T cell proliferation following infection is dependent on the type of pathogen. In the context of infection-related signals, maturation of DCs with pathogen-associated molecular patterns (PAMPs; e.g. TLR-agonists) is promising.[9,48]

Finally, cytokines, such as IL-15 and high-doses of IL-2, can provide the extra stimulus to enable mo-DCs to stimulate the proliferation of γδ T cells.[48] IL-15 is a pleiotropic cytokine, important for, among others, (γδ) T cell homeostasis.[56,57] Lack of IL-15 production by infected DCs results in the absence of γδ T cell effector functions, making the differentiation of central memory γδ T cells into effector memory cells difficult.[51] These data could support the implementation of IL-15 expressing DCs into clinical trials.[58-61]

3.2. IMPACT OF DCS ON EFFECTOR FUNCTIONS OF γδ T CELLS

The two main effector functions of γδ T cells, desirable in the context of cancer immunotherapy, are a direct cytotoxicity towards tumor cells and an immunomodulatory function through the secretion of pro-inflammatory cytokines including IFN-γ and TNF-α.

Table 2. DC-mediated γδ T cell activation

DC-type	culturing conditions	effect on γδ T cells	reference
MHC class II cells (DC)	+HMB-PP or IPP	(+) proliferation	[43]
blood DC	-	(+) proliferation	[41]
CD56$^+$ blood DC	+ zoledronate	(+) proliferation, (+) IFN-γ, TNF-α and IL-1β	[10]
DC	CD34$^+$-derived	(+) proliferation	[42]
immature mo-DC	GM-CSF + IL-4	(-) proliferation	[44,45]
	GM-CSF + IL-4 +BrHPP	(-) cytotoxicity	[46]
	GM-CSF + IL-4 + zoledronate	(+) proliferation	[44]
	GM-CSF + IL-4 + zoledronate	(+) IFN-γ and TNF-α	[9]
	GM-CSF + IL-4 + zoledronate	(+) killing THP-1	[45]
mature mo-DC	GM-CSF + IL-4 – IL-1β + TNF-α	(-) proliferation	[44]
	GM-CSF + IL-4 - IL-1β + TNF-α + zoledronate	(+) proliferation, (+) αβ T cell activation	[44]
	GM-CSF + IL-4 - TNF-a + IL-1b + IL-6 + PGE2	(-) IFN-γ, TNF-α	[9]
	GM-CSF + IL-4 - TNF-a + IL-1b + IL-6 + PGE2 + zoledronate	(+) proliferation tumor-antigen specific CD8$^+$ T cells	[47]
	GM-CSF + IL-4 - TNF-α + IL-1β + IFN-α + IFN-γ + poly(I:C)	(+) IFN-γ, TNF-α, (+) IL-10	[9]
	GM-CSF + IL-4 - MPLA + IFN-γ	(+) IFN-γ, TNF-α	[9]
	GM-CSF + IL-4 - TLR-matured	(+) killing Daudi	[9]
	GM-CSF+ IL-4 - LPS	(-) killing myeloma cells	[48]
	GM-CSF+ IL-4 – LPS + high dose IL-2	(+) proliferation	[48]
	GM-CSF+ IL-4 – LPS + low and high dose IL-15	(+) proliferation	[48]
	GM-CSF+ IL-4 – LPS + ibandronate	(+) IFN-γ, TNF-, (-) killing myeloma cells	[48]
	GM-CSF+ IL-4 - LPS + TNF-α	(-) cytotoxicity	[46]
	GM-CSF+ IL-4 - LPS + TNF-α + pamidronate	(+) IFN-γ, TNF-α	[46]
	GM-CSF + IL-4 - KLH + IFN-γ + LPS	(-) proliferation CD4$^+$ and CD8$^+$ T cells	[49]
	GM-CSF + IFN-α	(+) proliferation tumor-specific CD8$^+$ T cells	[50]
pathogen-infected DC	BCG-DC	(+) proliferation, (+) IFN-γ and TNF-α, (+) killing THP-1	[45]
	Brucella-DC	(+) proliferation CD4$^+$ T cells	[37]
	MTB-DC	(+) proliferation	[51]

	HIV-DC	(-) proliferation	[52]
pDC	R848-, CpG- or YF-17D-triggered	(+) IFN-γ	[53]

Abbreviations: (+), stimulation of γδ T cell function; (-), no effect on γδ T cell function; BCG, *Bacillus Calmette-Guérin*; BrHPP, bromohydrin pyrophosphate; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIV, human immunodeficiency virus; HMB-PP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IFN, interferon; IL, interleukin; IPP, isopentenyl pyrophosphate; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; mo-DC, monocyte-derived DC; MPLA, monophosphoryl lipid A; MTB, *Mycobacterium tuberculosis*; pDC, plasmacytoid dendritic cell; PG, prostaglandin; Poly(I:C), polyinosinic-polycytidylic acid ;TLR, toll-like receptor; TNF, tumor necrosis factor; YF, yellow fever

3.2.1. Immunomodulatory function

In line with the findings for γδ T cell proliferation, neither immature mo-DCs[45] nor their cytokine matured counterparts[9] are found to be capable of stimulating the cytokine secretion function of γδ T cells.

Likewise, pulsing DCs with zoledronate, which, as discussed above, results in production of phosphoantigens by DCs, enables their capacity to trigger the secretion of Th1 cytokines (e.g. IFN-γ, TNF-α and IL-1β) from γδ T cells.[10] A similar observation was made by Martino *et al.,* who showed that zoledronate-treated DCs can stimulate the production of IFN-γ and TNF-α by Vγ9δ2 T cells.[45] DCs stimulated with the bisphosphonate pamidronate[46] or with ibandronate[48] have also been shown trigger a Vγ9δ2 T-cell Th1 cytokine response, as defined by secretion of TNF-α and/or IFN-γ (Table2).

An infection can also function as an additional signal γδ T cells need to exert their secretory function. Thus IFN-γ and TNF-α secretion is observed with BCG-infected DCs.[45] Active infection is mandatory for Vγ9δ2 T cell triggering, as DCs infected with heat-killed BCG lose their incentive effect.[45] This ties in with the use of TLR ligand-based maturation cocktails in mo-DC generation protocols instead of the classic 'Jonuleit cocktail' (comprising TNF-α, IL-1β, IL-6 and prostaglandin (PG)E2).[62] TLR-matured DCs can namely potentiate the cytokine secretion function of γδ T cells.[9] Furthermore, lipoteichoic acids (TLR2 ligand),[63] poly(I:C) (TLR3 ligand), LPS (TLR4 ligand) and flagellin (TLR5 ligand)-mediated IFN-γ secretion of Vγ9δ2 T cells requires the presence of iDCs,[53] which was also previously observed with $CD11c^+$ iDC dependency of poly(I:C)-mediated activation of γδ T cells.[64] In line with the aforementioned, R848 (TLR7/8 ligand), CpG (TLR9 ligand) and vaccinal yellow fever virus strain (YF-17D)-triggered pDC induce Vγ9δ2 T cell IFN-γ production.[53] Generally the secretory immune stimulatory effect is associated with an upregulation of activation markers on the γδ T cells like CD25 and CD69,[9,45] which could be interesting for immunomonitoring of DC vaccination.

In addition to Th1 cytokines, γδ T cells have been demonstrated to become major producers of IL-17 in reaction to IL-1β produced by DCs after encounter of immunogenic dying tumor cells. Although the role of IL-17 in anti-tumor defense is still unclear, there is some evidence that IL-17 secretion by γδ T cells, leading to invasion of tumor-reactive CTL, is required for optimal anti-cancer immune response to cytotoxic chemotherapeutics.[65] On the other hand, IL-17-producing γδ T cells, support tumor growth by promoting angiogenesis.[66] This pro-tumor effect, associated with a poor clinical response, is more

pronounced in solid tumors comparing with hematologic malignancies.[67] IL-17 in the tumor micro-environment remains therefore controversial.

3.2.2. Cytotoxicity of γδ T cells

In context of cancer immunotherapy, the cytotoxic activity is an important effector function of γδ T cells. However, irrespective of the positive effect of co-culture with DCs on proliferation and cytokine secretion of γδ T cells, DCs, whether or not pulsed with ibandronate, did not induce an increase in cytotoxic activity against myeloma cells.[48] This is in line with findings from Devilder *et al.* who reported a potentiating effect of DCs on Th1 and Th2 cytokine responses of BrHPP-stimulated Vγ9δ2 T cells, but not on their cytotoxicity.[46] The cause of this discrepancy is unknown and warrants further investigation, especially given the fact that under some circumstances DCs are capable to enhance Vγ9δ2 T cell killing of tumor cells, for example against the AML cell line THP-1.[45] Moreover, zoledronate-exposed DCs increase the expression of cytotoxic effector molecules such as NKG2D on γδ T cells.[45] Also, TLR-activated DCs induce the degranulation of granzyme B leading to an improved killing of Daudi cells, as compared to classic DCs. These effects, moreover, are maintained long-term.[9]

3.2.3. γδ T cells influence on the adaptive arm of the immune system

Initially, the use of DCs as vaccine is based on their T cell stimulatory ability. Tumor antigen-specific $CD8^+$ CTLs, primed by DCs, are able to recognize tumor antigens expressed on malignant cells, leading to the killing of the latter. The question arises if γδ T cells can add to this effect. The majority of the reports state a valuable role for γδ T cell activation in the design of vaccine therapies for malignancy with regard to stimulation of the adaptive arm of the immune system. Indeed *Brucella*-infected DCs cultured in the presence of Vγ9δ2 T cells induce the amplification of naive $CD4^+$ T cells.[37] HLA-A2-positive $CD56^{high+}$mo-DCs successfully induce Mart-1-derived modified peptide (A27L)-specific $CD8^+$ T cells through preferential expansion of $CD56^+$ Vγ9δ2 T cells in the presence of A27L, zoledronate, and IL-2.[50] In connection herewith, stimulating tumor antigen-pulsed (MART-1-modified peptide) mo-DCs with zoledronate alone leads first to the activation of Vγ9δ2 T cells which in turn results as well in an amplified activation and proliferation of tumor antigen-specific $CD8^+$ T cells.[47] On the contrary, Traxlmayr *et al.* reported that γδ T-cells adversely regulate the proliferative capacity of $CD4^+$ and $CD8^+$ T-cells after stimulation with IL-12 secreting mo-DCs.[49] Overall, simultaneous activation of γδ T cells and αβ T cells improves the antigen-specific cytotoxic responses mediated by the latter.[44] From this it can be concluded that γδ T cells can indirectly contribute to the efficiency of DC based cancer vaccines by promoting the generation of tumor antigen-specific $CD8^+$ T cell responses.

4. MECHANISMS BEHIND DC-MEDIATED γδ T CELL ACTIVATION

A first potential key mechanism behind DC-mediated Vγ9δ2 T cell activation is the production and secretion of IPP by DCs. This is the case for the improved γδ T cell proliferation by zoledronate-pretreated mo-DCs.[68] Extracellular concentrations of IPP were found to be 1000 times higher than the intracellular levels.[69] The importance of IPP was confirmed by the abrogation of the proliferative effect by adding simvastatin or mevastatin to the pretreatment of the DCs.[44,68,69] Statins, a common group of cholesterol-lowering drugs, have an inhibiting effect on the mevalonate pathway by blocking the rate-limiting enzyme in the cholesterol synthesis, the HMG-CoA reductase. This results in a depletion of the starting products of IPP, impeding γδ T cell activation and the positive effect of the bisphosphonates.[70] Addition of mevastatin on the contrary has no effect on αβ T-cell counts.[44] It should however be pointed out that phosphoantigens do not solely influence DC-mediated γδ T cell activation.[45,51]

Since phosphoantigens are not responsible for the entire effect, there must be other growth factors, cytokines, or contact-dependent factors contributing to this crosstalk.[69] Soluble mediators described to induce γδ T cell activation are: IL-12,[9,71,72] IL-15,[45] IL-1β, TNF-α[72] and IFN-α/β.[53,64] IL-12 secretion abrogation by TLR-matured mo-DCs resulted in the almost complete absence of IFN-γ secretion by γδ T cells.[9] The importance of IL-12, along with IL-1β, TNF-α, secretion by BCG-DCs has been demonstrated by the increased production of IFN-γ, granzyme B, and the augmented cytotoxicity of Vγ9δ2 T cells against Daudi cells, pertaining to the IL-12 blocked conditions.[71,72] The IL-12 secretion by BCG blood DCs relies on IFN-γ production of memory $CD4^+$ T cells, reveling a complex biology between cells of the innate and adaptive immune system.[71] IL-12 however does not seems to have an indisputable positive effect. IL-12 secreting DCs, in contrast to DCs that are not enabled for IL-12 secretion, give rise to immune suppression by γδ T cells.[49] In this context the importance of the STAT3 pathway should be mentioned. The activating effect of STAT3 is at least in part mediated by increased IL-12 production and STAT3 silencing in mo-DCs enhances IFN-γ production of γδ T cells.[73] IFN-α/β, produced by $CD11c^+$ DCs, contribute to the activation of γδ T cells by poly(I:C).[64] Type I IFNs play also an important role in TLR-matured DC-γδ T cell activation, shown by an ±80% reduction of the induced IFN-γ secretion by blocking IFNAR2.[53]

Finally, transwell assays point out that both soluble and contact-dependent interactions play a role. The main activating and inhibitory γδ T cell receptors and their ligands are summarized in Table 1. Experiments with bacterial-infected IL-4 DCs, i.e. being pulsed with

BCG,[45] *Brucella*,[37] *B. burgdorferi*[74] or *M. tuberculosis,*[51] indicate that γδ T cell activation and proliferation by these DCs requires direct cell-to-cell contact. The latter is based on the expression of the activation and maturation markers CD25 and CD69, and the production of IFN-γ. Separation of γδ T cells and pamidronate stimulated mo-DCs by a semipermeable membrane completely reverses the γδ T cell activation, which argues against the implication of soluble factors.[75] The cytokine secretion by phosphoantigen-activated Vγ9δ2 T cells enhanced by immatue (i)DCs requires close cell-to-cell contact as well.[46] Contact-dependent interactions described in the literature include CD58-CD2, CD54-CD11a,[41] Fas/FasL[74] and CD80/86-CD28.[41,75] Moreover Takamizawa *et al*. discovered a possible role for interaction with HLA-DR.[41] Accordingly, the presence of the γδ TCR enables the recognition of peptidic and non-peptidic molecules too, whether or not in the context of MHC presentation.[3] Thus, the γδ TCR would play a vital role in recognizing a ligand, most likely a *M. tuberculosis*-derived phosphoantigen, on *M. tuberculosis*-infected DCs, resulting in Vγ9δ2 T cell proliferation.[51] As evident from the current literature, DC-mediated γδ T cell activation is apparently based on combinatory effects, in turn, dependent on the experimental setting.

5. CONCLUSION AND PERSPECTIVES

DCs have the capacity to harness γδ T cells for the development of anti-tumor immunity. Mechanisms directing DC-mediated γδ T cell activation are versatile and rely on combinatorial effects depending on the stimuli used for activation. In this context, DC vaccines hold potential to empower γδ T cells, assisting directly and indirectly the development of an anti-tumor immune response (Figure 1). Given the fact that the presence of activated γδ T cells in cancer patients is associated with a beneficial outcome,[16,22-24] monitoring γδ T cells would be an interesting parameter for evaluation of DC vaccines.

Importantly, this strategy must be examined with scrutiny. To date, research is predominantly performed on γδ T cells of healthy donors. γδ T cells of healthy donors and patients, however, may respond differently. While a strong proliferation of γδ T cells was observed in the majority of healthy donors after single phosphoantigen stimulation *ex vivo*, no such amplification was achieved with γδ T cells of cancer patients.[68,69] This weak response could be overcome by co-culturing γδ T cells with zoledronate-pretreated mo-DCs.[68,69] Immunophenotyping of the amplified γδ T cells revealed the pro-inflammatory state of these cells, based on the expression of costimulatory molecules (HLA-DR, CD86, CD80) and the absence of inhibitory molecules PD-1 and PD-L1.[69]

To get the full potential from DC vaccination for the treatment of cancer patients, some pitfalls should be overcome. Consideration should be given to the fact that conventional mo-DCs generally lack the ability to stimulate (patients') γδ T cells, and that additional/alternative signals are required. Moreover, DCs also have the potential of inducing adverse effects on anti-tumor immunity, like induction of γδ T reg cells, induction of immunosuppressive IL-10[33,76] or dampening of CD4/8 T cell proliferation.[49] It has been determined that co-culturing γδ T cells with DCs matured with an α-type one polarizing cocktail, results in the consistent production of the anti-inflammatory cytokine IL-10.[9] Therefore, one should not forget to look at the negative impact on immunostimulatory capacity, together with the positive effects.

Although there is mounting evidence for DC-mediated γδ T cell activation *in vitro*, to date only two clinical studies have monitored γδ T cells following DC vaccination.[49,77] Traxlmayr *et al.* reported a γδ T cell-mediated negative regulatory feedback mechanism triggered by IL-12 secreting DCs, potentially hampering the clinical effect of DCs.[49] Kitawaki *et al.* assessed the numbers of γδ T cells in peripheral blood and bone marrow, as well as serum IFN-γ serum levels, after vaccination with zoledronate/Wilms' tumor 1-pulsed DCs, but could not detect any changes.[77]

Based on our current knowledge, there is still no conclusive answer on how to generate a potent DC vaccine that harbors the ideal stimulatory triggers for γδ T cell anti-tumor activity. Bisphosphonate-pretreated mo-DCs respond to the fact that phosphoantigens are known activators of Vγ9δ2 T cells. The molecules, however, which are responsible for phosphoantigen presentation remain to date unknown.[11] Including infection-related signals in the protocol is a second way to optimize DC vaccines. Maturation cocktails with TLR-ligands have shown to induce γδ T cell cytokine secretion and granzyme degranulation.[9] As opposed to phosphoantigens, TLR-stimulation has the potential to stimulate both the Vδ1 and Vδ2 T cell subset.[78] Activation with TLR8 is of particular interest due to its potential to reverse immunosuppressive activity of γδ T cells. However, this effect has not been investigated so far through mo-DCs.[78] Finally, activating receptor-ligand interactions (Table 1) and secreting/presenting cytokines by DCs (*vide supra*), are possible mechanisms to harness γδ T cells in the anti-tumor DC vaccine effect. Here, a special mention of Il-15 is in place.[45,48,79] The latter can lead to the implementation of IL-15 expressing DCs in the clinic.[58,59]

To expand our knowledge on potentiating γδ T cell functions by DCs, evaluation of combinatorial vaccination strategies aiming at engagement of γδ T cells and inclusion of γδ T cell monitoring in DC-vaccination trials is warranted.

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CHAPTER 4:

DESIRABLE CYTOLYTIC IMMUNE EFFECTOR CELL RECRUITMENT BY INTERLEUKIN-15 DENDRITIC CELLS

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ABSTRACT

Success of DC therapy in treating malignancies is depending on the DC capacity to attract immune effector cells, considering their reciprocal crosstalk is partially regulated by cell-contact-dependent mechanisms. Although critical for therapeutic efficacy, immune cell recruitment is a largely overlooked aspect regarding optimization of DC vaccination. In this paper we have made a head-to-head comparison of interleukin (IL)-15-cultured DCs and conventional IL-4-cultured DCs with regard to their proficiency in the recruitment of (innate) immune effector cells. Here, we demonstrate that IL-4 DCs are suboptimal in attracting effector lymphocytes, while IL-15 DCs provide a favorable chemokine milieu for recruiting $CD8^+$ T cells, NK cells and γδ T cells. Gene expression analysis revealed that IL-15 DCs exhibit a high expression of chemokines involved in anti-tumor immune effector cell attraction, while IL-4 DCs display a more immunoregulatory profile characterized by the expression of Th2 and regulatory T cell-attracting chemokines. This is confirmed by functional data indicating an enhanced recruitment of granzyme B^+ effector lymphocytes by IL-15 DCs, as compared to IL-4 DCs, and subsequent superior killing of tumor cells by the migrated lymphocytes. Elevated chemokine (C-C motif) ligand (CCL)4 gene expression in IL-15 DCs and lowered CCR5 expression on both migrated γδ T cells and NK cells, led to validation of increased CCL4 secretion by IL-15 DCs. Moreover, neutralization of CCR5 prior to migration resulted in an important inhibition of γδ T cell and NK cell recruitment by IL-15 DCs. These findings further underscore the strong immunotherapeutic potential of IL-15 DCs.

1. INTRODUCTION

Active immunotherapy using tumor antigen-loaded DCs for anti-cancer vaccination has been under extensive investigation the past 20 years, and is currently being tested in among other phase 3 clinical trials.[1] DCs are sublime professional APCs, which can conveniently be manufactured from monocytes for therapeutic purposes. As a vaccine, they can enhance or induce *de novo* anti-tumor immune responses in cancer patients. Currently, the most widely adopted protocol generates so-called IL-4 DCs.[2] These mo-DCs are generated in the presence of GM-CSF and IL-4 for five days, followed by an activation step of two days with the Jonuleit cocktail consisting of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and PGE2.[2] Overall objective responses and survival benefits are, although undeniably present and comparable to some classic treatment strategies, considered rather modest.[1]

This has led to the development of a new generation of DC vaccines with improved potency.[3-6] IL-15 DCs,[7-10] replacing IL-4 with IL-15 for DC differentiation and using a TLR agonist-based maturation cocktail, have already proven themselves to outclass the conventional IL-4 DCs. This in terms of their capacity to induce both Th1 and CTL responses[7,9-11] and to potentiate innate NK cell and γδ T cell cytotoxicity.[12,13] Moreover, IL-15 DCs have intrinsic cytotoxic properties, allowing them to be listed as 'killer DCs'.[14]

To date, awareness is growing that DC-based immunotherapy should comprise more than vaccine-mediating effects on the adaptive immune system, i.e. aiming at induction of (tumor) antigen-specific CTLs and a Th1 response.[15] Tumors evading CTL recognition by downregulating major histocompatibility complex class I molecules can still be recognized and killed by NK cells[16] or γδ T cells.[17] Additionally, both cell types are important sources of pro-inflammatory cytokines, supporting *inter alia* DC and T cell functions.[16,17] This has recently been substantiated by preclinical data showing that when innate immunotherapy is successfully applied, subsequent long-term adaptive cancer immunity is effected.[18] Therefore, IL-15 DCs could represent an improved therapeutic vaccine component with regard to immunostimulatory activity and activation of both innate and adaptive anti-tumor arms. However, using DCs with optimal immunocompetence is, on its own, likely not sufficient for therapeutic effectiveness. Ideally, vaccine DCs should come in contact with the necessary effector cells to perform their directing and activating functions. Whereas the capacity of DCs to migrate towards the lymph nodes is routinely assessed, less consideration is devoted to their chemoattracting properties, leaving a gap in our understanding of DC functioning. Here we investigate the attraction of immune effector

cells by IL-4 DCs versus IL-15 DCs and provide new evidence of the superior immunotherapeutic potential of our short term-cultured IL-15 DCs over conventional IL-4 DCs as substantiated by DC features at the gene and protein levels, and by functional properties.

2. MATERIALS AND METHODS

2.1. ETHICS STATEMENT AND CELL MATERIAL

This study was approved by the Ethics Committee of the UZA (Edegem, Belgium) under the reference number B300201419756. All experiments were performed using blood samples from anonymous healthy volunteers supplied by the blood bank of the Red Cross (Mechelen, Belgium). PBMC were isolated by Ficoll density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare, Diegem, Belgium) and washed in phosphate buffered saline (Life Technologies, Merelbeke, Belgium) containing 1% EDTA (Merck, Darmstadt, Germany). Positive magnetic cell selection was used for purification of $CD14^+$ monocytes and γδ T cells, according to the manufacturer's instructions (Miltenyi Biotec, Amsterdam, The Netherlands), with minor modifications concerning the γδ T cell isolation protocol. Namely, the first effluent was re-administered onto the column before starting with the washing steps and these were augmented from three to five. Untouched NK cells were isolated from (CD14-depleted) PBMC using a human NK cell isolation kit (Miltenyi Biotec). Regular MACS buffer, containing EDTA, was used for all MACS separations. Untouched γδ T cells, for the functional evaluation of γδ T cells after migration, were isolated with the EasySep™ Human Gamma/Delta T Cell Isolation Kit (Grenoble, France), according to the manufacturer's instructions. (CD14-depleted) PBMC underwent one freeze-thaw cycle to enable the use of autologous wash-out supernatant (*vide infra*). Cryopreservation of PBMC had an insignificant negative effect on the migratory capacity (data not shown). The Burkitt's lymphoma tumor cell line Daudi was kindly provided to us by the laboratory of Prof. Kris Thielemans (Free University of Brussels, Brussels, Belgium) and the chronic myeloid leukemia tumor cell line K562 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA; catalogue number: K-562 ATCC® CCL243™).

2.2. DENDRITIC CELL CULTURE

IL-15 DCs were prepared as per our previously reported rapid DC culture protocol.[11,12,14] Briefly, monocytes were seeded in RPMI (Life Technologies) supplemented with 2.5% hAB (Invitrogen, Merelbeke, Belgium) at a final concentration of 1.0-1.2x106 cells/mL.

Differentiation was induced with 800 IU/mL granulocyte macrophage colony-stimulating factor and 200 ng/mL IL-15 (Immunotools, Friesoythe, Germany). A TLR-activating maturation cocktail, comprising R848 (3 µg/mL; Alexis Biochemicals, San Diego, USA), tumor necrosis factor-α (2.5 ng/mL), interferon-γ (250 ng/mL; Immunotools) and PGE2 (1 µg/mL; Pfizer, Puurs, Belgium), was added after 24-48 hours of differentiation for 18-20 hours. All components were bought from Invitrogen, unless stated otherwise. Control 7-day IL-4 DCs from the same blood donors were prepared as previously described in detail.[11] All subsequent experiments were performed using the obtained activated IL-15 DCs and IL-4 DCs. For the collection of x-hour wash-out supernatant, mature DCs were harvested, washed thoroughly and resuspended in fresh medium, RPMI + 2.5% hAB, at a concentration of $1x10^6$ cells/mL. After x hours of culturing in low absorbing polypropylene tubes, cell-free supernatant was collected and frozen at -20°C until further use.

2.3. CELLULAR RNA EXTRACTION, PREPARATION, AND HYBRIDIZATION ON MICROARRAYS

Total RNA was extracted from $7\text{-}11x10^6$ mature IL-15 DCs and IL-4 DCs of three independent donors using RLT lysis buffer (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. RNA quantity and purity were evaluated spectrophotometrically by Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA), while the quality was assessed by the Agilent 2100 bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA). Only samples with good RNA yield and no RNA degradation (28S:18S > 1.7 and RNA integrity number > 6) were retained for further experiments. Labelling of samples and hybridization on the Human Genome U133 plus 2.0 microarray chipswere performed according to the manufacturer's protocols (Affymetrix, Santa Clara, CA).

2.4. CELLULAR MICROARRAY DATA ANALYSIS

The high-throughput microarray data are accessible through GEO Series accession number GSE79184. Data analysis was performed using AMDA software.[19] To filter out noise, an Inter Quartile Range > 0.2 was applied. In order to define a set of differential expressed genes, a Linear Model for Microarray Data[20] with a False Discovery Rate correction (Benjamini-Hochberg)[21] was implemented, selecting probe sets with an adjusted p-value ≤ 0.001 and a fold change ≥ 3 among the two conditions. Hierarchical clustering was performed through MultiExperiment Viewer[22] using the Log2 of the ratio between expression signals and the global median expression for each gene, and setting Euclidean distance as dissimilarity measure and Average linkage as linkage method.

2.5. CELL MIGRATION ASSAY

Transwell chemotaxis assays were performed using 24-well transwells with 5 µm pore size polycarbonate membrane. $1x10^6$ PBMC, $0.1x10^6$ purified NK cells or $0.1x10^6$ purified γδ T cells were seeded in the upper wells and lower wells were filled with autologous 48-hour wash-out supernatant. RPMI + 2.5% hAB in the lower compartment served as a negative control, representing the random background migration of immune cells. Cell migration was allowed for three hours at 37°C/5% CO_2, whereupon migrated cells were collected from the lower compartment. After washing, cells were resuspended in a fixed volume and counted flow cytometrically on a FacsAria II (Becton Dickinson [BD], Erembodegem, Belgium) at a continuous flow rate. Migration was calculated as % increase of migration (number of migrated cells in the specific condition / number of migrated cells in the negative control) x 100. To examine the role of the chemokine receptor CCR5, cells were pre-incubated with neutralizing anti-CCR5 mAbs (10 µg/mL, R&D; Abingdon, United Kingdom) or corresponding IgG2b isotype controls 1 hour prior to migration.

2.6. FLOW CYTOMETRIC IMMUNOPHENOTYPING

To determine circulating peripheral blood cell subtypes, migrated cells from PBMC were stained with the following mAbs: γδ TCR-FITC (Miltenyi), CD14-FITC, CD56-PE, CD3-PerCP-Cy5.5, CD11c-V450, CD8-PB (Life Technologies), CD19-APC and CD4-APC-H7. The gating strategy can be consulted in Figure 1. To assess the phenotypic (chemokine receptor) profile of isolated γδ T cells and NK cells pre- and post-migration the ensuing mAbs were used: γδ TCR-FITC (Miltenyi), CD56-FITC, IL-15Rα-PE (R&D), CD195-PE-Cy7, CD3-PerCP, δ1 TCR-vioblue (Miltenyi), CD197-V450, δ2 TCR-APC (Miltenyi), CD193-APC and CD192-AF647. For evaluation of intracellular granzyme B expression, brefeldin A (1 µL/mL; BD) and monensin (0.67 µL/mL; BD) were added to the harvested cells and incubated for 3 hours at 37°C/5% CO_2. PBMC were then washed and incubated with surface Abs CD56-PE, CD8-PerCP, γδ TCR-APC (Miltenyi) and CD3-APC-H7 for 30 minutes at 4°C. Subsequently, cells were fixed and permeabilized, using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, Vienna, Austria). Intracellular granzyme B-BV421 was added for 1 hour at 4°C. In all applications the Live/Dead® fixable dead cell stain (Invitrogen) was used to allow discrimination between viable and non-viable cells. All samples were acquired on a FACSAria II flow cytometer and mAbs were purchased from BD, unless stated otherwise. Corresponding species- and isotype-matched antibodies were used as controls.

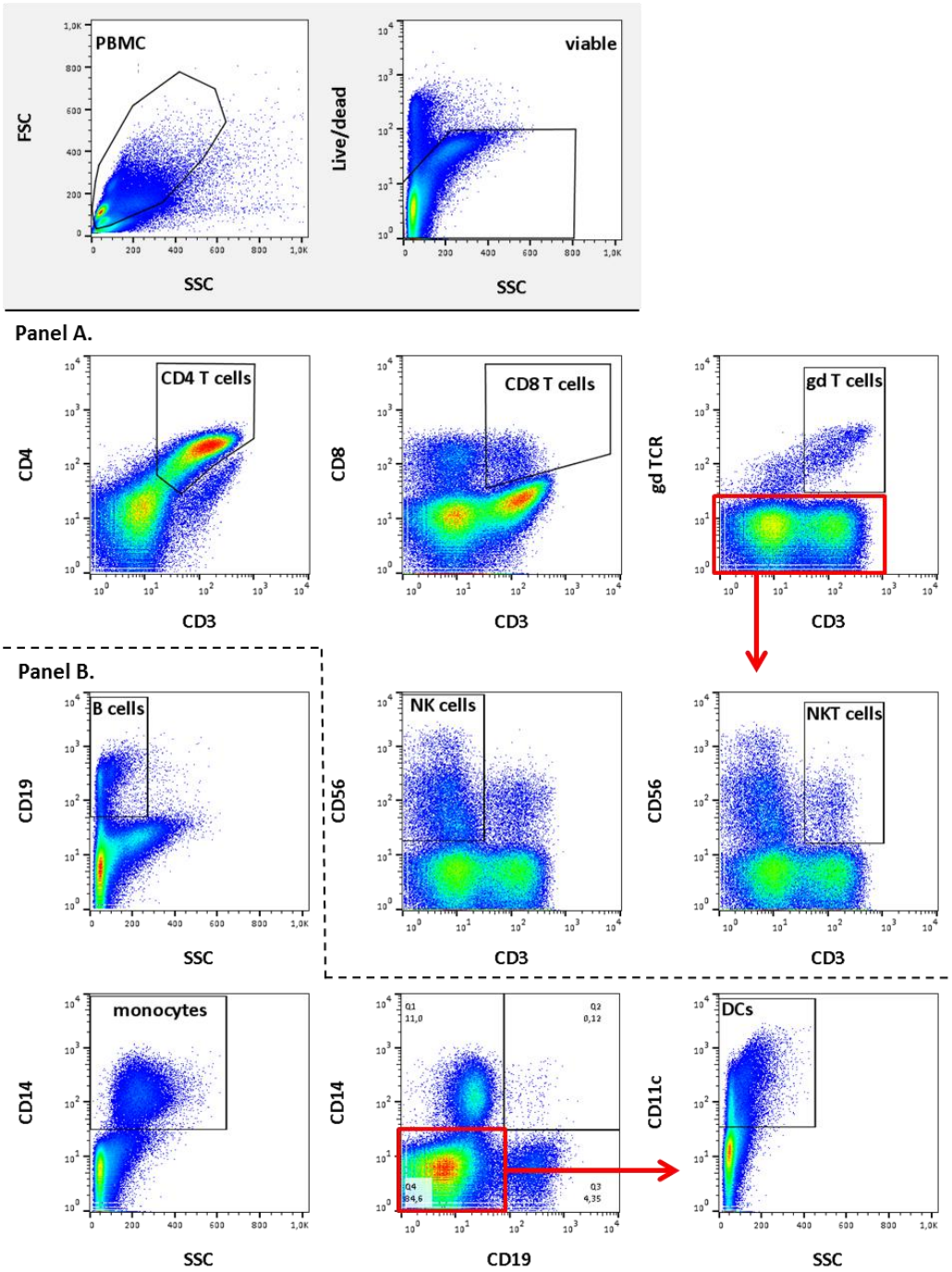


Figure 1. Gating strategy for determination of cell subsets within unfractioned PBMC. Initial gating is done on FSC and SSC, eliminating debris, followed by the exclusion of dead cells. Panel A) Within the viable cell gate, $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), γδ T cells ($CD3^+$ γδ TCR^+), and NK cells ($CD3^-CD56^+$) are identified. Subsequently, NKT cells are defined as $CD3^+CD56^+$ in the γδ TCR^- gate. Panel B) Viable monocytes are $CD14^+$, B cells are $CD19^+$ and DCs are selected as $CD11c^+$ cells within the $CD14^-CD19^-$ gate.

2.7. CYTOKINE SECRETION ASSAY

Secretion of the chemokine CCL4 by IL-15 DCs and IL-4 DCs was determined in 1:2 diluted 4-, 12-, 24- and 48-hour wash-out supernatant of both DC types by means of a human CCL4 ELISA (Peprotech, Rocky Hill, NJ, USA), according to the manufacturer's instructions.

2.8. CYTOTOXICITY ASSAY

The killing capacity of recruited γδ T cells and NK cells was determined using a flow cytometry based protocol. Hereto, a three-hour cell migration assay (see above) with $1x10^6$ isolated γδ T cells or NK cells was performed, after which the cells from the lower well were harvested and counted. Target cells (Daudi or K562) were labeled with PKH67 green fluorescent cell linker (Sigma-Aldrich, Diegem, Belgium), according to manufacturer's instruction, and put together with migrated γδ T cells or NK cells at an E:T cell ratio of 5:1 γδ T cells or 1:1 NK cells, respectively. After 4 hour-coculture, samples were stained with Annexin V-APC (BD) and PI (BD), followed by acquisition on a FACSAria II flow cytometer (BD). Cytotoxicity was calculated based on the percentage of viable (Annexin V^-/PI^-) $PKH67^+$ tumor cells, using the following equation: % killing = 100 % - (% viable tumor cells with effector cells/% viable tumor cells without effector cells).

2.9. STATISTICS

Flow cytometry data were analyzed using FlowJo (v10; Treestar, Ashland, OR, USA). GraphPad Prism software (v5.0; San Diego, CA, USA) was used for statistical calculations, including testing to ascertain Gaussian distribution of the data, and artwork. P-values < 0.05 were considered statistically significant.

3. RESULTS

3.1. IL-15 DCs AND IL-4 DCs ATTRACT DISTINCT CELL POPULATIONS

To explore the migratory capacity of immune cells towards chemoattractant agents secreted by IL-15 DCs and IL-4 DCs, a three-hour migration assay towards 48-hour wash-out supernatant of autologous activated DCs was performed. PBMC were recruited by either DC preparation, corresponding with an increase of migration ± standard deviation (SD) of 190 ± 95 % and 193 ± 97 %, respectively (n = 11). Immunophenotyping of the migrated cells demonstrated that both $CD8^+$ T cells and γδ T cells were significantly more

recruited by IL-15 DCs in comparison with the IL-4 DCs (Figure 2). Concerning $CD8^+$ T cell migration, an increase of 236 ± 50 % and 173 ± 40 % was observed towards IL-15 DCs and IL-4 DCs, respectively. For the γδ T cells an increase of 276 ± 57 % and 195 ± 73 % was true. Both NK cells and $CD3^+$ $CD56^+$ γδ TCR^- NKT cells were attracted by IL-15 DCs and IL-4 DCs (Figure 2). This resulted in an increase of migration of 233 ± 84 % and 222 ± 130 % of NK cells, and 330 ± 130 % and 268 ± 149 % of NKT cells towards IL-15 DC and IL-4 DC wash-out supernatant, respectively.

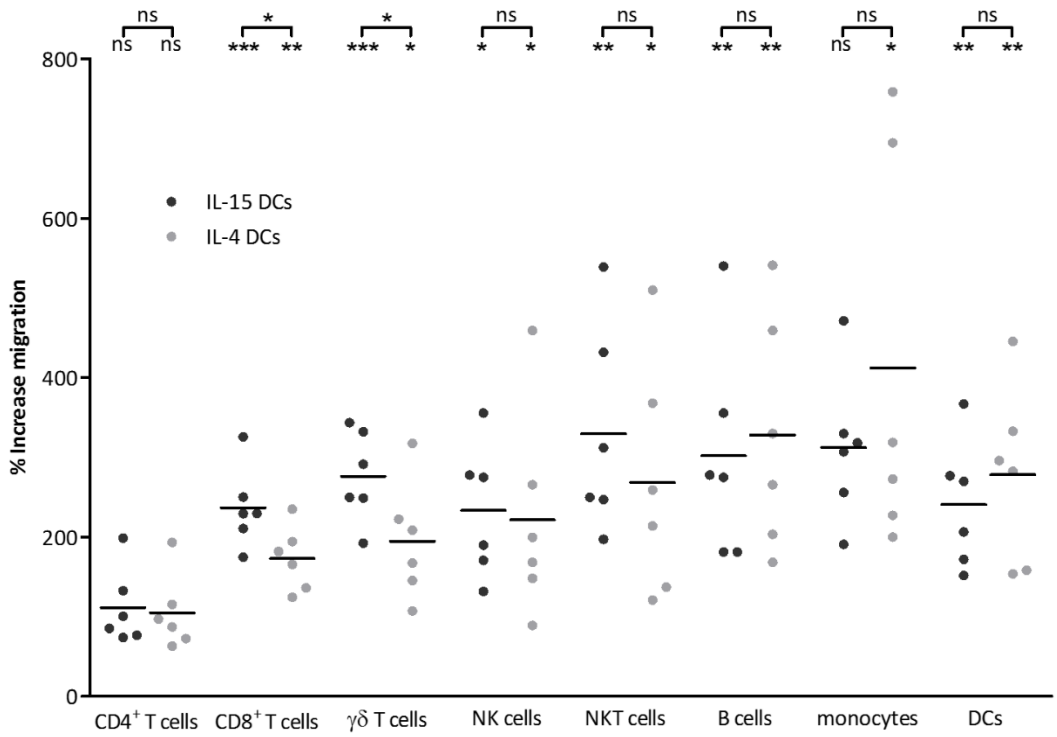
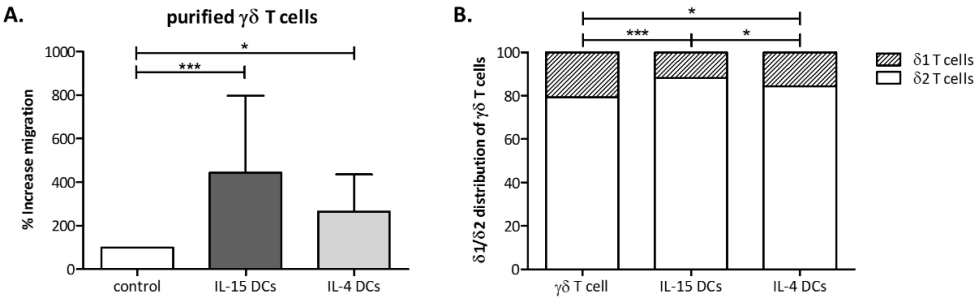


Figure 2. Immune effector cells are recruited by IL-15 DCs. 48-hour wash-out supernatant of IL-15 DCs and IL-4 DCs was used in a three-hour transwell chemotaxis assay with PBMC. Scatter dot plots (line at mean) represent the % increase of migration, calculated as (number of migrated cells in the specific condition / number of migrated cells in the negative control) x 100, of viable migrated PBMC subsets (n=6), with the negative control representing spontaneous migration towards medium. Immune cells recruited by IL-15 DC and IL-4 DC wash-out supernatant are depicted as dark grey and light grey spheres, respectively. Repeated measures ANOVA with Bonferroni's Multiple Comparison Testing was used for statistical analysis. Statistics depicted on top of the brackets represent differences between migration towards supernatant of IL-15 DCs versus IL-4 DCs, whereas statistics of migration towards each DC type as compared to the medium control is noted below the brackets. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$, ns, $p > 0.05$

Of the evaluated cell populations, both IL-15 DCs and IL-4 DCs were able to attract $CD19^+$ B cells and $CD14^-$ $CD19^-$ $CD11c^+$ blood DCs, whereas $CD14^+$ monocytes only migrated towards IL-4 DC wash-out supernatant (Figure 2). $CD4^+$ T cell counts were comparable in all conditions.

3.2. IL-15 DCs RECRUIT PURIFIED γδ T CELLS AND NK CELLS

Further elaborating on the overall higher innate effector cell recruiting potential of IL-15 DC supernatant, transwell migration assays with purified γδ T cells (Figure 3A) and NK cells (Figure 4A) support the superior chemoattractive capacity of IL-15 DCs. Unstimulated peripheral blood γδ T cells showed an increase of migration of 443 ± 354 % towards IL-15 DCs, while IL-4 DCs only effectuated an increase of migration of 264 ± 172 %. Both main subtypes, Vδ1 and Vδ2, of blood γδ T cells were attracted by IL-15 DCs and IL-4 DCs, with a predominance of the Vδ2 fraction. This was pointedly higher after attraction by IL-15 DCs (Figure 3B). Analyzing the basal chemokine receptor profile (Figure 3C), γδ T cells were weakly positive for C-C chemokine receptor 2 (CCR2) (1.0 ± 1.4 %), CXCR3 (2.4 ± 1.4 %) and IL-15Rα (1.5 ± 1.2 %), and positive for CCR5 (18.9 ± 8.3 %) and CCR7 (10.5 ± 6.7 %). After migration towards IL-15 DCs, a significantly lower percentage of CCR2 and CCR5-positive γδ T cells was observed, whereas only a lower expression of CCR5 was detected upon IL-4 DC-mediated migration.



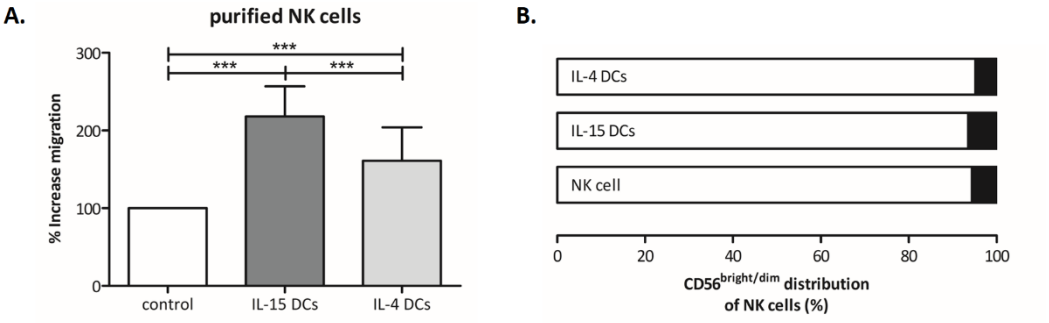
C.

% (±SD)	CD192 (CCR2)	CD195 (CCR5)	CD197 (CCR7)	CD183 (CXCR3)	IL-15Rα
γδ T cell	1.038 (±1.444)	18.90 (±8.337)	10.50 (±6.738)	2.422 (±1.373)	1.462 (±1.225)
IL-15 DCs	0.073 (±0.080)*	7.514 (±3.789)***	8.501 (±6.437)	2.227 (±2.237)	0.840 (±0.951)
IL-4 DCs	0.167(±0.120)	11.04 (±5.446)***	8.692 (±6.891)	2.165 (±2.314)	1.453 (±1.412)

*, statistically significant as compared to γδ T cell

Figure 3. DC-mediated migration and phenotype analysis of purified γδ T cells. A. Percentage of isolated γδ T cell migration towards medium (control), 48-hour wash-out supernatant of IL-15 DCs (IL-15 DCs) and IL-4 DCs (IL-4 DCs). Data were calculated as percentage of migrated γδ T cells relative to background migration and show mean values (+ SD) of eleven different donors. **B.** Bar graphs illustrate the relative distribution of Live/$dead^-$ $CD3^+$ γδ TCR^+ TCR-$δ2^+$ and Live/$dead^-$ $CD3^+$ γδ TCR^+ TCR-$δ1^+$ subsets of purified γδ T cells (γδ T cell) and following DC-mediated migration (n = 7). **C.** Percentage surface

expression of chemokine receptors CCR2, CCR5, CCR7 and CXCR3, and IL-15Rα on isolated Live/dead$^-$ CD3$^+$ γδ TCR$^+$ T cells before and after migration (n = 6-10). For δ1/δ2 distribution and CCR5/CCR7 expression) a repeated measures ANOVA with Bonferroni's Multiple Comparison Test was used, for γδ T cell migration, CCR2/CXCR3/IL-15Rα expression a Friedmann Test with Dunn's Multiple Comparison Test was used. ***, $p < 0.001$, *, $p < 0.05$



C.

% (±SD)	CD192 (CCR2)	CD195 (CCR5)	CD197 (CCR7)	CD183 (CXCR3)	IL-15Rα
NK cell	0.323 (±0.377)	4.363 (±3.869)	1.374 (±0.731)	0.506 (± 0.860)	7.366 (±2.948)
IL-15 DCs	0.302 (±0.239)	1.209 (±0.585)**	1.183 (±0.598)	0.165 (± 0.175)	2.713 (±1.457)**
IL-4 DCs	0.251 (±0.305)	1.374 (±0.808)*	0.941 (±0.833)**	0.158 (±0.242)	3.673 (2.246)*

*, statistically significant as compared to NK cell

Figure 4. DC-mediated migration and phenotype analysis of purified NK cells. A. Migration capacity of purified NK cells towards the chemokine milieu of IL-15 DCs and IL-4 DCs is depicted as % increase migration (+ SD) as compared to the negative control (n = 11). **B.** Bar graphs represent the relative distribution of Live/dead$^-$ CD3$^-$CD56^{bright} and Live/dead$^-$ CD3$^-$CD56^{dim} subsets in isolated NK cells prior to migration (NK cell) and in NK cells migrated towards IL-15 DC (IL-15 DCs) and IL-4 DC (IL-4 DCs) wash-out supernatants. **C.** Percentage surface expression of chemokine receptors CCR2, CCR5, CCR7 and CXCR3, and IL-15Rα on isolated NK cells before and after migration (n = 8). Repeated measures ANOVA with Bonferroni's Multiple Comparison Test (NK cell migration, IL-15Rα expression), Friedmann Test with Dunn's Multiple Comparison Test (CD56 distribution, chemokine receptor expression). ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$

Like γδ T cells, purified peripheral blood NK cells were recruited by both IL-15 DCs (% increase of migration = 218 ± 39 %) and IL-4 DCs (161 ± 43 %), the latter however to a considerably lower degree as compared to IL-15 DCs (Figure 4A). Based on the CD56 surface expression, both DC types were capable of recruiting the two major NK cell subsets (Figure 4B), though a small enrichment of CD56^{bright} NK cells could be observed towards IL-15 DCs. The chemokine receptors CCR5 (6.8 ± 3.5 %), CCR7 (16.6 ± 6.5) and IL-15Rα (46.0 ± 35.4) were predominantly expressed by CD56^{bright} NK cells relative to CD56^{dim} NK cells expressing 2.5 ± 0.8 % CCR5, 0.9 ± 0.5 % CCR7 and 5.6 ± 1.3 % IL-15Rα, respectively (data not shown).

Yet, expression of CCR2 and CXCR3 on purified NK cells was absent (Figure 4C). After migration a decline in surface CCR5 and IL-15Rα was observed on NK cells migrated towards IL-15 DC and IL-4 DC wash-out supernatant, and CCR7 was downregulated on NK cells recruited by IL-4 DCs (Figure 4C). Lastly, non-migrated γδ T cells and NK cells, harvested from the transwell insert after the three-hour migration assay, displayed a comparable receptor profile relative to unstimulated peripheral blood γδ T cells and NK cells. γδ T cells and NK cells exposed to 48-hour wash-out supernatant, however, demonstrated a receptor expression profile similar to migrated γδ T cells and NK cells (Figure 5).

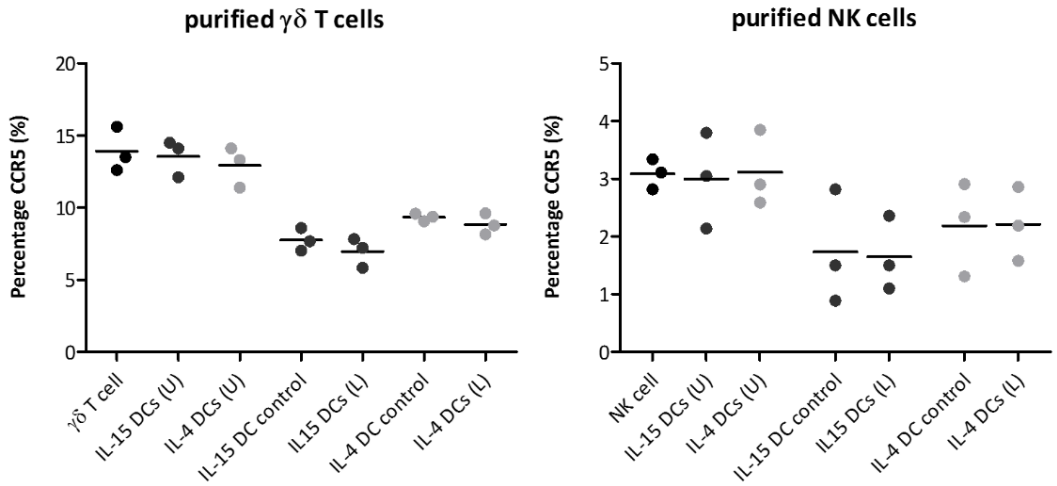


Figure 5. Influence of DC supernatant on purified γδ T cell and NK cell CCR5 expression. Percentage surface expression of CCR5 was assessed flow cytometrically on isolated viable $CD3^+$ γδ TCR^+ T cells (γδ T cell) and $CD3^-CD56^+$ NK cells (NK cell), immune cells exposed for 3 hours to 48-hour wash-out supernatant of IL-15 DCs (IL-15 DC control) or IL-4 DCs (IL-4 DC control), and immune cells harvested from the lower well (L) or transwell insert (U) after a three-hour migration assay towards IL-15 DC (IL-15 DCs) or IL-4 DC (IL-4 DCs) wash-out supernatant.

3.3. DIFFERENTIAL CHEMOKINE GENE EXPRESSION

Gene expression analysis between IL-15 DCs and IL-4 DCs revealed a distinct dissimilitude between both activated DC types at different levels, with changes of genes involved in the chemokine signaling pathway being the most significant. Based on attraction of (innate) immune effector cells by DCs, 6 chemokine genes with differential expression between IL-15 DCs and IL-4 DCs were selected (Table 1) out of 18 genes (Figure 6) belonging to the chemokine family. Overall, IL-15 DCs exhibit a high expression of chemokines involved in

anti-tumor immune effector cell attraction, in particular CCL2, CCL4, CCL7, chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10 and CXCL11, whereas IL-4 DCs display a more immunoregulatory profile characterized by high expression of Th2 and regulatory T cell attracting chemokines, notably CCL17 and CCL22.

Table 1. List of chemokines involved in anti-tumor immune effector cell attraction with differential gene expression between IL-4 DCs and IL-15 DCs.

chemokine	Chemokine receptor	Chemokine GenBank ID	Fold change	Function (references)
CCL2	CCR2 CCR4	S69738	54.1	"killer DC" homing to tumor sites,[23] NK cell,[24] NKT cell[25] and Vδ1 T cell[26] recruitment
CCL4	CCR5	NM_002984	15.7	$CD4^+$ and $CD8^+$ T cell-mediated immunity,[27,28] NK cell,[29] and Th1 γδ T cell[30] recruitment
CCL7	CCR2	NM_006273	117.2	recruitment activated NK cells and T cells[31,32]
CXCL9	CXCR3	NM_002416	325.5	Th1, $CD8^+$ T cell,[33] NK cell[34] and NKT cell[35] recruitment
CXCL10	CXCR3	NM_001565	587.8	Th1,[36] $CD8^+$ T cell,[33,37] γδ T cell,[38] NK cell [39,40] and NKT cell[41] recruitment
CXCL11	CXCR3	AF030514	31.6	Th1, $CD8^+$ T cell,[33] NK cell and NKT cell[42] recruitment

Fold change represents ratio in expression signal between mature IL15 DCs *vs.* IL-4 DCs. Abbreviations: DC, dendritic cell; Th1, T helper type 1; NK cell, natural killer cell; NKT cell, natural killer T cell

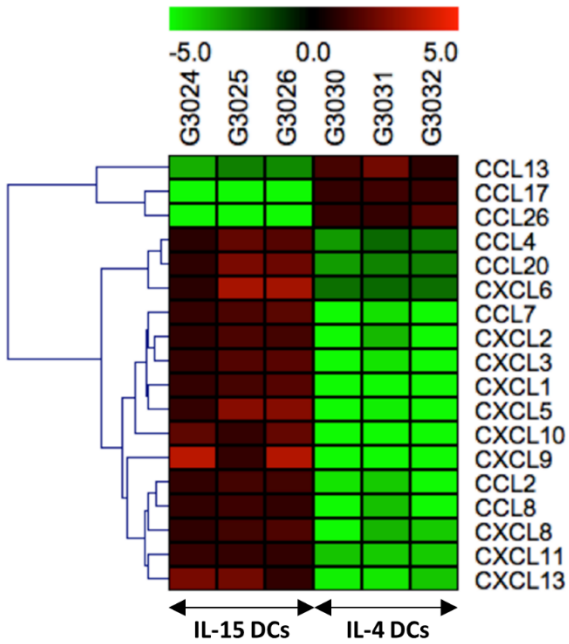


Figure 6. Hierarchical cluster analysis of 18 genes belonging to the chemokine family, distinctly different between IL-15 DCs and IL-4 DCs. The heat map represents the differential expressed genes patterns between IL-15 DC samples (left) and IL-4 DC samples (right) of three independent donors. The value range represents the Log2 of the ratio between expression signals and the global median expression for each gene. Red color indicates up-regulated genes and green color down-regulated genes.

3.4. IL-15 DC-DEPENDENT EFFECTOR CELL RECRUITMENT IS PARTIALLY CCL4-CCR5 MEDIATED

Taking into account the lowered CCR5 expression on both migrated γδ T cells and NK cells, the higher CCL4 chemokine gene expression in IL-15 DCs and the described effects of CCL4 on effector cells,[27,29,30,43,44] we further evaluated the involvement of the CCL4-CCR5 axis in the chemoattractive activity of IL-15 DCs. Using a CCL4 ELISA, the higher gene expression of CCL4 in IL-15 DCs was validated at the protein level in 48-hour wash-out supernatant, demonstrating a significant higher secretion by IL-15 DCs (3819 ± 1600 pg/mL) then by IL-4 DCs (1687 ± 1244 pg/mL) (Figure 7A). CCL4 secretion kinetics indicate that the main body of CCL4 in 48-hour wash-out supernatant of both DC types accumulates in the first 24 hours after DC harvest. Still, CCL4 secretion continues after 24 hours (Figure 7B). Subsequently, we demonstrated that neutralization of CCR5 on PBMC prior to migration results in a significant inhibition of migration of γδ T cells and NK cells towards IL-15 DCs, whereas this was not observed for IL-4 DC-mediated migration (Figure 7C). It can therefore be concluded that the CCL4-CCR5 signaling pathway is to some extent responsible for the IL-15 DC-mediated effector cell recruitment.

3.5. CYTOTOXIC CAPACITY OF MIGRATED EFFECTOR CELLS

To evaluate the functional consequences of the different recruitment abilities of IL-4 DCs and IL-15 DCs, we first evaluated the intracellular granzyme B expression on migrated PBMC. After migration a consistent enrichment of granzyme B^+ cells was observed towards either DC type. It concerned preferential recruitment of cytotoxic effector cells, since granzyme B expression in PBMC remained unaffected after exposure to 48-hour wash-out supernatant itself (Figure 8A). Phenotyping of the different granzyme B^+ PMBC populations indicated a higher proportion of granzyme B^+ $CD8^+$ T cells, γδ T cells and NK cells migrating towards IL-15 DCs, whereas IL-4 DCs only significantly recruited granzyme B^+ $CD8^+$ T cells (Figure 8B). Further building on these results, we investigated whether the increased recruitment of granzyme B^+ cells could also be translated into functional cytotoxic activity against tumor cells (Figure 8C). Performing a 4-hour cytotoxicity assay with migrated cells from a 3-hour migration assay, purified γδ T cells and NK cells attracted by secreted factors of IL-15 DCs showed a significantly higher killing capacity against tumor cells as compared to their non-migrated counterparts. Migrated γδ T cells and NK cells recruited by IL-4 DCs were equally cytotoxic as non-migrated effector cells. This supports the aforementioned (micro-array) results, stating that IL-15 DCs are superior in the recruitment of anti-tumor effector cells as compared to IL-4 DCs, which endorses the suitability of IL-15 DCs as a tumor vaccine.

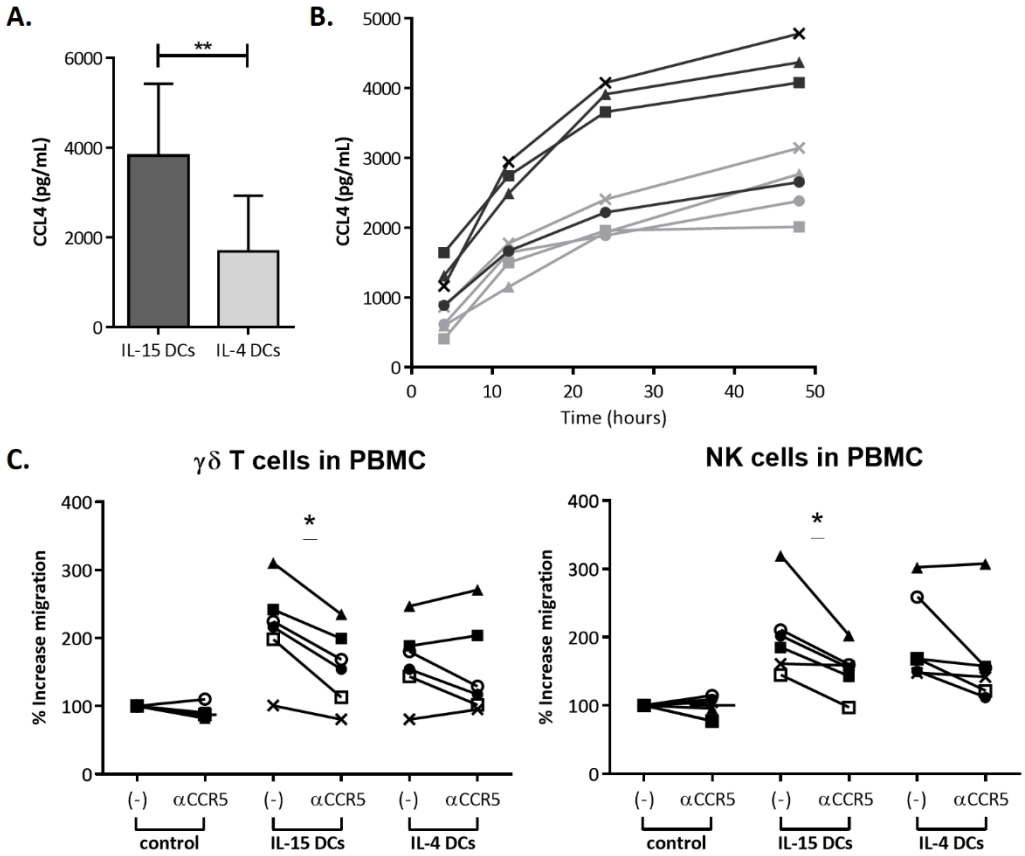


Figure 7. CCR5-dependent recruitment of effector immune cells by IL-15 DCs. A. Quantitative comparison of CCL4 secretion by IL-15 DCs and IL-4 DCs as measured by ELISA in 48-hour wash-out supernatant of IL-15 DCs and IL-4 DCs. Concentration (pg/mL + SD) is shown of duplicate conditions for 10 donors. Paired T test. **B.** Time course of CCL4 secretion (pg/mL) by IL-15 DCs (dark grey lines) and IL-4 DCs (light grey lines) determined by ELISA in 4-, 12-, 24- and 48-hour wash-out supernatant (in duplicate) of 4 different donors (unique symbols). **C.** PBMC were cultured in the absence (-) or presence of neutralizing anti-CCR5 mAbs (αCCR5) for 1 hour prior to a three-hour chemotaxis assay towards medium (control), 48-hour wash-out supernatant of IL-15 DCs (IL-15 DCs) or IL-4 DCs (IL-4 DCs). Data of 6 independent donors (unique symbols) were calculated as percentage of migrated γδ T cells and NK cells normalized to the negative control. Wilcoxon matched-pairs signed rank test. Different donors were used for experiment B and C. **, $p < 0.01$, *, $p < 0.05$

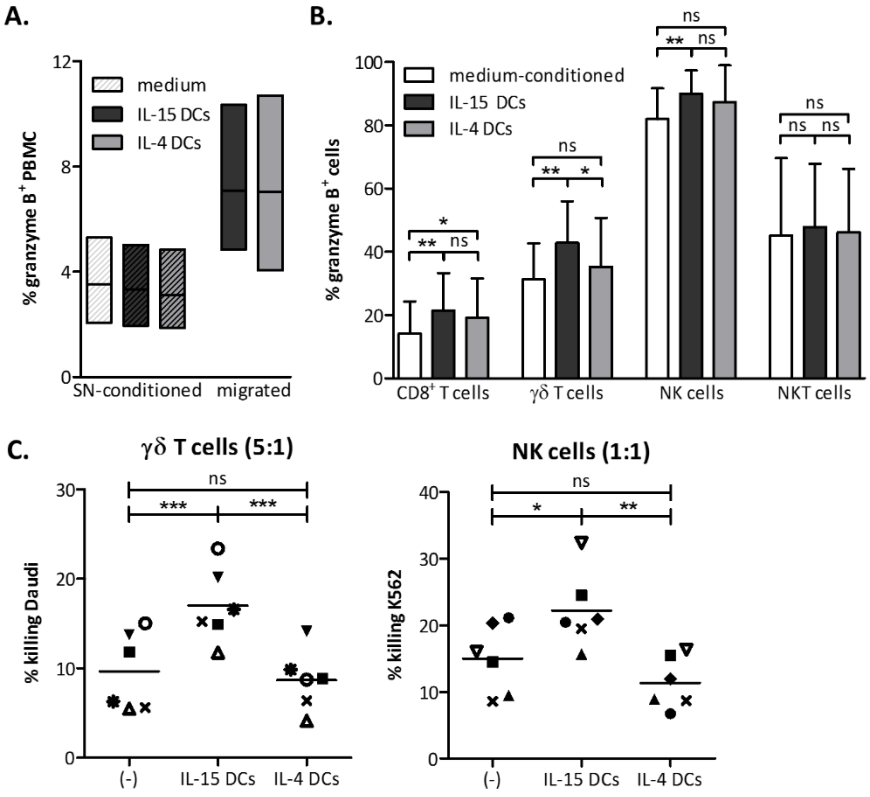


Figure 8. IL-15 DCs superiorly attract cytotoxic immune effector cells A. Floating bars (min to max, line at mean) represent % granzyme B^+ cells in PBMC (white bar), PBMC exposed for 3 hours to 48-hour wash-out supernatant of IL-15 DCs (striped dark grey bar) or IL-4 DCs (striped light grey bar), and PBMC following IL-15 DC- (dark grey bar) or IL-4 DC-mediated (light grey bar) migration (n = 3). **B.** Representation of % granzyme B^+ cells (+ SD) within the different effector cell subsets in full PBMC fraction (white bars) and following IL-15 DC- (dark grey bars) or IL-4 DC-mediated (light grey bars) migration (n = 8). **C.** Cytotoxicity was determined of migrated purified γδ T cells and NK cells towards 48-hour wash-out supernatant of IL-15 DCs (IL-15 DCs) or IL-4 DCs (IL-4 DCs). Non-migrated purified cells (-) were used to define control killing capacity. Daudi and K562 target cells were added at an E:T ratio of 5:1 γδ T cells and 1:1 NK cells, respectively. Percentage tumor cell killing was determined by Annexin-V/PI staining after 4 hours and calculated using the formula specified in "Materials and methods". Donors are represented by unique symbols (n = 6). Repeated measures ANOVA with Bonferroni's Multiple Comparison Test. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$, ns, $p > 0.5$

4. DISCUSSION

The past decade has witnessed the advent of a new generation of DC-based vaccines with improved potency. Herewith, IL-15 DCs represent a promising cancer vaccine, stimulating both innate and adaptive anti-tumor immune effector cells.[11,12,14] Innate immunity may be critical for activation and polarization of adaptive immune responses, and therefore for success of DC-based vaccines.[16-18,45] Expressing CCR7,[11] IL-15 DCs have the clinical potential to migrate from the site of injection to the lymph nodes where they can stimulate antigen-specific T cells, as well as interact with NK cells and γδ T cells. However, the effectiveness of IL-15 DCs, and generally for all DC vaccines, might in the end rely on their ability to secrete the appropriate chemokines, allowing them to effectively recruit, engage, and activate (γδ) T cells and NK cells. To this extent, we made a head-to-head comparison of IL-15 DCs and conventional IL-4 DCs with regard to their proficiency in the recruitment of (innate) effector cells.

Our data show that both IL-15 DCs and IL-4 DCs are endowed with potent immune cell recruiting capacities, but attract distinct PBMC populations. First and foremost, the most common cytolytic effector cells, in particular $CD8^+$ T cells, γδ T cells and NK cells, are superiorly recruited by IL-15 DCs. This is congruent with other data underpinning the weak ability of conventional IL-4 DCs to induce effector leukocyte migration.[46,47] Both IL-15 DCs and IL-4 DCs fail to attract $CD4^+$ T cells, which is, however, consistent with the *in vivo* situation in the lymph nodes. Here naïve $CD4^+$ T cells approach DCs along random trajectories, without any apparent directed motion toward DCs.[48] Next, B cells are recruited by both IL-15 DCs and IL-4 DCs. The biological and clinical relevance of humoral immune responses against tumor antigens remains, however, controversial to date.[49] In light of the booked successes with monoclonal antibody-based therapeutics, to improve among other cellular anti-tumor immunity, attraction of B cells by DCs holds a potential benefit.[50,51] Monocytes, on the other hand, are merely attracted by IL-4 DCs. The implications concerning DC vaccination will depend on the subtype to which these monocytes belong,[52,53] considering their dual role in cancer.[54] Interestingly, both IL-15 DCs and IL-4 DCs are able to attract autologous peripheral blood DCs. This is of importance as activated host DCs are capable of transducing the anti-tumor response to other immune cells, sustaining anti-tumor immunity.[55-57]

Focusing on the cytolytic effector cells in PBMC, our microarray data show significant differences in RNA levels between IL-15 DCs and IL-4 DCs for the $CD8^+$ T cell attracting chemokines CCL4[27,28,58] and CXCL9-11[36,37,59] suggesting their involvement in the superior

attraction of $CD8^+$ T cells by IL-15 DCs in comparison to IL-4 DCs. Additional functional downstream analyses are warranted to confirm their attribution. The same applies for the chemokines involved in the DC-mediated NKT cell attraction. The migratory potential of NKT cells towards DCs has so far sparsely been investigated, with only one prior report demonstrating their migratory responsiveness to the chemokine milieu of poly(I:C)-matured IL-4 DCs and to a lesser extent of conventional IL-4 DCs.[42] Since the first-mentioned DC type produces much higher levels of the CXCR3 ligands CXCL9-11, a causal relationship is possible, although no direct evidence is provided through, for example, neutralization experiments.[42] Since NKT cells are also more prone to migrate towards IL-15 DCs, as compared to IL-4 DCs, and our micro-array data suggest enhanced CXCL9-11 secretion by IL-15 DCs, it is tempting to draw a parallel between the two results. However, it is known that the majority of human NKT cells not only express the chemokine receptor CXCR3, but also CCR2, CCR5 and CXCR4.[60] Considering we also found enhanced RNA levels of CCL2, CCL4 and CCL5 in IL-15 DCs, all ligands of the aforementioned receptors, these chemokines could therefore as well contribute to the improved attraction of NKT cells by IL-15 DCs to a greater or lesser extent.

Going further into detail on the innate immune effector lymphocyte attraction by DCs, our results on DC-mediated recruitment of γδ T cells are in line with the few recently available data of Massa *et al.*, demonstrating no attraction of γδ T cells by conventional IL-4 DCs.[46] IL-4 DCs stimulated with TLR3-agonist poly(I:C) or TLR4-agonist MPLA were, however, able to attract IFN-γ-producing γδ T cells. Unlike our IL-15-differentiated TLR7/8-agonist R848-matured DCs, their TLR7/8 agonist CL097-matured IL-4 DCs did not significantly recruit γδ T cells, suggesting that IL-15 during differentiation is a key requisite. Our findings on DC-mediated recruitment of NK cells are, however, inconsistent with those of Gustafsson *et al.,* who could not detect migration of NK cells towards conventional IL-4 DCs.[47] Yet, when DCs were matured with poly(I:C), DC-mediated NK cell migration did occur, exemplifying involvement of TLR-engagement.[47] In line with these findings, several other research groups demonstrated NK cell migration towards TLR4-agonist (LPS)-matured DCs,[40] TLR9-agonist (CpG)-stimulated plasmacytoid DCs[61] and TLR2/4-agonist *Mycobacterium bovis*-infected DCs.[62] Although one study found no significant increase in NK cell migration in response to stimulation with TLR7/8-activated pDC,[61] a possible involvement of R848 in the IL-15 DC-induced NK cell migration cannot be precluded. R848-treated monocyte-derived DCs have previously been shown to secrete chemokines involved in NK cell migration, such as CCL2-5 and the CXCR3-binding chemokine CXCL10.[63,64] These chemokines can, additionally, be responsible for the increased γδ T cell recruitment. This is in line with our current findings, showing that R848-matured IL-15 DCs have an increased gene expression of CCL4 and CXCL10. Taken together, the TLR7/8 signal could prepare the IL-15 DCs for strong NK cell and γδ T cell recruitment, while IL-15 has been shown to induce secretion of

γδ T cell- and NK cell-attracting chemokines by DCs (such as CCL2[65] and CCL5[65,66]) and by T cells (CCL4)[67]. Differentiation with IL-15 may therefore underlie the upregulation of CCL2 and CCL4 gene expression in IL-15 DCs as compared to conventional IL-4 DCs. Furthermore, soluble IL-15, which is significantly secreted by IL-15 DCs (275.0 ± 184.5 pg/mL in 48-hour wash-out supernatant of $1x10^6$ IL-15 DCs ; unpublished data), has been suggested to be chemotactic for γδ T cells and NK cells.[68,69]

To further elaborate on the potential mechanisms of recruitment, the complete blood γδ T cell fraction was screened for chemokine receptor expression. We observed a high expression of CCR5 and CCR7, and a lower expression of CCR2, CXCR3 and IL-15Rα. Upon migration towards IL-15 DCs, downregulation of CCR2 and CCR5 suggests their involvement, based on 'receptor sequestration', a process whereby the surface expression of chemokine receptors is reduced after binding of their ligands, possible within minutes of agonist exposure.[70] Providing evidence for the presence of the respective chemokines in the wash-out supernatant and binding to their receptor, the same chemokine receptor downregulation is observed on non-migrated immune cells exposed to 48-hour wash-out supernatant as such. CCR2 expression has been associated with effector γδ T cells, whereas CCR5 have been found preferentially on Th1 cells producing IFN-γ, which is desired for an anti-tumor immune response.[30] CCR5 expression is restricted to Vγ9Vδ2 T cells,[71] which could explain the more outspoken enrichment of this subset after migration towards IL-15 DCs. Preferential recruitment of Vγ9Vδ2 T cells would be an asset to DC vaccines in the quest of promoting DC vaccine immunogenicity for improved eradication of tumor cells.[17,72-75] The expression of chemokine receptors on NK cells was less prominent, of which CCR5 clearest. In analogy with γδ T cells, NK cells show a significant lower CCR5 surface expression after IL-15 DC-mediated migration. While CCR5 can occur on both the $CD56^{bright}$ and $CD56^{dim}$ NK cell fraction, the expression is most distinct on $CD56^{bright}$ NK cells.[76,77] This could explain the modest enrichment of $CD56^{bright}$ NK cells after IL-15 DC-mediated migration. Since IL-15 is capable of attracting NK cells,[68] the observed downregulation of IL-15Rα could imply a contribution of this cytokine in the NK cell migration.

Here, we propose a CCL4-CCR5-dependent mechanism that underlies the superior IL-15 DC-mediated recruitment of innate and adaptive cytolytic effector cells. This interaction has been described to play a key role as a mechanism whereby immune cells organize themselves in the fight against cancer. It has been demonstrated that CCR5 is required to guide naive $CD8^+$ T cells to CCL3/CCL4-secreting DC-$CD4^+$ T cell complexes,[58] orchestrating communication between DCs, $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes.[27] Moreover, CCR5-dependent migration of NK cells towards TLR2/4-agonist-matured DCs has previously been described[43] and cytokine-induced killer (CIK) cells use CCR5 signaling

in the contact-dependent information exchange with DCs.[78] Furthermore, neutralization of CCL4 in the supernatant of virally activated plasmacytoid DCs resulted in > 80% inhibition of NK cell chemotaxis.[44] In connection herewith, we show that NK cells are recruited by IL-15 DCs, at least in part, through activation of the CCL4-CCR5 signaling pathway, and we are the first to demonstrate that γδ T cells are attracted by DCs via a CCR5-mediated mechanism.

The increased expression of CCL4 by IL-15 DCs, as compared to the IL-4 DCs, could therefore be an important and preferable element in the development of highly potent DC vaccines. Especially since the, at first glance, small differences in immune cell attraction between IL-15 DCs and IL-4 DCs have the potential to result in truly biological effects, being the recruitment of granzyme B^+ effector lymphocytes by IL-15 DCs and subsequent significantly improved killing of tumor cells.

5. CONCLUSION

Our results show that IL-15 DCs are superior to IL-4 DCs in terms of attraction of all important anti-tumor effector lymphocytes and that at least γδ T cells and NK cells exhibit enhanced cytotoxic function upon migration. Furthermore, our data demonstrate involvement of the CCL4-CCR5 signaling pathway in the improved capacity of IL-15 DCs to recruit anti-tumor immune effector lymphocytes, by means of increased expression and secretion of CCL4 by IL-15 DCs. In addition to the previously demonstrated superior T cell- and NK cell stimulatory properties and direct tumor cell killing capacity of IL-15 DCs,[11,12,14] these findings further underscore their strong immunotherapeutic potential as next-generation DC-based vaccines.

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CHAPTER 5:

INTERLEUKIN-15-CULTURED DENDRITIC CELLS ENHANCE ANTI-TUMOR GAMMA DELTA T CELL FUNCTIONS THROUGH IL-15 SECRETION

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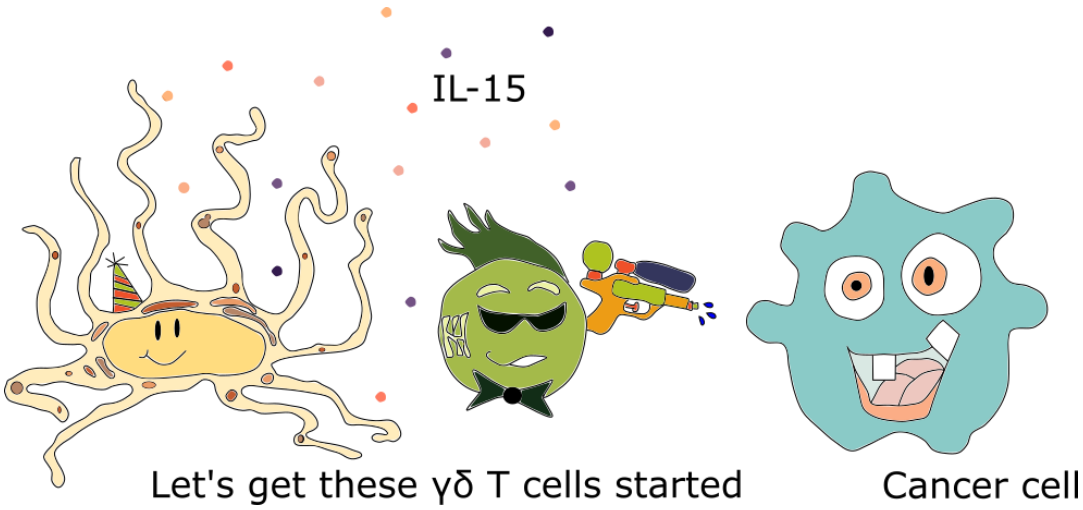
ABSTRACT

DC vaccination can be an effective post-remission therapy for AML. Yet, current DC vaccines do not encompass the ideal stimulatory triggers for innate γδ T cell anti-tumor activity. Promoting type 1 cytotoxic γδ T cells in patients with AML is, however, most interesting, considering these unconventional T cells are primed for rapid function and exert meaningful control over AML. In this work we demonstrate that IL-15 DCs have the capacity to enhance the anti-tumoral functions of γδ T cells. IL-15 DCs of healthy donors and of AML patients in remission induce the upregulation of cytotoxicity-associated and co-stimulatory molecules on the γδ T cell surface, but not of co-inhibitory molecules, incite γδ T cell proliferation and stimulate their IFN-γ production in the presence of blood cancer cells and phosphoantigens. Moreover, the innate cytotoxic capacity of γδ T cells is significantly enhanced upon interaction with IL-15 DCs, both towards leukemic cell lines and allogeneic primary AML blasts. Finally, we address soluble IL-15 secreted by IL-15 DCs as the main mechanism behind the IL-15 DC-mediated γδ T cell activation. These results indicate that the application of IL-15-secreting DC subsets could render DC-based anti-cancer vaccines more effective through, among others, the involvement of γδ T cells in the anti-leukemic immune response.



1. INTRODUCTION

AML is a clonal neoplasm derived from myeloid progenitor cells with a high mortality rate.[1,2] Although most AML patients will achieve remission with induction chemotherapy, the majority of them will eventually relapse within 2 years. This is due to the persistence of residual leukemic (stem) cells, known as MRD.[3,4] Therefore eradication of MRD is a therapeutic priority in the treatment of AML. Despite recent advances in the biologic characterization of AML, standard treatment has remained largely unchanged. Hence, novel therapies to delay, or at best prevent, relapse and prolong survival are urgently warranted. Given the high suitability of AML cells for immune intervention, DC vaccination is subject of intense examination in the MRD setting.[5-7] DC vaccine strategies aim to generate an anti-tumor immune attack, by harnessing both the innate and adaptive arms of the immune system.[6,8] This dual action is of interest, considering that both arms of the immune system are unequivocally important and cooperate in the generation of an effective anti-tumor immune response.[9,10] For example, it has been shown that γδ T cells and $CD8^+$ T cells synergistically target cancer stem cells.[9]

γδ T cells, an unconventional T cell subset in the twilight zone between innate and adaptive immunity, have the ability to provide strong and sturdy anti-tumor responses.[11,12] Contrary to αβ T cells, activation of γδ T cells does not rely on antigen presentation by major histocompatibility complexes. On the other hand, γδ T cell activation can be induced by T cell receptor-dependent recognition of phosphoantigens, such as IPP. This intermediate of the mevalonate pathway is commonly overexpressed in AML blasts due to aberrations contributing to transformation.[13,14] Moreover, given their direct killing capability, antigen-presenting capacity and skill to mobilize other components of the immune system,[8,12] it is no coincidence that a recent meta-analysis of gene expression data from over 18000 cancers, including hematological malignancies, identified the presence of γδ T cells to be the most significant factor associated with favorable prognosis.[15] In line with this, γδ T cells exert meaningful control over AML.[16] Harnessing γδ T cells by a DC vaccine is therefore an interesting approach to tackle MRD and to maximize the anti-tumor potency of vaccination (recently reviewed)[8]. However, little is known about DC-mediated γδ T cell activation and the widely used DC vaccines, commonly referred to as IL-4 DCs, have been found to be largely ineffective at stimulating γδ T cells.[8]

IL-4 DCs are generated by stimulating monocytes with IL-4 and GM-CSF for 5 days, followed by a maturation step of 1-2 days with a cytokine-based cocktail.[17] We previously established a novel monocyte-derived DC generation protocol with improved potency by

employing IL-15 and a TLR ligand, both generating strong immunostimulatory signals.[17,18] These so-called IL-15 DCs have already proven themselves superior to the classic IL-4 DCs based on their direct cytolytic activity against tumor cells and their capacity to stimulate adaptive[17] and innate[19] anti-tumor immunity. Moreover, $CD8^+$ T cells and natural killer cells respond to the chemokine milieu created by IL-15 DCs, whereas IL-4 DCs are suboptimal in attracting effector lymphocytes.[20] This is of importance considering that vaccine DCs can only perform their directing and activating functions *in vivo*, provided that the effector cells come in their vicinity. γδ T cells too are more prone to migrate towards IL-15 DCs,[20] suggesting the possibility of interaction between both cell types.

In the current study, we characterized the IL-15 DC-mediated γδ T cell activation on both the phenotypic and functional level and investigated the potential mechanisms involved. With this we focused on DC vaccination as a therapeutic approach to surmount MRD in AML patients.

2. MATERIALS AND METHODS

2.1. ETHICS STATEMENT AND CELL MATERIAL

This study was approved by the Ethics Committee of the UZA (Edegem, Belgium) under the reference number B300201419756. Primary cells were isolated from buffy coats of healthy volunteers supplied by the Red Cross Blood Transfusion Center (Mechelen, Belgium). Immune cells of AML patients (Table 1) were obtained as residual material of the WIDEA dendritic cell vaccination trial (NCT01686334) or obtained via the division of Hematology of the UZA. Informed consent was received from all patients for being included in the study. PBMCs were isolated by Ficoll density gradient centrifugation. γδ T cells were isolated using a negative (Easysep, Cologne, Germany) or positive (Miltenyi, Leiden, The Netherlands) immunomagnetic cell selection kit for cytokine production determination and cytotoxicity assays, respectively. The Burkitt's lymphoma tumor cell line Daudi - a known target for γδ T cells - was kindly provided to us by the laboratory of Prof. Kris Thielemans (Free University of Brussels, Brussels, Belgium). The chronic myeloid leukemia cell line in blast crisis K562 was purchased from the American Type Culture Collection (Rockville, MD, USA) and the AML cell lines NB4 and THP-1 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Table 1. Patient characteristics

	UPN1	UPN2	UPN3	UPN4
Sex	F	F	M	F
Age at diagnosis	74	87	62	59
WHO type	AML NOS, AML with maturation	AML with myelodysplasia-related changes	AML NOS, Acute monoblastic / monocytic leukemia	AML with t(8;21)(q22;q22);RUNX1-RUNX1T1
Prior treatment	Induction (eMICE); Consolidation (mini-ICE)	6 x Decitabine	Induction I (Ida-AraC); Induction II (Dauno-AraC)	Induction I (Ida-AraC); Induction II (Dauno-AraC)
disease stage	CR1	CR1	CR1	CR1

Remark: UPN1 and UPN2 were included in the WIDEA dendritic cell vaccination trial (NCT01686334). Inclusion criterion; completion of at least one cycle of induction chemotherapy and one cycle of consolidation chemotherapy, resulting in morphological complete remission. UPN3 and UPN4 were not included in the WIDEA vaccination trial. Blood was drawn prior to the start of the first cycle of consolidation chemotherapy.
Abbreviations**:** F, female; M, male; eMICE, elderly mitoxantrone + arabinoside-c + etoposide; mini-ICE, idarubicin + arabinoside-C + etoposide; Ida-AraC, idarubicin + arabinoside-C; Dauno-AraC, daunorubicine + arabinoside-c; CR1, first complete remission; R1, first relapse; PB, peripheral blood; UPN, unique patient number

2.2. DENDRITIC CELL CULTURE

Monocyte-derived IL-15 DCs were prepared conforming to our previously described 48-hour culture protocol;[18,20] Positively selected $CD14^+$ monocytes (Miltenyi) are cultured in Roswell Park Memorial Institute medium (Life Technologies) with 2.5% hAB (Life Technologies) at a final concentration of $1.2x10^6$ cells/mL. To generate mature IL-15 DCs, a 28-hour differentiation step using GM-CSF (800 IU/mL; Life Technologies) and IL-15 (200 ng/mL; Immunotools) is followed by maturation induction with R848 (3 µg/mL; Alexis Biochemicals, San Diego, USA), IFN-γ (250 ng/mL; Immunotools), TNF-α (2.5 ng/mL; Life Technologies) and PGE2 (1 µg/mL; Pfizer, Puurs, Belgium) for 20 hours. To collect 48-hour wash-out supernatant, IL-15 DCs are harvested, thoroughly washed and reseeded in fresh medium at a concentration of $1x10^6$ cells/mL in low absorbing polypropylene tubes. After 48 hours, cell-free supernatant is collected and frozen at -20°C, until further use.

2.3. CO-CULTURE SET-UP

PBMCs, unless stated otherwise, were cultured with IPP (122 µM; Tebu-bio, Le-Perray-en-Yvelines, France), autologous IL-15 DCs and/or tumor cells (K562, Daudi) at an E:T ratio of 1:(1:)10. Unstimulated cells were used as negative control. With regard to cultures with

immune cells of AML patients (Table 1), the same culture set-up was used let alone the use of peripheral blood lymphocytes (PBLs) instead of PBMCs.

2.4. IMMUNOPHENOTYPING

γδ T cells were phenotyped after 48-hour co-culture using the following mAbs: γδ TCR-FITC (Miltenyi), CD86-FITC, CD80-PE, PD-1-PE, NKG2D-PE, CD16-PB, BTLA-BV421, γδ TCR-APC (Miltenyi), NKp30-AF647 and CD3-APC-H7. Unless specified otherwise, all mAbs were purchased from BD (Erembodegem, Belgium). Live/Dead® Fixable Aqua Stain (Invitrogen, Merelbeke, Belgium) was used to exclude dead cells from analysis. Corresponding species- and isotype-matched antibodies were used as controls.

2.5. PROLIFERATION ASSAY

Proliferation of γδ T cells was quantified by the degree of CFSE (Invitrogen) dye dilution. At different time points, γδ T cell proliferation was measured as the proportion of CFSE-diluted cells within the viable $CD3^{+}\gamma\delta TCR^{+}$ gate (Live/Dead® Fixable Aqua Stain, CD3-PerCP [BD], γδ TCR-APC). Unstimulated CFSE-labeled cells served as a non-dividing control.

2.6. CYTOKINE PRODUCTION

IFN-γ, TNF-α, IL-4, IL-10 and IL-17 (BD) production by γδ T cells was assessed intracellularly after overnight co-culture. Cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, Vienna, Austria). The IL-15 concentration in 48-hour wash-out supernatant of IL-15 DCs was quantified using electrochemiluminescent detection on a SECTOR3000 (Meso Scale Discovery [MSD], Rockville, MD, USA).

2.7. DEFINING CONTACT- AND IL-15-DEPENDENCY

To examine the role of cell-cell contact, IL-15 DCs were separated from γδ T cells by a 0.4 µm pore size Transwell insert (Elscolab, Kruibeke, Belgium). In specific experiments, 10 µg/mL anti-IL-15 neutralizing mAbs (R&D, Minneapolis, Canada) or corresponding isotype control mAbs were added to investigate the involvement of IL-15. IL-15 DCs were incubated for one hour with the above mAbs before starting co-culture to ensure adequate blocking of IL-15. The mAbs remained present during the further course of the co-culture.

2.8. γδ T CELL-MEDIATED CYTOTOXICITY ASSAYS

In order to measure the tumoricidal capacity of (un)stimulated γδ T cells, a flow cytometric assay was set-up as previously described with minor modifications.[21-24] $0.2x10^6$ isolated γδ T cells were cultured for 24-36 hours in the presence or absence of IPP (122 µM) and/or autologous IL-15 DCs at a DC : γδ T cell ratio of 1:10 in 200 µL RPMI (Invitrogen) supplemented with 10% FBS (Invitrogen). Subsequently, tumor cells (Daudi, K562, NB4 and THP-1) and fresh allogeneic primary AML blasts (unique patient number [UPN]a/b) were labeled with CellTracker™ Blue CMF2HC Dye (Invitrogen) and served as 'target cells'. They were added to the γδ cell +/- DC (co-)cultures for four hours at an E:T ratio of 2:1 in a final volume of 300 µL RPMI + 10% FBS. Cultures with allogeneic AML material were stained with CD33/CD34 to discriminate between AML blasts and the rest of the PBMC fraction of UPNa/b. Cell death was quantified after Annexin-V-APC (BD) and PI staining. Specific target cell killing was calculated according to the formula: % killing = 100 – ([(% Annexin-V^-/PI^- target cells with γδ T cells / % Annexin-V^-/PI^- target cells without γδ T cells) x 100].

2.9. STATISTICS

All flow cytometry data were acquired on a FACSAria II flow cytometer (BD) and analyzed using FlowJo (v10; Treestar, Ashland, OR, USA). For statistics and illustrations GraphPad Prism software (v5.0; San Diego, CA, USA) was used. To validate the Gaussian distribution of data sets, they had to pass Shapiro-Wilk test for normality. Dissimilarities were predefined to be statistically significant when p-values < 0.05. All data are depicted as means ± standard error of the mean.

3. RESULTS

3.1. IL-15 DCs INDUCE THE UPREGULATION OF CYTOTOXICITY-ASSOCIATED AND CO-STIMULATORY MOLECULES ON THE γδ T CELL SURFACE, BUT NOT CO-INHIBITORY MOLECULES

First, phenotypic characterization of γδ T cells was performed after a 48-hour culture period, either unstimulated or with the addition of IPP and/or IL-15 DCs (Table 2; % and Δ mean fluorescence intensity [MFI]). γδ T cells exposed to IL-15 DCs were found to express higher levels of markers related with γδ T cell cytotoxicity (CD16) and co-stimulatory molecules (CD80, CD86). Further upregulation of CD16, CD80, CD86 and the natural cytotoxicity receptor NKp30 was observed with the combination of IL-15 DCs with IPP. In

contrast, the expression of the co-inhibitory receptors and exhaustion-associated molecules BTLA and PD-1 on γδ T cells were not or only limited influenced by IL-15 DCs. The addition of IPP, however, resulted both in a significant induction and upregulation of PD-1 on γδ T cells.

Table 2. γδ T cell phenotype of healthy donors after 48 hours of culture with IPP, IL-15 DCs or both

%	Resting	IPP	IL-15 DCs	IPP + IL-15 DCs
CD16	12.36 ± 3.45 %	15.39 ± 3.72 %	16.99 ± 4.16 %	17.26 ± 4.19 % **
NKG2D	62.52 ± 11.40 %	66.30 ± 10.94 %	63.42 ± 11.20 %	66.27 ± 11.18 %
NKp30	9.328 ± 2.403 %	15.95 ± 2.59 %	17.16 ± 3.08 %	20.70 ± 2.58 % *
BTLA	47.78 ± 7.24 %	46.30 ± 7.07 %	46.48 ± 7.23 %	42.35 ± 7.91 %
PD-1	6.738 ± 1.545 %	19.27 ± 2.37 % **	11.42 ± 1.48 %	20.84 ± 3.19 % *
CD80	0.7833 ± 0.2737 %	2.892 ± 1.174 %	6.067 ± 2.033 % *	9.115 ± 3.912 % **
CD86	3.132 ± 0.937 %	15.69 ± 6.63 %	13.28 ± 2.71 %	20.32 ± 4.49 % ***
Δ MFI	**Resting**	**IPP**	**IL-15 DCs**	**IPP + IL-15 DCs**
CD16	237.5 ± 103.3	296.1 ± 118.0	376.4 ± 162.0 *	379.8 ± 174.2 *
NKG2D	8750 ± 4599	8871 ± 4482	8786 ± 3931	9662 ± 4407
NKp30	58.38 ± 12.64	88.17 ± 10.82	98.00 ± 16.82	114.7 ± 12.5 *
BTLA	80.33 ± 9.14	95.52 ± 41.69	83.70 ± 9.84	85.40 ± 18.97
PD-1	65.38 ± 9.20	174.2 ± 28.6 *	108.0 ± 10.0	187.9 ± 32.8 *
CD80	5.450 ± 1.743	35.37 ± 13.36	69.12 ± 20.72 *	89.48 ± 36.14 *
CD86	19.48 ± 4.62	59.77 ± 16.87	69.77 ± 9.63 *	83.77 ± 8.15 **

Abbreviations: ΔMFI, difference in mean fluorescence intensity between the marker and corresponding isotype control. Asterisks indicate a statistically significant difference in cell surface marker expression between stimulated γδ T cells and resting γδ T cells (n = 6; Friedmann test with Dunn's Multiple Comparison Test; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

3.2. SOLUBLE FACTORS SECRETED BY IL-15 DCs PROMOTE γδ T CELL PROLIFERATION

Next, we established the influence of IL-15 DCs on resting γδ T cells in full PBMC fraction through a standard 5-day proliferation assay (Figure 1A-B). IL-15 DCs induced significant γδ T cell proliferation, i.e. 25.13 ± 1.33 % after five days. This proliferative response was doubled (54.98 ± 6.25 %) in the presence of IPP. However, there was no clear proliferative reaction of the γδ T cells to IPP stimulation only after five days. When looking at a longer period, i.e. eight and twelve days, IPP addition does result in γδ T cell proliferation (day twelve; 33.60 ± 1.25 %). Moreover, regarding these later time points, it is evident that the stimulatory effect of IL-15 DCs is relevant for the generation of high numbers of γδ T cells long-term, namely 63.37 ± 8.81 % γδ T cell proliferation after a twelve-day co-culture (Figure 1D). To end, a similar degree of γδ T cell proliferation was observed after five days,

irrespective of whether IL-15 DCs were separated from the PBMCs by a transwell membrane or not. This indicating that soluble factors secreted by IL-15 DCs were responsible for the observed proliferative response (Figure 1C).

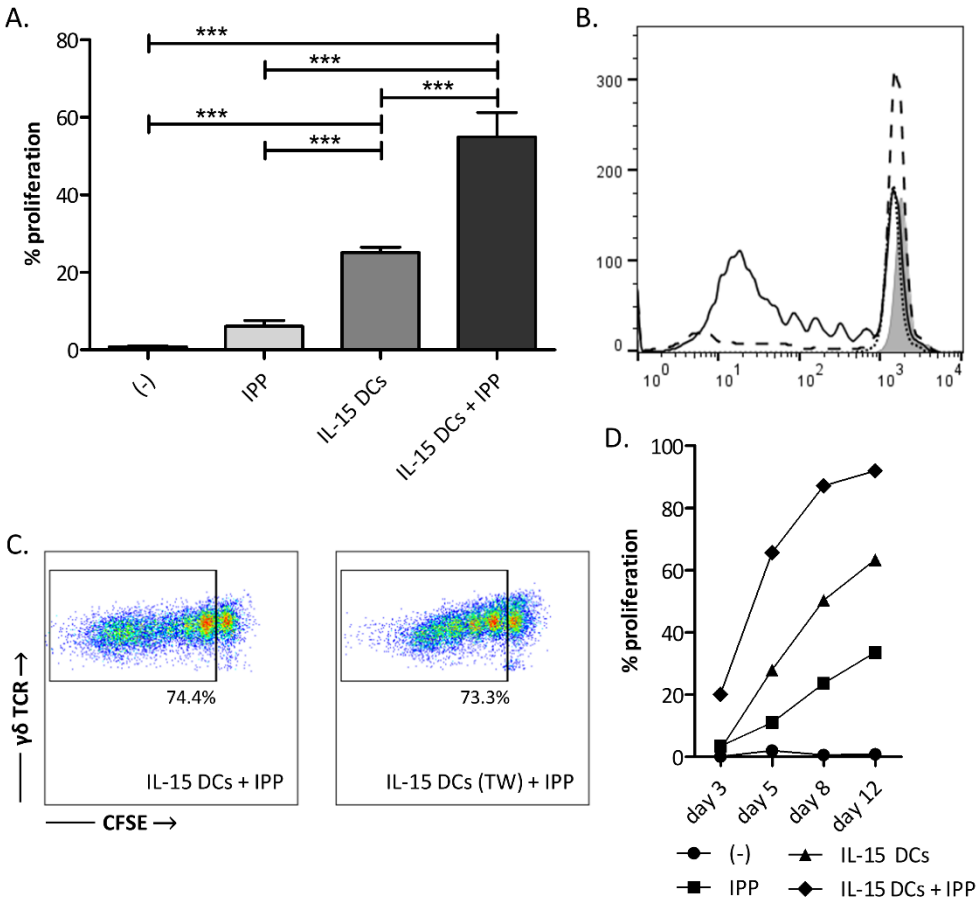


Figure 1. IL-15 DCs induce γδ T cell proliferation in a contact-independent manner. A. γδ T cells in full PBMC fraction were stimulated with IPP, IL-15 DCs (E:T ratio = 1:10) or IL-15 DCs + IPP for 5 days. Unstimulated PBMCs (-) were used as negative control. γδ T cell proliferation, as assessed by reduction in CFSE staining, was determined by flow cytometry. The percentage of cells to have undergone at least one cell division is shown (n = 12). One-Way ANOVA with Bonferroni's Multiple Comparison Test. **B**. The histogram overlay shows CFSE dilution of viable γδ T cells within unstimulated PBMCs (filled grey), PBMCs stimulated with IPP (dotted line), IL-15 DCs (dashed line) and IL-15 DCs + IPP (full line) for one representative donor. **C.** Dot-plots of one representative donor (n = 3) showing the CFSE-dilution of proliferated γδ T cells in PBMCs stimulated with IL-15 DCs + IPP with and without IL-15 DCs in transwell (TW). **D.** γδ T cell proliferation over time (experimental set-up in line with panel A; n = 3). ***, $p < 0.001$

3.3. IL-15 DCs STIMULATE IFN-Γ PRODUCTION IN γδ T CELLS IN THE CONTEXT OF A LEUKEMIC ENVIRONMENT

One of the desirable effector functions of γδ T cells in cancer immunotherapy is the secretion of pro-inflammatory cytokines, such as IFN-γ and TNF-α. Therefore, we measured the intracellular IFN-γ production in γδ T cells after overnight stimulation (Figure 2A-B). Stimulation of γδ T cells with IPP and/or tumor cells without DCs, failed to trigger IFN-γ production in γδ T cells. IL-15 DCs alone also did not provide the necessary signals for IFN-γ production. On the other hand, when IL-15 DCs were added together with signals of malignancy (presence of IPP and tumor cells) a strong IFN-γ response was observed in γδ T cells. As shown in Figure 2A, stimulation with IL-15 DCs + IPP + tumor cell lines resulted in 13.12 ± 2.68 % (for K562 cell line) and 7.35 ± 1.24 % (for Daudi cell line) IFN-γ^+ γδ T cells. TNF-α production was absent in all situations, as well as the Th2-promoting cytokine IL-4, the Th17-promoting cytokine IL-17 and the anti-inflammatory cytokine IL-10 (data not shown). In analogy with the induction of γδ T cell proliferation, the stimulation of IFN-γ production in γδ T cells by IL-15 DCs was found to be contact-independent (Figure 2C).

3.4. ACTIVATION OF γδ T CELLS BY IL-15-DCs IS DEPENDENT ON SOLUBLE IL-15

As apparent from the previous experiments, IL-15 DCs clearly induce γδ T cell proliferation and IFN-γ production by contact-independent mechanisms. Therefore, we endeavored to identify the soluble factor(s) produced by the IL-15 DCs triggering γδ T cell activation. We previously performed a cDNA microarray analysis of (im)mature IL-15 DCs and "gold-standard" IL-4 DCs of 3 independent healthy controls.[20] Interestingly, introducing IL-15 during *in vitro* differentiation of monocytes, results in the generation of iDCs producing this pro-inflammatory cytokine themselves. On the RNA level (GenBank ID: NM_000585), we detected a fold change difference of 3.6 in expression signal between immature IL-15 DCs (Probe signal: 142) vs. IL-4 DCs (Probe signal: 40). In concordance with these data, we have shown that mature IL-4 DCs do not secrete IL-15.[23] Subsequently, we examined the IL-15 secretion of IL-15 DCs. The concentration of IL-15 in 48-hour wash-out supernatant of $1x10^6$ IL-15 DCs was found to be 275 ± 107 pg/mL (Figure 3A). To clarify the involvement of this pleiotropic cytokine, IL-15 effects were canceled out using neutralizing mAbs (Figure 3B-C). IL-15 DC-mediated γδ T cell proliferation was reduced by approximately 60 % upon IL-15 neutralization. Concerning IFN-γ production, blocking IL-15 significantly reduced the ability of γδ T cells to produce IFN-γ upon stimulation with IL-15 DCs in a malign environment.

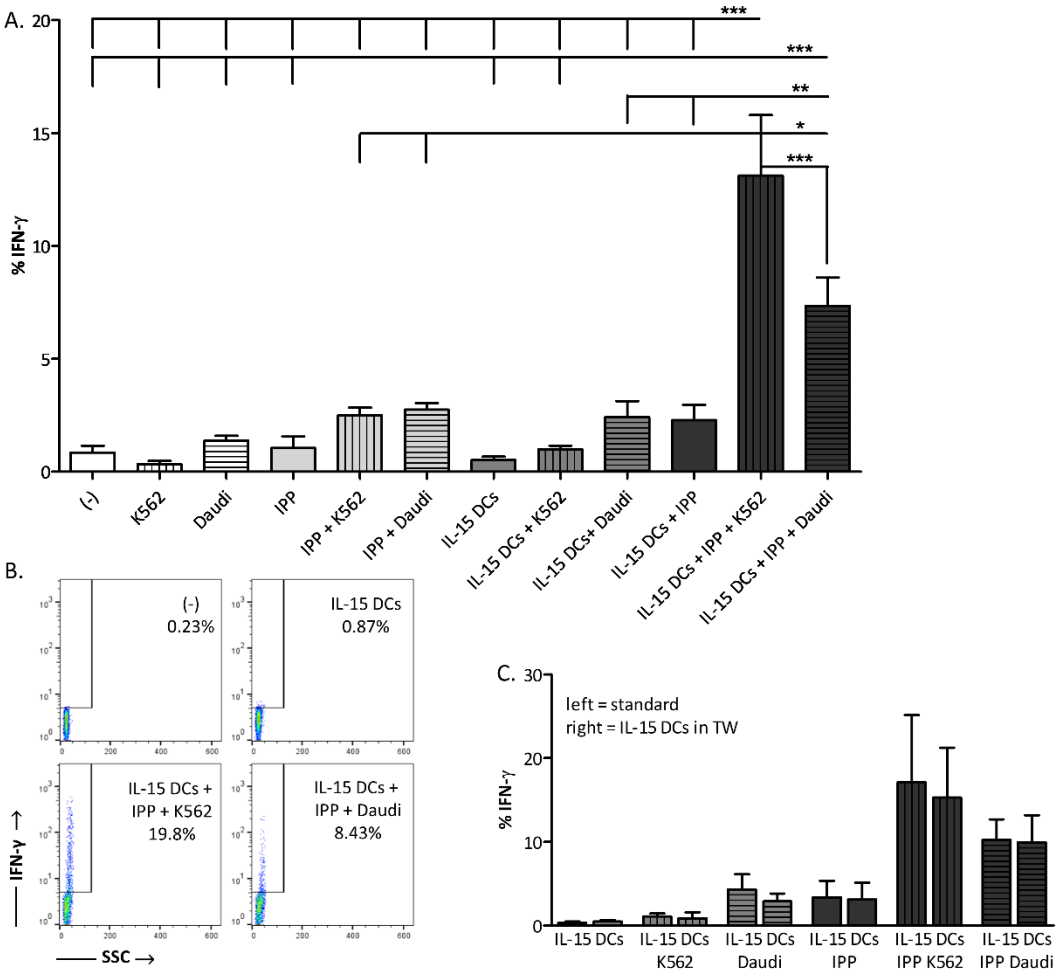


Figure 2. IL-15 DCs stimulate γδ T cell IFN-γ production in the presence of a leukemic environment through a contact-independent mechanism. A. γδ T cells in full PBMC fraction were examined for IFN-γ production by intracellular staining after overnight stimulation with IPP, IL-15 DCs (E:T ratio = 1:10), tumor cells (E:T ratio = 1:10) and all possible combinations. Resting γδ T cells (-) were used as negative point of reference. Data are depicted as the mean of ten independent donors. One-Way ANOVA with Bonferroni's Multiple Comparison Test. **B.** Representative FACS dot plots of one healthy donor out of ten showing IFN-γ production by γδ T cells. **C.** In parallel, the same experiment was carried out except that IL-15 DCs were separated from the cultures by 0.4 µm transwell inserts (TW; n = 3).***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.5$

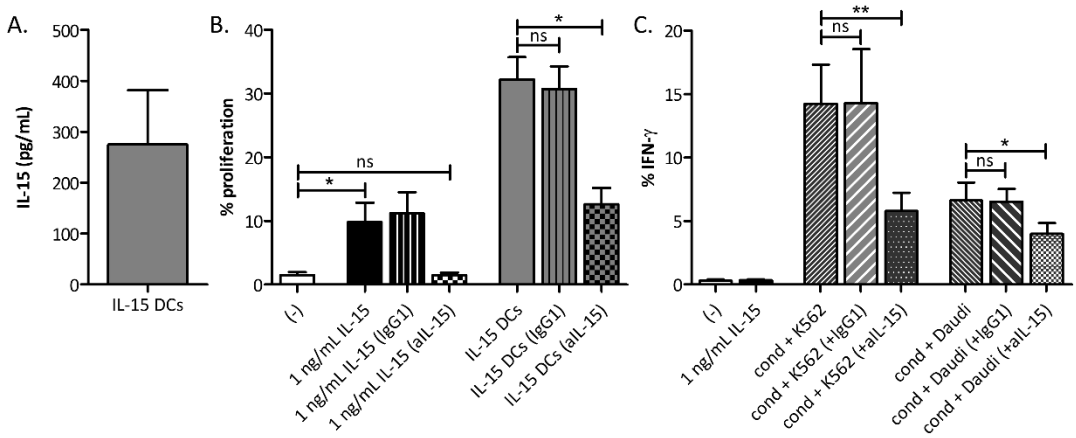


Figure 3. IL-15, secreted by IL-15 DCs, provides an important signal for DC-mediated γδ T cell proliferation and IFN-γ production. A. Representation of the IL-15 secretion level (pg/mL), as determined by MSD immunoassay, in 48-hour wash-out supernatant of IL-15 DC cultures ($1x10^6$ cells/mL; n = 3). **B.** CFSE-labeled PBMCs were co-cultured with 1ng/mL recombinant IL-15 or IL-15 DCs (E:T ratio = 1:10). In certain conditions, neutralizing IL-15 mAbs (aIL-15) or isotype control mAbs (IgG1) were added to the IL-15 DC cultures one hour prior to the addition of PBMCs. Percentages refer to the proportion of γδ T cells that proliferated within 5 days (n = 6). Friedman test with Dunn's Multiple Comparison Test. **C.** After overnight culture of γδ T cells in full PBMC fraction with IL-15 DCs + IPP (= cond) and K562 or Daudi tumor cells (E:T ratio = 1:10), with anti-IL-15 mAbs (aIL-15) or with isotype control mAbs (IgG1), intracellular IFN-γ production was measured with flow cytometry in γδ T cells (n = 6). Friedman test with Dunn's Multiple Comparison Test. **, $p < 0.01$; *, $p < 0.5$; ns, $p > 0.5$

3.5. IL-15 DCs POTENTIATE ΓΔ T CELLS FROM HEALTHY DONORS TO KILL LEUKEMIC CELL LINES AND ALLOGENEIC PRIMARY AML BLASTS

After establishing the stimulatory effect of IL-15 DCs on γδ T cell proliferation and pro-inflammatory cytokine production, we looked at their ability to improve γδ T cell lysis of a panel of leukemic tumor cell lines and primary AML blasts. As shown in Figure 4, resting (unstimulated) γδ T cells failed to lyse Daudi tumor cells (1.10 ± 1.07 %), had a low cytotoxic activity against K562 (5.75 ± 2.23 %) and killed the AML tumor cell lines NB4 (10.13 ± 1.67 %) and THP-1 (13.71 ± 4.04 %), and primary blasts (UPNa; 17.12 ± 6.07 %, UPNb; 24.77 ± 4.96 %) to some degree. This 'limited' cytotoxic capacity is significantly enhanced against all targets by the addition of IL-15 DCs; Daudi = 11.31 ± 2.00 %, K562 = 13.83 ± 1.95 %, NB4 = 18.18 ± 2.49, THP-1 = 25.46 ± 3.56 %, UPNa = 29.99 ± 4.48 % and UPNb = 33.93 ± 3.11 %. Additional stimulation with IPP has little influence on the overall lytic activity of γδ T cells.

As regards primary AML blasts of donor UPNa, even a small but noticeable negative effect on killing was observed with IPP stimulation.

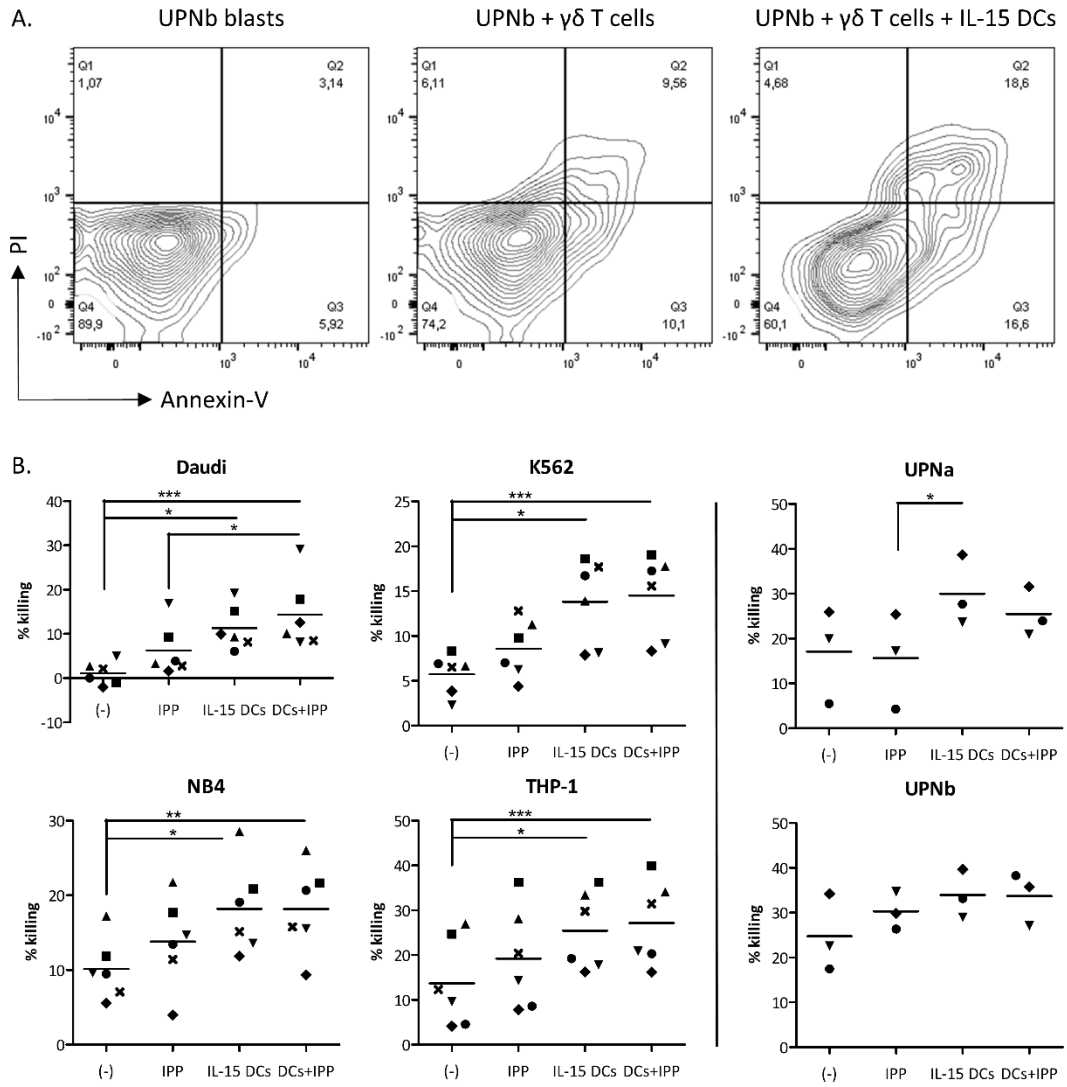


Figure 4. The cytotoxic capacity of γδ T cells is radically increased by IL-15 DCs. A. Representative example of primary AML blast killing (UPNb) from one out of three donors. **B.** Cytotoxicity was determined of γδ cell +/- IL-15 DC (co-)cultures with or without IPP after 24-36 hours. Unstimulated γδ T cells (-) were used to define basal killing capacity. Target cells were added at an E:T ratio of 2:1. Percentage tumor cell killing was ascertained by Annexin-V/PI staining after 4 hours and calculated using the formula specified in "Materials and methods". Donors are represented by unique symbols. Friedman test with Dunn's Multiple Comparison Test. ***, $p < 0.001$, **, $p < 0.01$; *, $p < 0.5$

3.6. γδ T CELLS FROM AML PATIENTS IN FIRST COMPLETE REMISSION ARE ROBUSTLY ACTIVATED BY IL-15 DCs

Next, we sought to investigate whether γδ T cells present in the peripheral blood of treated AML patients (Table 1) could be efficiently stimulated by IL-15 DCs. Therefore, the abovementioned experiments were repeated with monocyte-depleted PBLs (containing γδ T cells) from AML patients in complete remission. Since previous experiments in healthy donors were carried out with full PBMCs, we first established the equivalence of both experimental designs with immune cells of healthy donors (Figure 5).

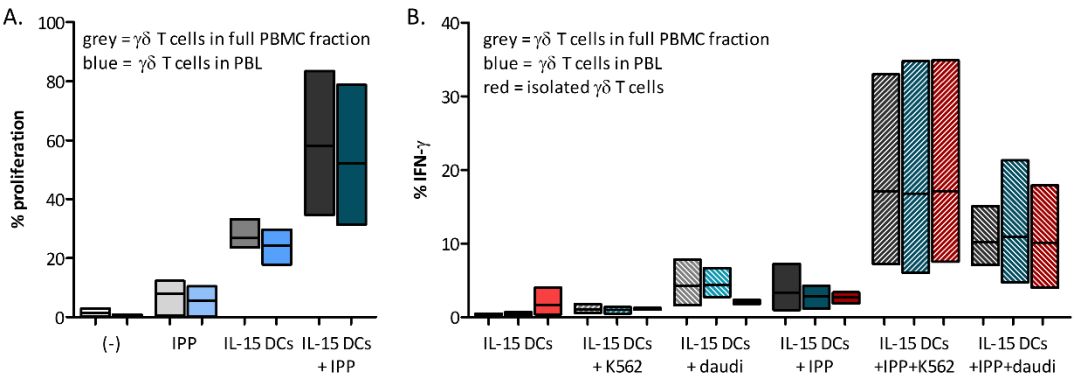


Figure 5. Direct comparison between γδ T cell activation in full fraction PBMC, $CD14^+$ cell-depleted PBMC (PBL) and isolated γδ T cells. **A.** Proliferative response of γδ T cells after 5-day culture (n = 5) and **B.** intracellular IFN-γ production after overnight culture (n = 3), presented as floating bars (min to max - line at mean).

At the outset, we looked at the cytotoxic phenotype of γδ T cells of AML patients (after activation) (Table 3; % and ΔMFI). The expression level of CD16, both basally as well as after exposure to IL-15 DCs, was similar to that of healthy donors. Interestingly, whereas IL-15 DCs of healthy volunteers did not affect the expression of NKG2D, we noticed a trend towards upregulation of this marker on γδ T cells of AML patients upon co-culture with IL-15 DCs. Of note, UPN2 had a low basal expression of both markers, but showed a similar reaction to activation as compared to γδ T cells from healthy donors. In UPN4, baseline CD16 and NKG2D expression was already high, making it difficult to observe any additional activation. Strikingly, in all patients a robust, more than 2-fold, induction of NKp30 expression on the γδ T cell surface was observed upon IL-15 DC stimulation (21.73% in IL-15 DC-stimulated γδ T cells versus 9.5% in unstimulated γδ T cells). Secondly, expression of co-stimulatory and co-inhibitory molecules was examined (Table 3; % and ΔMFI). Upregulation of CD80 on the γδ T cell surface was observed in 3 of the 4 AML patients (mean; > 6-fold increase in CD80 expression upon stimulation with IL-15 DCs as compared to baseline) and

CD86 in all 4 patients (mean; 23.18 % versus 8.21 % baseline). Concerning PD-1 and BTLA, PD-1 expression was virtually absent on γδ T cells of AML patients in remission (mean; 1.058 %), in contrast to healthy donors (mean; 6.738 %), whereas the BTLA expression was slightly more pronounced (mean; 59.78 % versus 47.78 %). The expression of both molecules was not affected by IL-15 DCs, i.e. mean expression of 2.188 % PD-1 and 60.73 % BTLA. For all markers the influence of the addition of IPP on their surface expression was comparable with the effect of IPP stimulation on healthy donor γδ T cells.

Table 3. γδ T cell phenotype of AML patients 48-hours after co-culture with IPP, IL-15 DCs or both

%	Resting	IPP	IL-15 DCs	IPP + IL-15 DCs
CD16	25.92 ± 18.12 %	25.21 ± 15.87 %	31.50 ± 16.36 %	33.55 ± 14.07 %
NKG2D	45.91 ± 21.39 %	47.51 ± 19.27 %	49.27 ± 20.06 %	52.79 ± 19.79 %
NKp30	9.500 ± 2.359 %	20.02 ± 6.94 %	21.73 ± 1.24 %	32.70 ± 5.20 %
BTLA	59.78 ± 5.41 %	66.93 ± 7.90 %	60.73 ± 6.80 %	65.18 ± 7.77 %
PD-1	1.058 ± 0.248 %	6.213 ± 4.212 %	2.188 ± 0.818 %	13.76 ± 6.098 % *
CD80	1.465 ± 0.581 %	6.670 ± 2.981 %	9.875 ± 5.415 %	11.45 ± 6.73 % *
CD86	8.205 ± 5.252 %	19.73 ± 8.87 %	23.18 ± 10.54 %	32.15 ± 1218 % **
Δ MFI	**Resting**	**IPP**	**IL-15 DCs**	**IPP + IL-15 DCs**
CD16	174.7 ± 138.1	227.0 ± 176.2	280.7 ± 189.8	334.0 ± 226.1 *
NKG2D	7170 ± 2522	10237 ± 3279	9177 ± 3453	12324 ± 4861
NKp30	60.75 ± 3.199	113.0 ± 35.39	132.8 ± 27.19	187.3 ± 40.16
BTLA	150.3 ± 44.83	200.4 ± 62.20	168.2 ± 55.47	197.3 ± 71.39
PD-1	16.50 ± 11.68	101.0 ± 39.79	38.75 ± 19.04	430.0 ± 290.7
CD80	10.73 ± 2.414	95.55 ± 39.21	99.48 ± 48.84	120.9 ± 59.81
CD86	29.75 ± 16.87	66.15 ± 27.68	96.40 ± 45.45	129.7 ± 46.12 *

Abbreviations: ΔMFI, difference in mean fluorescence intensity between the marker and corresponding isotype control. Asterisks indicate a significant difference in cell surface marker expression between stimulated γδ T cells and resting γδ T cells (n = 4; Friedmann test with Dunn's Multiple Comparison Test; *, $p < 0.05$).

3.7. FUNCTIONALITY OF IL-15 DC-ACTIVATED γδ T CELLS OF AML PATIENTS IN REMISSION

Finally, we aimed to examine the functionality of γδ T cells of AML remission patients upon IL-15 DC stimulation. The percentage of proliferated γδ T cells after 5-day co-culture with IL-15 DCs was 30.57 ± 1.33% in AML patients (Figure 6A), comparing favorably to the proliferative response in healthy donors. However, in the other 2 patients (UPN3 and UPN4), who had only completed induction chemotherapy at the time of the blood draw, IL-15-DC stimulation on its own already led to a noticeable IFN-γ production by γδ T cells but with a diminished response to stimulation with additional IPP and tumor cells. With

regard to IFN-γ production by γδ T cells (Figure 6B), we observed an enhanced IFN-γ production, i.e. 15-20% intracellular IFN-γ expression, upon stimulation with IL-15 DCs in a leukemic environment in AML patients that were brought into complete remission after induction/consolidation chemotherapy (UPN1/2).

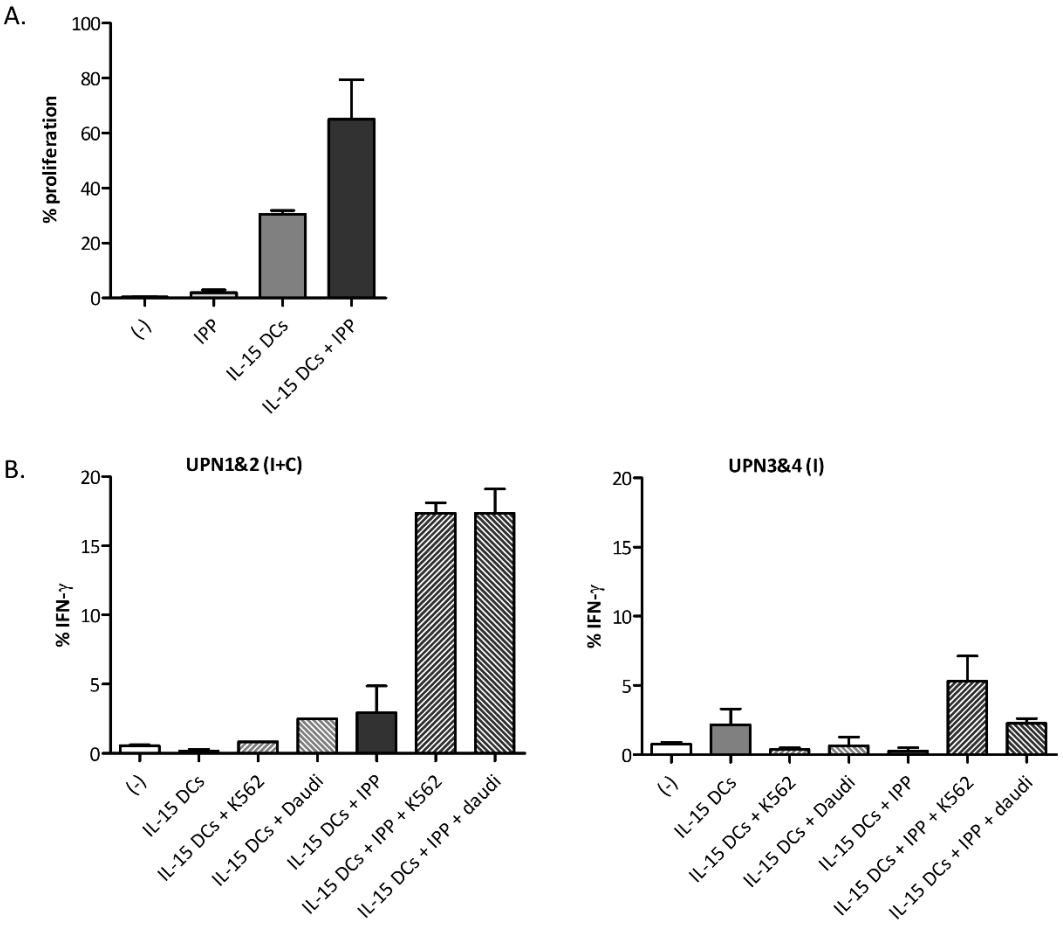


Figure 6. Functional activation of γδ T cells of AML patients by IL-15 DCs. A. Bar graphs depict γδ T cell proliferation upon 5-day culture (-) with IPP, IL-15 DCs (E:T ratio = 1:10) or IL-15 DCs + IPP. **B.** Percentage IFN-γ-positive γδ T cells after overnight culture (-) with IPP, IL-15 DCs (E:T ratio = 1:10) and/or tumor cells (E:T ratio = 1:10) measured by flow cytometry for four AML patients in first complete remission. Remark: the IFN-γ production by γδ T cells of UPN1&2 and UPN3&4 are shown separately, as they differ from each other in response to stimuli. Blood from UPN1&2 and UPN3&4 was drawn after termination of induction and consolidation chemotherapy (I+C) and induction chemotherapy (I), respectively. No statistics were performed due to limited numbers.

4. DISCUSSION

The clinical implementation of γδ T cells in cell-based cancer immunotherapy holds encouraging perspectives regarding the treatment of (advanced) malignancies.[11,25] This prospect is not a remote possibility anymore, evinced by the burst of several new biotech companies aiming at bringing γδ T cells into the clinic, including Gadeta (Utrecht, The Netherlands), GammaDelta Therapeutics (London, UK), Incysus (Hamilton, Bermuda) and Lymphact (Coimbra, Portugal). In this study, we aimed at harnessing γδ T cells in the anti-tumor immune response of DC vaccination, a promising and active immunotherapeutic modality, amongst others, used in the MRD setting of AML.[5,6] We recently showed that WT1 mRNA-electroporated conventional IL-4 DCs prevent or delay relapse in 43% of AML patients in remission after chemotherapy.[5] However, since the IL-4 DC vaccine only weakly recruits γδ T cells[20] and generally fails to potentiate γδ T cell functions,[8] this missing interaction could aid to optimize DC vaccine immunopotency, promoting cytotoxic and long-lasting anti-tumor immunity. Here, we show that our immunogenic IL-15 DC vaccine is able to activate γδ T cells (of AML patients in remission) and that this is attributable to a large extent to the secretion of IL-15.

First, we looked at the phenotypic features of γδ T cells upon interaction with IL-15-DCs. This has highlighted that γδ T cells of AML patients are prone to IL-15 DC stimulation, particularly in the presence of IPP. The latter is evidenced by the marked upregulation of NKp30 on the γδ T cell surface. This natural cytotoxicity receptor has been shown to be the main contributor to TCR-independent leukemia cell recognition by γδ T cells, even enabling resistant primary lymphocytic leukemia targeting.[26] Interestingly, CD80 and CD86, specific APC cell markers involved in co-stimulatory signaling to αβ T cells, are clearly upregulated on γδ T cells after interaction with IL-15 DCs. This could be of importance, considering γδ T cells have been shown to adopt an APC phenotype upon activation and to effectively (cross-)present peptide antigens.[27,28] Moreover, the upregulation of CD16 on the cell membrane enables ADCC and the efficient 'licensing' of γδ T-APCs.[29]

Subsequently, we looked at negative co-stimulatory markers associated with T cell exhaustion, i.e. BTLA and PD-1.[30] These inhibitory molecules were not, or only to a limited extent, induced by IL-15 DCs on γδ T cells. On the other hand, we are the first to show that IPP causes PD-1 expression. This is of importance in view of the increased IPP expression by cancerous cells and the compelling evidence on the stimulatory effect of IPP for γδ T cell activation and expansion.[8] Interestingly, γδ T cells are able to surmount (in part) the inhibitory effects mediated by PD-1 by means of TCR triggering.[31] Indeed, sensitization of

tumor cells with zoledronate, a nitrogen-containing bisphosphonate resulting in the accumulation of IPP through the inhibition of FPP synthase,[13] overcomes the inhibitory effect of PD-1. Therefore, the relationship between IPP signaling and PD-1 expression/functioning warrants further investigation. Besides, DC therapy in concert with anti-PD-1 therapeutics is a combination to be investigated, possibly rendering AML cells more susceptible to immune attack. This especially considering the expression of PD-1 ligands is a well-recognized mechanism by which AML cells inhibit anti-leukemic immune cell responses.[32,33] The results of a clinical trial combining anti-PD-1 therapeutics with a DC-based AML vaccine are therefore awaited.[34]

To date, the capacity of circulating DCs to trigger γδ T cell activation is largely unknown. Notwithstanding, we and others have shown that autologous *ex vivo* generated IL-4 DCs, used routinely for clinical studies, are inefficient in mobilizing γδ T cells[20] and unable to induce γδ T cell proliferation and effector functions, and that additional/alternative signals are required.[35] In this study we provide evidence that IL-15 DCs are able to induce autologous γδ T cell proliferation and a Th1-like polarization profile and that these features were conserved in AML patients who are in complete remission. Perhaps even more important, IL-15 DCs are able to significantly upgrade γδ T cell cytotoxicity against leukemic cell lines and primary AML blasts. This makes the IL-15 DC vaccine an all-round activator of the cytotoxic immune effector response, to wit γδ T cells, NK cells[19] and conventional T cells.[17] The interesting observation that γδ T cells from AML patients before consolidation chemotherapy exhibited a different functional profile with regard to IFN-γ production as compared to that of patients after a consolidation regimen needs to be confirmed in a larger cohort of AML remission patients. This might highlight the importance of timing of administration of γδ T cell-activating immunotherapeutic strategies in AML.[36]

Future work will also need to reveal if patients would benefit of the addition of IPP to the vaccine or if there is sufficient IPP present on the leukemic residual cells to enhance γδ T cell activation. Seminal work of Gundermann *et al.* has already shown that if AML cells are pretreated with zoledronate, they display a significantly augmented predisposition to γδ T-cell-mediated cytotoxicity. The latter suggests a potential benefit of the addition of IPP to the vaccine or the combination with a nitrogen-containing bisphosphonate.[13,37] This is supported by our current data showing an enhanced proliferative response and IFN-γ production by γδ T cells to IL-15 DC stimulation in combination with IPP. At the same time, no apparent effect could be detected of the addition of a phosphoantigen as regards γδ T cell cytotoxicity.

Of note, phosphoantigens, such as IPP, activate γδ T cells in a TCR-mediated manner, whereby the expression of ubiquitous butyrophilin (BTN) proteins CD277/BTN3A on stimulator cells were shown to be indispensable.[38-41] In accordance with other data showing that BTN3 molecules are widely expressed by immune cells, some tumor cell lines and even monocyte-derived DCs,[42] it can be assumed that the presentation of IPP to γδ T cells was not hampered in our experiments. Of note, since BTN3A is a determining factor of γδ T cell-recognition of AML cells and agonistic BTN3A mAbs have been show to circumvent primary AML blast resistance to allogeneic γδ T cell lysis, combination therapy is here too something to consider.[43]

Finally, focusing on the IL-15 DC-mediated γδ T cell activation itself, the stimulatory effect seemed to be mainly attributable to the secretion of IL-15 by the IL-15 DC vaccine. IL-15 is a well-documented regulator of homeostasis and activator of both innate and adaptive immunity.[44] Its non-redundant role is reflected by the fact that $CD8^+$ memory T cells and NK cells are absent in IL-15-deficient environments,[45,46] and the lack of IFN-γ-producing γδ T cells in IL-$15^{-/-}$ mice.[47] Moreover, the anti-tumor effect of IL-15 on the immune system has been well-documented in experimental systems.[48-50] Concerning IL-15-mediated γδ T cell activation, our findings are in line with data describing γδ T cell proliferation in rhesus macaques after continuous administration of IL-15[51] and an increase in absolute γδ T cell counts, accompanied by an upsurge in proliferating γδ T cells, in the first-in-human trial of recombinant IL-15 in cancer patients.[52] Previously, we have shown that IL-15 supports γδ T cell proliferation *ex vivo*, and that the addition of IL-15 to γδ T cell cultures results in a more pronounced Th1 polarization and an increased cytotoxic capacity of γδ T cells.[53] In addition, pediatric thymic tissue has been used to unravel the process that drives human γδ T cell differentiation toward anti-tumor lymphocytes. They have confirmed that IL-15 signaling is sufficient to guide human γδ T cells along the Th1 pathway, coinciding with a strong killing capacity of leukemia cells.[54] Moreover, the lack of IL-15 production by M. *tuberculosis*-infected DCs leads to deficient γδ T cell effector functions, impeding the conversion of central memory γδ T cells into effector memory cells.[55] Although IL-15 is a promising immunotherapeutic candidate, and currently tested in clinical trials in AML (National Cancer Institute Trial IDs; 2016LS056 and 2016LS058), the stability and side effects of recombinant IL-15 are some of the bottlenecks to be overcome.[44] The targeted release of IL-15 by IL-15 DCs might therefore offer an important advantage over the systemic delivery of recombinant IL-15, namely avoiding substantial toxicity.[52]

In summary, we have shown that the IL-15 DCs are able to harness γδ T cells in their anti-tumoral activity via secretion of soluble IL-15. These data support the implementation of IL-15-expressing DCs into future clinical trials.

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CHAPTER 6:

CD56 IN THE IMMUNE SYSTEM: MORE THAN A MARKER FOR CYTOTOXICITY?

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ABSTRACT

Over the past years, the phenotypic and functional boundaries distinguishing the main cell subsets of the immune system have become increasingly blurred. In this respect, CD56 (also known as neural cell adhesion molecule [NCAM]) is a very good example. CD56 is the archetypal phenotypic marker of natural killers cells, but can actually be expressed by many more immune cells, including αβ T cells, γδ T cells, DCs and monocytes. Common to all these CD56-expressing cell types are strong immunostimulatory effector functions, including Th1 cytokine production and an efficient cytotoxic capacity. Interestingly, both numerical and functional deficiencies and phenotypic alterations of the $CD56^+$ immune cell fraction have been reported in patients with various infectious, autoimmune or malignant diseases. In this review, we will discuss our current knowledge on the expression and function of CD56 in the hematopoietic system, both in health and disease.

1. INTRODUCTION

NCAM, also known as CD56, is a member of the immunoglobulin superfamily engaged in both so-called homophilic and heterophilic interactions. Three main isoforms exist of CD56 (NCAM-120, NCAM-140, and NCAM-180), all generated by alternative splicing from one single gene, differing in their intracellular domain length.[1] CD56 is often considered a marker of neural lineage commitment due to its discovery site.[2] However, CD56 expression is also found in, among others, the hematopoietic system. Here, the expression of CD56 is most stringently associated with, but certainly not limited to, NK cells (Figure 1).[3]

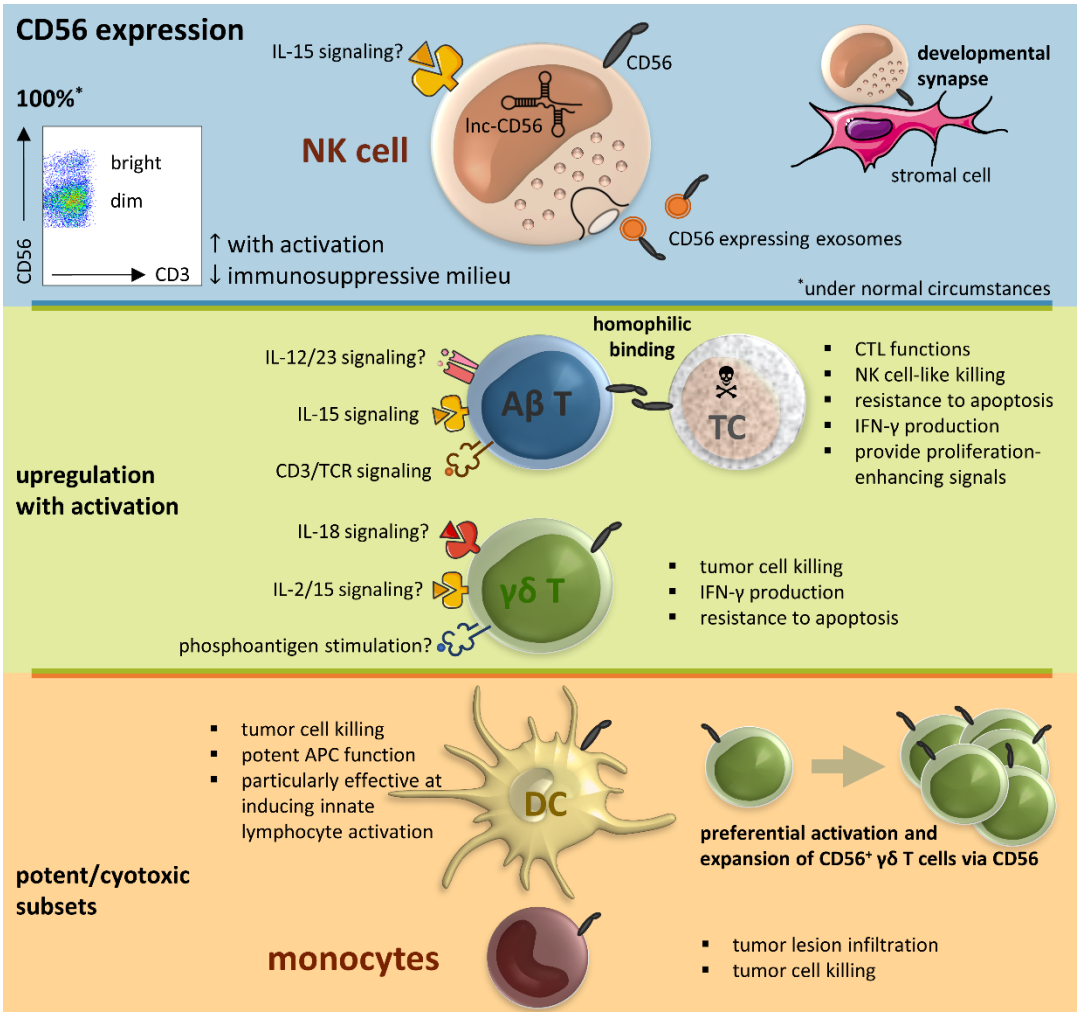

Figure 1. CD56 in the immune system. Abbreviations: αβ T, αβ T cell; γδ T, γδ T cell; APC, antigen-presenting cell; CTL, cytotoxic T cell; DC, dendritic cell; lnc, long non-coding; NK cell, natural killer cell; SC, stressed cell; TCR, T cell receptor

CD56 has been detected on other lymphoid cells, including γδ T cells and activated $CD8^+$ T cells, as well as on DCs.[4-6] Also, in the bone marrow, at the site where hematopoiesis occurs, CD56 fulfills a pivotal role. Mesenchymal stromal cells provide niches for hematopoietic stem cells by, inter alia, the expression of adhesion molecules comprising CD56, maintaining long-term hematopoiesis.[7,8] On the other hand, aberrant CD56 expression is seen in a range of hematological malignancies (e.g. MM, leukemia)[9,10] as well as solid tumors (e.g. lung cancer, ovarian cancer, neuroblastoma).[11-13] Moreover, numerical and functional deficiencies and phenotypic alterations of $CD56^+$ immune cells have been reported in patients with various infectious, autoimmune or malignant diseases (Table 1).

2. CD56 EXPRESSION ON HUMAN IMMUNE CELLS IN HEALTH AND DISEASE

2.1. NK CELLS

NK cells are prototypic members of the innate lymphoid cell (ILC) family and characterized in humans by expression of the phenotypic marker CD56 in the absence of CD3. They are usually further divided into two subsets based on their surface level expression of CD56.[3] Whereas most NK cells in peripheral blood are $CD56^{dim}$, $CD56^{bright}$ NK cells are more abundant in tissues. Until recently it was widely believed that $CD56^{bright}$ NK cells were superior at producing pro-inflammatory cytokines and $CD56^{dim}$ NK cells were described as the more cytotoxic subset. Rather, $CD56^{bright}$ NK cells respond better to soluble factors, while the $CD56^{dim}$ subset responds better to receptors binding ligands anchored on other cells.[14] NK cells have a central role in the cellular immune response, comprising tumor-cell surveillance as demonstrated in the setting of HSCT. The discovery of donor-derived alloreactive NK cells present in T-cell-depleted HLA haplo-identical grafts for HSCT was a milestone in the field of NK cell therapy.[15-17]

To date the reason why NK cells, and immune cells in general, express CD56 remains unanswered. However, there is a clear relationship with the degree of activation. Expression of CD56 can therefore be used as a phenotypic activation marker, similar to the use of CD69 and HLA-DR.[18-20] For example, upon DC vaccine-mediated activation, both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets upregulate their expression of CD56.[18,20] The same applies for expanded NK cells prepared for adoptive transfer using artificial APCs.[19] It should therefore come as no surprise that regulatory DCs, in contrast to their mature immunostimulatory counterparts, lack the ability to upregulate the expression of CD56 on $CD56^{dim}$ NK cells.[42]

Table 1. CD56 expression on immune cells in disease

Disease	Cell type	Effect	Reference
Cancer			
Cancer (general)	Monocytes	↑ higher numbers	21
Metastatic melanoma	αβ T cells	↑ in patients responding to anti-PD-1 therapy	22
(Advanced) solid tumors	αβ T cells	↑ after immunoradiotherapy → injecting $CD56^+$ CTLs ↑ complete remissions	23
Bladder cancer	NK cells	↑ in Bacillus Calmette–Guérin-treated patients	24
Head and neck squamous cell carcinoma	Dendritic cells	$CD56^+$ DC subsets are absent in metastatic lymph nodes	25
Hepatocellular carcinoma	αβ T cells	$FOXP3^+CD3^+CD4^+CD56^+$ tumor-infiltrating lymphocytes inversely correlate with survival	26
Hepatic metastases of colonic origin	γδ T cells	↑ expression of CD56	27
Papillary thyroid carcinoma	Monocytes	$CD14^+CD56^+$ monocytes infiltrate into tumor lesions	21
Acute myeloid leukemia	γδ T cells	↑ expression of CD56	4
Asymptomatic myeloma	NK cells	↑ expression in patients receiving low-dose lenalidomide and DC therapy	28
Infectious diseases			
HIV	NK cells	dysfunctional $CD56^-$ NK cells	29
	αβ T cells	$CD56^+$ $CD8^+$ T cell depletion, but not in natural virus suppressors	30
	γδ T cells	$CD56^+$ γδ T cell depletion, but not in natural virus suppressors	31
Chronic hepatitis C	NK cells	dysfunctional $CD56^-$ NK cells	32
	γδ T cells	↑ expression of CD56	33
Cytomegalovirus	NK cells	dysfunctional $CD56^-$ NK cells	34
Hantavirus	NK cells	dysfunctional $CD56^-$ NK cells	35
Autoimmune disorders			
Crohn's disease	Monocytes	↑ higher numbers	36
Multiple sclerosis	αβ T cells	myelin-specific $CD56^+$ $CD4^+$ T cells kill oligodendrocytes	37
	αβ T cells	Fingolimod ↑ $CD56^+$ T cells in peripheral blood	38
Systemic sclerosis	αβ T cells	↑ $CD56^+$ CTL recruitment to affected tissues	39
Ocular myasthenia gravis	NK cells	dysfunctional $CD56^-$ NK cells	40
Psoriasis	αβ T cells	↑ $CD56^+$ CTL recruitment to affected tissues	39
Rheumatoid arthritis	Monocytes	↑ higher numbers → treatment with etanercept ↓ = better response	41

Moreover, this applies not only for DC-mediated NK cell stimulation. Upon activation $CD56^{dim}$ NK cells can adopt a $CD56^{bright}$-like immunophenotype or upregulate their CD56 expression in general.[24,28,43,44] Conversely, NK cells exposed to an immunosuppressive

milieu downregulate their CD56 expression, concomitant with abolition of their cytotoxicity.[45] This is, among others, demonstrated for factors present in plasma of chronic lymphocytic leukemia patients.[45] In support of the functional role of CD56, exosomes released by NK cells express CD56 as well.[46] Since exosomes are cell-derived vesicles, conceivably having a role in the immune response, the expression of CD56 serves by all odds a purpose, just like the enclosed killer proteins (i.e. FasL, perforin).[46] One possible role could be for example the adhesion of the NK cell exosomes to target cells.[47]

Lastly, it should be noted that there also exists a $CD56^-$ NK cell subpopulation, namely $CD3^-CD4^-CD14^-CD19^-CD16^+NKp46^+$ lymphocytes.[32,48] While $CD56^-$ NK cells are rarely found in healthy individuals,[32] elevated levels of $CD56^-$ NK cells are commonly found in patients with several pathological conditions, including HIV,[29] chronic hepatitis C,[32] human cytomegalovirus[34] and hantavirus infections,[35] autoimmune disorders,[40] and following HSCT.[49,50] In all these diverse settings, $CD56^-$ NK cells are reported to be dysfunctional or impaired with reference to their cytolytic capacity and cytokine production. Unfortunately, aging per se may also have a deleterious effect on the functional capacity of NK cells. A redistribution of NK cell subsets is confirmed in the elderly, whereby the proportion of the dysfunctional $CD56^-$ NK cell subset is increased.[51] Altogether these data emphasize the association between CD56 expression and NK effector function.

2.2. Aβ T CELLS

The cell-mediated adaptive immune response is primarily attributable to conventional T cells. CD56 expression on these αβ T cells is, similarly to NK cells, associated with potent effector function in the human intestine, liver and peripheral blood.[52-54] More specifically, CD56 surface expression on T cells correlates well with expression of CD16, NKG2A/D, NKp44/46, CD122 and DNAM-1, a high intracytoplasmic perforin and granzyme B content, and $CD8^+$ CTL functions.[30,52,53,55-57] Moreover, $CD56^+$ T cells are able to exert NK cell-like killing activity in a proinflammatory milieu.[52,55] This property is mainly due to killer-cell immunoglobulin–like receptor $(KIR)^+$ cells within the $CD56^+$ T cell fraction.[55] All the aforementioned suggests a link between CD56 acquisition by T cells with increased TCR-mediated and NK-like cytotoxic potential. Since CD56 also correlates with the expression of the anti-apoptotic protein Bcl-2, increased resistance to apoptosis is advocated.[52] Secondly, $CD56^+$ T cells share with NK cells the capacity to produce IFN-γ upon IL-15 or IL-12+IL-18 treatment.[53] This pro-inflammatory cytokine production is also seen after stimulation with other immune activating signals such as stimulation of CD3,[6] engagement of the cell adhesion molecule CD2 (LFA-1)[54] or the presence of infectious pathogens. For example, $CD56^+$ T cells produce IFN-γ in the presence of *Bacillus Calmette–Guérin*, as well

as in response to *Salmonella typhimurium*–infected macrophages.[53] This pro-inflammatory cytokine response is, on the other hand, barely detectable in their $CD56^-$ counterparts.[53] Furthermore, regulatory (IL-10) and Th2 (IL-4 and IL-5) cytokine production by $CD56^+$ T cells is marginal.[6,54] Next, mucosal $CD56^+$ T cells exhibit a compromised proliferation potential as compared to their $CD56^-$ counterparts, characteristic for their mature state.[54] Nevertheless, $CD56^+$ T cells provide proliferation-enhancing signals following global activation to other immune cells, mediating-immune responses in a contact-dependent manner.[54]

These properties make $CD56^+$ T cells attractive potential targets for therapy for infectious and immune-mediated diseases as well as cancer. For example, in metastatic melanoma a distinct population of T cells with high expression of HLA-DR and CD56 increased by 9-fold in patients responding to anti-PD-1 therapy.[22] In line with this, immunoradiotherapy augmented the abundance of circulating $CD8^+CD56^+$ cells in advanced cancer patients.[23] Interestingly, injecting patients who failed to respond to immunoradiotherapy with $CD56^+$ CTLs into their recurrent metastatic lesions resulted in 59% of complete remissions at these sites.[23] On this note we would like to touch on CIK cells who fall within this group of NK cell-like T lymphocytes, whereby the $CD56^+$ CIK cells represent the cell type with the highest tumor killing abilities.[58,59] Although CIK cells are beyond the scope of this review, we would like to direct the reader to Schmeel *et al.* (2014)[58] and Mesiano *et al.* (2012)[59] for a comprehensive review.

Proceeding to infectious diseases, CD56-expressing $CD8^+$ T cells were found to be depleted in HIV^+ patients (on therapy) but not in natural virus suppressors, i.e. elite patients suppressing HIV replication without antiretroviral therapy.[30] This could be of importance bearing in mind that antiretroviral therapy to date, showing effective HIV suppression and reconstitution of CD4 T cells, still fails to restore $CD8^+$ T cell lytic effector functions needed to eradicate the viral reservoir. One reason behind this loss of $CD56^+$ T cells could be the high expression of the exhaustion marker TIM-3 on $CD56^+$ T cells of HIV patients, whereas this is not seen for $CD56^+$ T cells of elite patients. Immune exhaustion is therefore a potential mechanism for preferential depletion of $CD56^+$ $CD8^+$ T cells.[30]

Regarding autoimmune diseases, patients suffering from systemic sclerosis, mainly those with active/late capillaroscopic patterns or with severe lung impairment, have decreased numbers of circulating $CD56^+$ CTLs as compared to healthy individuals.[60] Even though different mechanisms may be involved, this decline in $CD56^+$ T cells in the peripheral blood results at least in part from the recruitment and/or trafficking of these cells to the affected tissues, where they cause endothelial cell injury and apoptosis.[39] The decrease in $CD56^+$ T

cell counts is furthermore not only a characteristic of scleroderma but also of other autoimmune disease, for example, of psoriasis.[39] In addition, in multiple sclerosis, cytotoxic myelin-specific $CD56^+$ $CD4^+$ T cells were described, killing oligodendrocytes in a NKG2C-mediated manner.[37] It should therefore be noted that not only $CD56^+$ CTLs, but also cytotoxic $CD56^+$ $CD4^+$ T cells do exist, lying at the root of the pathogenesis of disease. As a consequence, the impact of (novel) treatment strategies for multiple sclerosis on the $CD56^+$ immune cell fraction is of importance. Fingolimod, a sphingosine 1-phosphate receptor modulator, is indicated as single disease-modifying therapy in highly active relapsing remitting multiple sclerosis patients. Notably, it turns out that fingolimod therapy increases the frequency of $CD56^+$ T cells in peripheral blood in multiple sclerosis patients, particularly during relapses.[38]

In analogy with NK cells, aging induces weakening of the adaptive immune system, generally termed as immunosenescence.[61,62] This is accompanied by an accumulation of cells combining features of both the innate and adaptive arms of the immune system, most likely to compensate for functional defects of conventional NK and $CD8^+$ T cells with age. In contrast to exhausted T cells, senescent terminally differentiated CD45RA ($CD8^+$) T (TEMRA) cells are effector T cells with complete competence. They develop features of NK cells comprising the upregulation of NK cell receptors, including CD56. TEMRA cells use their acquired NK cell machinery to maintain rapid effector functions throughout life, tackling the increased burden of tumors and infections in the elderly.[61,62]

As a final remark, not every $CD56^+$ T cell exhibits by definition immune invigorating capacity. In the tumor bed of patients with hepatocellular carcinoma, a $FOXP3^+CD3^+CD4^+CD56^+$ population with immunosuppressive function has been found (similar to regulatory T cells).[26] In comparison, $FOXP3^+$ cells were rarely detected in the $CD3^+CD56^+$ population from adjacent non-cancerous tissues and were completely absent from normal liver tissues.[26] The prevalence of $FOXP3^+CD4^+CD56^+$ T cells in TILs was moreover found to be inversely correlated with patient survival.[26] Besides $FOXP3^+$ TILs, other immune cell subsets may as well exert a powerful regulatory/suppressive influence upon the cell-mediated immune response. In human glioblastoma, a significant proportion of TILs were $CD3^+CD4^+CD56^+$ immunosuppressive T cells.[63] Here too, only a minority of $CD3^+$ peripheral lymphocytes expressed CD56.[63] Other non-glial intracranial tumors, including meningioma and metastatic non-small cell lung cancer, showed however no accumulation of $CD4^+CD56^+$ in the tumor.[63] It is therefore necessary to take into account that the favorable profile of the $CD56^+$ T cells is possibly depending on the T cell subset and/or is sensitive to changes by the tumor microenvironment, with variation between cancer types. Concerning the latter, special emphasis should be given to the expression levels of CD56

on the tumor cells, considering glioblastoma is known to express CD56 whereas for example non-small cell lung cancer generally does not.

2.3. γδ T CELLS

γδ T cells are the prototype of 'unconventional' T cells, defined by the expression of a TCR composed of a γ and δ chain. Although they only constitute less than 5% of all T lymphocytes, their role in the immune system should not be underestimated. γδ T cells have a wide range of functional properties including innate killing, tumor tropism, the support of DC, T cell, and NK cell functions, and, as recently demonstrated, also antigen presentation skills.[64-67] Moreover, the proportion of $CD56^+$ γδ T cells appears to be determined by their level of activation.[4,27]

Different signals are able to induce γδ T cell activation including phosphoantigens, cytokines, activating receptors and TCR-mediated signals.[66,68-70] As leading example, stimulation of isolated γδ T cells with IPP, a mevalonate-derived isoprenoid phosphoantigen, and the cytokines IL-2 or IL-15 leads to a significant upregulation of CD56 concomitant with CD69 and HLA-DR.[4] Activation of γδ T cells with IL-15 and isoprenoid pyrophosphates induces furthermore the expression of IL-15Rα, CD96, CD161 and perforin, all markers of cytotoxic cells.[71] Stimulation of γδ T cells from HIV^+ donors with IL-18 and IPP, results not only in γδ T cell proliferation, but also in the higher expression of CD56, NKG2D, and CD107a.[72] The cytotoxic phenotype of all these $CD56^+$ effector γδ T cells puts forward that CD56 may be a marker of true effector γδ T cells. It is indeed expressed on a potently cytotoxic subset of human γδ T cells. CD56-expressing, but not $CD56^-$, IPP-expanded γδ T cells kill head and neck squamous cell carcinoma through the perforin-granzyme pathway.[73] Yet, CD56 neutralization itself did not affect $CD56^+$ γδ T cell-mediated killing of tumor cells.[73] In addition to cytotoxicity, $CD56^+$ effector γδ T cells rapidly produce large amounts of IFN-γ upon stimulation,[71] and have an increased resistance to Fas ligand and chemically induced apoptosis.[73] Strikingly, expression of CD56 is strongest in non-proliferating γδ T cells and gradually disappears with the number of cell divisions.[71] Taken together, CD56 defines γδ T cells with increased anti-tumor activity, identifying a robust γδ T cell subset for effective cancer treatment.

In the context of immunotherapy, expanded γδ T cells for adoptive transfer exhibit an enhanced CD56 expression as well.[4] However, this observation was only made in healthy donors. Conversely, γδ T cells of AML patients exhibit already elevated levels of CD56, and after expansion, even a down-regulation of CD56 is observed.[4] The increased expression of

CD56 by γδ T cells is also seen in patients with solid tumors, for instance in γδ T cells associated with hepatic metastases of colonic origin,[27] and infectious disease, including chronic hepatitis C virus infection.[33] Intriguingly, while the proportion of $CD56^+$ γδ T cells dramatically drops in patients with HIV disease, and does not return to normal levels even after prolonged antiretroviral therapy, natural viral suppressors have unaffected $CD56^+$ γδ T cell levels and function similar to those of healthy controls.[31]

2.4. DENDRITIC CELLS

Given their primary function to capture, process and present antigens to T cells, DCs play a critical role in stimulating adaptive (antigen-specific) immunity. In general, lytic effector functions are not classically attributed to DCs. However, it is has been demonstrated that both pDCs and mDCs can adapt a $CD56^+$ phenotype and acquire cytotoxic functions.[5] Plasmacytoid DCs activated by the tick-borne encephalitis vaccine früh sommer meningoencephalitis display high CD56 expression, coinciding with elevated expression of programmed death-ligand 1, granzyme B, TRAIL and effector functions. Interestingly, neutralizing CD56 did not result in diminished specific lysis of tumor cells.[74] Furthermore, $CD56^+$ monocyte-derived IL-15 DCs possess a more pronounced lytic effector function towards tumor cells as compared to their $CD56^-$ counterparts, accompanied by elevated TRAIL and granzyme B levels, as well as a superior antigen-presenting capacity.[75] Similarly, $CD56^+$ IFN-α DCs exhibit a TRAIL-mediated cytolytic activity against tumor cells.[76] Apart from exerting a direct cytolytic activity, $CD56^+$ DCs have potent antigen-presenting capacity as well,[74-76] and are particularly effective at inducing innate lymphocyte activation.[18,77,78] Unfortunately, malignant cells seem to disable these DC subsets.[25,76] $CD56^+$ expression on pDCs and mDCs is down-regulated subsequent to contact with head and neck squamous cell carcinoma cells *in vitro* and $CD56^+$ DC subsets are absent in metastatic lymph nodes.[25,76]

2.5. MONOCYTES

One important function of monocytes is their contribution to the renewal of DCs (and some tissue macrophages) predominantly under inflammatory conditions. $CD14^+CD56^+$ monocytes, which could function as potential precursors of $CD56^+$ DCs, have been found in human peripheral blood.[21] They are able to infiltrate into tumor lesions and have a direct cytolytic activity towards malignant cells upon activation.[21] Interestingly, cancer patients, both with solid tumors and hematological malignancies, reveal much higher numbers of this monocyte subset as compared to healthy controls.[21] However, a negative correlation exists between the magnitude of tumor spread and the amount of $CD56^+$ monocytes.[21] This possibly points to the fact that these cells might play a role in immune tolerance or, in

analogy with DCs, that CD56$^+$ monocytes are being down-regulated by the tumor environment.[79] Consonant with the increased prevalence of CD56$^+$ monocytes in cancer patients, this subset is also found elevated in autoimmune diseases, such as Crohn's disease and rheumatoid arthritis.[36,41] From a therapeutic point of view, patients with rheumatoid arthritis treated with etanercept, a TNF-α inhibitor, show a decline in the CD56$^+$ monocyte subset, associated with a better response to treatment.[41] This is in line with the assumption that CD56$^+$ monocytes are being part of the activated cellular immune response.

3. CD56 EXPRESSION IN OTHER SPECIES

Given the fact that many laboratory animals are used as preclinical models for human disease or basic biomedical research, a brief overview of CD56 expression in other species is warranted, especially since patterns of CD56 expression differ markedly from those found in humans. NK cells of rodents for example, the most commonly used small animals, do not express CD56 at all. Instead, they are generally identified by their expression of DX5/CD49b or NKR-P1C in mice, or NKR-P1A in rats, in the absence of CD3.[80] Of note, recent data indicate a developmental or lineage relationship between mouse ILCs and human blood CD56^{bright} NK cells, while signature genes of mouse NK cells turned out to be enriched in human CD56^{dim} NK cells.[81] However, the current debate on NK cell vs ILC types[82,83] is beyond the scope of this review. Despite exhibiting far greater similarities to human NK cells, as compared to rodent NK cells, NK cells of non-human primates, such as Cynomolgus and Rhesus Macaques, generally lack CD56 expression as well.[84,85] On the other hand, the majority of monocytes of non-human primates do express CD56.[85,86] Considering CD56 protein expression on immune cells seems to be highly interspecies-dependent, it remains to be addressed whether there is a specific functional role for this molecule on (human) immune cells.

4. UPREGULATION, BINDING INTERACTIONS AND FUNCTIONALITY

As stated above, there is a body of evidence that CD56 expression is often associated with activation or cytotoxicity in immune cells. This brings forward the question as to whether CD56 is merely an indicator/marker of an activated cell state or if it is actively involved in immune effector function. Moreover, in view of future target discovery for immunotherapy, it is interesting to elucidate which molecular processes give rise to the expression of this molecule. In this context it has been shown that the proportion of the subset of T cells expressing surface CD56 was drastically reduced in IL-12/23 axis–deficient

patients, suggesting that the presence of IL-12/23 is mandatory for the expansion of $CD56^+$ T cells.[53] Of course, this finding does not prove that IL-12/23 stimulation leads to the direct upregulation of CD56, especially since these patients did not show alterations in their $CD56^{dim/bright}$ NK cells subsets.[53] On the other hand, culturing NK cells with IL-15 leads to an upregulation of CD56 on both $CD56^{bright}$ and $CD56^{dim}$ NK cells, concomitant with enhanced expression of NK cell activating receptors NKG2D, NKp30, and NKp46.[87] The same shifts in phenotype are also observed in allogeneic HSCT patients, whereby the greatest changes were observed in the early post-transplant months, a period in which IL-15 levels peak.[87] In addition, there is evidence that CD56 expression on $CD56^-$ T cells can be induced by IL-15 stimulation.[52] Purified $CD8^+CD56^-$ T cells express *de novo* CD56 after 12-days of culture with IL-15.[52] Likewise, umbilical cord blood T cells acquire CD56 after culture in IL-15.[56] Therefore, with some certainty, it can be stated that the pleiotropic cytokine IL-15, a well-documented regulator of homeostasis and activation of both innate and adaptive immunity, induces the expression of CD56 on immune cells bearing the IL-2/IL-15Rβ unit such as NK cells and T cells.[88,89] However, the exact mechanism remains unclear. Besides, based on the available literature on this subject, it is unlikely that IL-15 is the only factor capable of having a direct effect on the expression on CD56. For example, similar effects on CD56 expression have been described for CD3/TCR-mediated activation of T cells.[6]

On a molecular level, long non-coding (lnc) RNAs orchestrate genetic regulatory outputs, participating in cell differentiation and function.[90] Recently lncRNA AB128931 or lnc-CD56 has been discovered in NK cells, positively correlating with CD56 expression.[91] Also, lnc-CD56 knockdown reduces CD56 transcription, providing evidence that lnc-CD56 functions as a positive regulator of CD56.[91] Additional data are however required to unequivocally confirm the roles for this lncRNA in CD56 expression by immune cells in general.

Lastly, from a functional viewpoint, unfortunately, to date, very little is known regarding the functional role of CD56 on immune cells. One key function in the development of NK cells is the CD56-driven migratory behavior of NK cells on stromal cells, forming a developmental synapse.[92] NK cells acquire motility with progressive maturation, correlated with the expression of CD56 on developing NK cells. Blocking of CD56 therefore perturbs both NK cell motility and maturation.[92] $CD56^+$ immune cells are also able to form strong immune synapses with each other through CD56 binding. For example $CD56^+$ DCs have been shown to induce the preferential activation and expansion of $CD56^+$ γδ T cells via CD56.[93] In particular, homophilic interaction between CD56 molecules on $CD56^+$ cells can be formed, including immune cells but also for example tumor cells. In this way, $CD56^+$ CIK are able to kill $CD56^+$ leukemic cells.[94] This implies that knocking down CD56 on effector

cells makes them less cytotoxic against a $CD56^+$ target cells and, conversely, that downregulating CD56 on target cells impedes CD56-mediated lysis.[94]

5. CONCLUSION AND FUTURE PERSPECTIVES

Hematopoietic expression of CD56 seems to be confined to activated immune cells exhibiting some level of cytotoxic properties. It is therefore tempting to speculate that CD56 is not merely a phenotypic marker of NK cells. Aβ T cells, γδ T cells, DCs, monocytes, and possibly even more cells of the immune system can upregulate or neo-express CD56 when activated. This implies some concerns regarding current scientific research. For example, it would be recommended to reconsider the use of negative selection kits making use of CD56 as a depletion marker for NK cells. Chances are that besides the removal of NK cells also other valuable (activated) $CD56^+$ white blood cell types will be depleted impacting on the applied assay. Furthermore, in current clinical trials cancer patients with $CD56^+$ tumors are being treated with Lorvotuzumab mertansine, a CD56-targeting antibody-drug.[95] Although in preclinical studies Lorvotuzumab was shown to be promising in the treatment of CD56-positive tumors, these results were not entirely translatable to the human situation. A major adverse event was the rate of infection and infection-related deaths upon addition of Lorvotuzumab to etoposide/carboplatin therapy in patients with small cell lung cancer.[95] The combination of agents with known hematologic toxicities is a risk, but potentially the depletion of $CD56^+$ immune cells holds an even greater risk. It is evident that more research is warranted on the role of CD56 expression in cells of the immune system. Clarifying the upregulation and functional role of CD56 on immune cells should therefore be considered a priority, considering current and future immune therapeutic options will most likely benefit from it.

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CHAPTER 7:

CD56 HOMODIMERIZATION AND PARTICIPATION IN IMMUNE EFFECTOR CELL ACTIVATION AND TUMOR CELL ERADICATION: A ROLE FOR INTERLEUKIN-15

This chapter has been submitted for publication:

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ABSTRACT

A particularly interesting marker to identify anti-tumor immune cells is the neural cell adhesion molecule (NCAM), also known as CD56. Namely, hematopoietic expression of CD56 seems to be confined to activated immune cells exhibiting varying levels of cytotoxic properties. Nevertheless, current knowledge on the expression and functional role of CD56 is very fragmented. Therefore, we sought to elucidate the role of CD56 expression on various killer immune cells. First, we identified the high motility NCAM-120 molecule to be the main isoform expressed in immune cells. Next, through neutralization of surface CD56, we were able to *(I)* demonstrate a direct involvement of CD56 in tumor cell lysis exerted by CD56-expressing killer cells such as natural killer cells, gamma delta (γδ) T cells and interleukin (IL)-15-cultured dendritic cells (DCs) and *(II)* reveal a putative crosstalk mechanism between IL-15 DCs and CD8 T cells, suggesting CD56 as a co-stimulatory molecule in their cell-to-cell contact. Moreover, by means of a proximity ligation assay, we visualized the CD56 homophilic interaction amid cancer cells and between immune cells and cancer cells. Finally, by blocking the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3K)-Akt pathway, we confirmed our hypothesis that IL-15 stimulation directly leads to CD56 upregulation. In conclusion, these results underscore the previously neglected importance of CD56 expression on immune cells, benefiting current and future immune therapeutic options.

1. INTRODUCTION

CD56 or NCAM is expressed in nearly all tissues. Depending on the cell type and its surroundings, three main isoforms can be generated from one single gene by alternative splicing. Based on their apparent molecular weights, they are termed NCAM-120, NCAM-140 and NCAM-180. Whereas NCAM-140 and NCAM-180 are transmembrane proteins, NCAM-120 is glycosylphosphatidylinositol (GPI)-anchored with no intracellular residues.[1] The highest CD56 levels are found in the central and peripheral nervous system, where it plays an important role mediating intracellular signal transduction and stabilizing synaptic contacts.[2] By contrast, the function of the CD56 protein in the hematopoietic system is largely undetermined, but its expression seems to be confined to activated immune cells exhibiting some level of cytotoxic properties, recently reviewed by Van Acker *et al.*.[3] Although known for its role as the archetypal phenotypic marker of NK cells, CD56 can actually be expressed by many more immune cell subsets including NKT cells, γδ T cells, CD8 T cells, CIK cells, DCs and monocytes. Alluding to a central role of the $CD56^+$ immune cell fraction, both functional and numerical aberrations affecting this subset are observed in a wide variety of pathologies, comprising several malignant diseases.[4-6]

Interestingly, CD56 was found to be expressed by our next-generation IL-15 DC vaccine, differentiated with IL-15 instead of IL-4, and was absent on conventional IL-4 DCs.[7] In turn, IL-15 DCs secrete IL-15 themselves.[8] NK cells activated by IL-15 DCs significantly upregulate CD56, both on the $CD56^{bright}$ and $CD56^{dim}$ NK cell subpopulations.[9] In addition, CD56 expression is significantly higher on IL-15- and IPP-stimulated γδ T cells as compared to IL-2/IPP-stimulated or unstimulated γδ T cells.[10] We therefore hypothesize that IL-15 signaling augments CD56 expression, which is further supported by data from other groups.[11,12] For example, IL-15 can convert central memory γδ T cells into potent $CD56^+$ effector cells with the ability to rapidly produce large amounts of IFN-γ and kill tumor cells.[13] Correia *et al*. reported that after 12-days of culture with IL-15, purified $CD8^+CD56^-$ T cells expressed *de novo* CD56.[14] It is known that IL-15 is capable of enhancing the cytotoxic effector functions of lymphocytes through three pathways; i.e. *(I)* the JAK/STAT pathway, *(II)* the MAPK pathway and *(III)* the PI3K–Akt pathway.[15] To advance our understanding of the individual contribution of each pathway to the enhanced activation and cytotoxic state of the γδ T cells and their expression of CD56, all three pathways were blocked separately.

An initial impetus pursuing the clarification of the upregulation and functional role of CD56 on immune cells was taken in this paper. In the first part we looked at the CD56 phenotype and isoforms of different immune cell subsets on the molecular and protein level. Next, we

investigated if CD56 is actively involved in the cytotoxic capacity of $CD56^+$ effector cells by means of neutralizing CD56 in a killing assay against a panel of $CD56^+$ tumor cell lines. Furthermore, we looked for definitive proof of the existence of a CD56-CD56 interaction on the surface of immune cells and tumor cells by means of Duolink® Technology. In the second part we examined if IL-15 and, more broadly, cytokines of the common gamma-chain cytokine family play a central role in the activation of immune cells and their upregulation of CD56.

2. MATERIALS AND METHODS

2.1. CELL LINES AND PRIMARY CELLS

The AML cell line NB4 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The SH-SY5Y and SH-SY5Y-eGFP[16] neuroblastoma cell lines were kindly provided to us by the laboratory of Prof. Dewilde (University of Antwerp, Belgium) and the MM cell line U266 was a gift from Dr. Germeraad (Maastricht University, The Netherlands). PBMC were isolated from buffy coats of healthy donors, provided by the Red Cross donor center (Red Cross-Flanders, Mechelen, Belgium). Immune cells subsets for downstream molecular biology applications were sorted on a FACSAria II flow cytometer (BD; Erembodegem, Belgium) based on the following markers within the viable cell population (Live/Dead® Fixable Aqua Stain; Thermo Fisher Scientific, Waltham, MA, USA); NK cells (CD3-PerCP-Cy5.5$^-$ [BD], CD56-PE$^+$ [BD]), γδ T cells (CD3-PerCP-Cy5.5$^+$ [BD], γδ TCR-APC$^+$ [Miltenyi, Bergisch Gladbach, Germany] and $CD8^+$ T cells (CD3-PerCP-Cy5.5$^+$ [BD], CD8-PB$^+$ [Thermo Fisher Scientific]. Flow cytometric evaluation of CD56 membrane expression of the different subsets was performed at the same time. Monocytes were isolated with anti-CD14-conjugated CliniMACS microbeads (REF 272-01; Miltenyi). For cytotoxicity experiments, NK cells were isolated with an NK cell isolation kit (REF 130-092-657; Miltenyi) and CD8 T cell purification was performed with the REAlease CD8 microbead kit (Miltenyi) followed by a CD56 enrichment step with CD56 microbeads (Miltenyi). CD8 T cells were 61% CD56-positive (range 45-89%).

2.2. IL-15 DC CULTURE

Monocyte-derived IL-15 DCs were generated according to our previously reported protocol comprising a rapid (28-hour) differentiation of monocytes with GM-CSF and IL-15, followed by a maturation step of 20 hours by triggering of the TLR7/8 signaling pathway.[8,17,18]

2.3 ΓΔ T CELL EXPANSION PROTOCOL

γδ T cells were expanded from PBMC as previously described.[10] Briefly, PBMC were seeded at a final concentration of $1x10^6$ cells/mL in in RPMI supplemented with 10% hAB (Thermo Fisher Scientific), zoledronate (5 µM; Stemcell, Cologne, Germany), IL-2 (100 IU/mL; ImmunoTools, Friesoythe; Germany) and IL-15 (10 ng/mL, ImmunoTools). Cell cultures were maintained at a cell density of $0.5–2 \times 10^6$ cells/mL and replenished every 2 to 3 days with IL-2/IL-15-supplemented medium. After 14 days, γδ T cells used in functional assays were on average 94% pure and 57% positive for CD56. The donors used for the Duolink® assay had a mean culture purity and CD56 expression level of 96% and 45%, respectively.

2.4. RNA EXTRACTION AND REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was extracted from 250 000 cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction. Subsequent quantification and purity evaluation was performed on a NanoDrop spectrophotometer (Thermo Fisher Scientific). First-strand cDNA synthesis was conducted with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), of which 10 ng was taken for each quantitative-PCR (qPCR) analysis. SYBR Green technology was used for relative mRNA quantification by qPCR on a CFX96 C1000 thermal cycler (Bio-Rad). The PrimePCR cycling protocol consisted of an initial pre-incubation of 30 s at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The following primers were used to amplify CD56 isoform specific transcripts: *120 kDa CD56* (F-CACAGCCATCCCAGCAACCTTG, R-TCCAAAGGGGGCACTGATCTT), *140 kDa CD56* (F-CACTGACGGAGCCCGAGAAG, R-TCATGCTTTGCTCTCGTTCTCC), *180 kDa CD56* (F-GACCCCAGATATTGACCTTGC, R-CCTTCTCGGTCTTTGCTGGC). The primers of the CD56 isoforms have been described previously.[19] Three housekeeping genes (*B2M*, *SDHA* and *TBP*) were carefully chosen from a list of 8 reference targets, also including *ACTB*, *IPO8*, *PGK1*, *RPL13* and *RPS14*, using geNorm in qBase+ (geNorm V < 0.15; Biogazelle, Gent, Belgium). Inter-run calibrators were included. All primers were purchased from Bio-Rad. Fold-changes were calculated using the comparative Cq method, scaled to $CD14^+$ cells.

2.5. CYTOTOXICITY ASSAY

Determination of the (innate-like) cytotoxic capacity of different immune cells was performed by means of a 4-hour flow cytometry-based lysis assay against PKH67-labeled (Thermo Fisher Scientific) $CD56^+$ cell lines; NB4, SH-SY5Y and U266. To specify the direct killing capacity of isolated NK cells, CD8 T cells and expanded γδ T cells, they were co-cultured at an E:T ratio of 5:1, whereas IL-15 DCs were cultured at an E:T ratio of 20:1. To

investigate the involvement of CD56, 100 µL of the neutralizing GPR165 mAbs-containing supernatant (IgG2a)[19,20] was added to specific conditions. To assess the involvement of CD56 in immune effector cell activation by ($CD56^+$) IL-15 DCs, NK cells / γδ T cells / CD8 T cells were cultured overnight at a 1:1 E:T ratio with IL-15 DCs, whether or not in the presence of anti-CD56 neutralizing antibodies or isotype controls. Subsequently, cultures were washed and PKH67-positive target cells were added at a 5:5:1 ratio for four hours. Target cell death was determined by combined Annexin-V-APC (BD) / PI (Sigma-Aldrich) staining. Specific target cell killing was calculated using the following formula: % killing = 100 − [(% Annexin-V^-PI^- target cells with effector cells / % Annexin-V^-PI^- target cells without immune cells) x 100].

2.6. BLOCKING OF THE DISTINCT IL-15 SIGNALING PATHWAYS

Il-15 signaling pathways were blocked using either; *(I)* CAS 285986-31-4 (1 µM; Merck, Burlington, MA, USA) as a STAT5 Inhibitor, *(II)* Trametinib (1µM; GSK1120212; Selleckchem, Munich, Geramany) as a highly selective inhibitor of both MEK1 and MEK2, and *(III)* Afuresertib (2 µM; GSK2110183, Selleckchem) as a highly selective inhibitor of Akt. Concentrations of the different inhibitors were first titrated to ascertain preservation of cell viability. One hour prior to the addition of IL-15 (10 ng/mL), PBMC ($1x10^6$ cells/mL) were exposed to the above mentioned inhibitors to ensure adequate blocking. NK cells and monocytes were analyzed after 48 hours, whereas γδ T cells and CD8 T cells were assessed after 1 week. For immunophenotypic analysis, cells were stained with following fluorochrome-labeled mAbs: CD3-FITC (Immunotools), CD8-FITC, CD14-FITC, γδ TCR-FITC (Miltenyi), CD69-PE, CD314-PE, CD3-PerCP-Cy5.5, CD56-BV421, CD3-APC, CD253-APC, γδ TCR-APC (Miltenyi), CD178-BV786 and HLA-DR-APC-H7 (or isotype controls). Fc receptor blocking was done with mouse gamma globulins (Jackson ImmunoResearch, West Grove, PA, USA) for 10 minutes prior to staining with mAbs. For the combined cell surface and intracellular perforin and granzyme B staining, Brefeldin A (1 µL/mL; Golgi-Plug, BD) was added to the cultures for four hours prior to membrane staining. Cultures were fixed and permeabilized with the Foxp3 Transcription Factor Staining Buffer Set (Invitrogen, Merelbeke, Belgium) and stained with granzyme B-BV421 and perforin-AF647. All mAbs were purchased from BD, unless stated otherwise. Additionally, dead cells were excluded using Live/Dead® Fixable Aqua Stain (Invitrogen).

2.7 PROXIMITY LIGATION ASSAY

To study CD56 homodimerization, Duolink©-PLA analysis was performed. Per 24-well, 150.000 SH-SY5Y-eGFP cells were plated for three days on round 12 mm cover glasses

(VRW, Radnor, PA, USA). Subsequently, 750.000 immune cells, i.e. IL-15 DCs, NK cells or γδ T cells, were added to the wells and co-incubated for 20 minutes. Fixation of the co-cultures was performed with 4% formaldehyde. Goat anti-human CD56 mAbs (AF2408, 1:200; R&D, Minneapolis, MN, USA) were labeled with the Duolink® *in situ* probemaker PLUS or MINUS. Identification of immune cells relied on staining with CD45 mAbs (YAML501.4, 1:500; Thermo Fisher Scientific) in combination with Cy™5 AffiniPure donkey anti-rat IgG mAbs (1:100; Jackson ImmunoResearch). Samples were amplified with the Duolink® In Situ Detection Reagents Red (#DUO92008; Merck), generating red fluorescent spots if two CD56 proteins are located in a radius of 40 nm (dimerization). The cover slips were mounted with Duolink® *in situ* mounting media with DAPI. Images were captured on a Nikon TI-E inverted confocal microscope (Nikon, Tokyo, Japan) and analyzed with ImageJ.

2.8. STATISTICAL ANALYSIS

All flow cytometry data were acquired on a FACSAria II flow cytometer (BD) and analyzed using FlowJo (v10; Treestar, Ashland, OR, USA). Graphpad Prism software (v7.00; Graphpad, San Diego, CA, USA) was used for statistical analysis and artwork. The Shapiro-Wilk normality test was performed to test if the data sets came from a Gaussian distribution.

3. RESULTS

3.1. CD56 ISOFORM EXPRESSION IN IMMUNE CELLS

Expression of CD56 is found on a variety of immune cells (Figure 1). Considering human NK cells are generally characterized by a $CD56^{+}CD3^{-}$ phenotype, all NK cells express some level of CD56. A significant portion of γδ T cells (26.13 ± 6.86 %), $CD8^{+}$ T cells (27.08 ± 15.45 %), monocytes (11.50 ± 3.68 %) and monocyte-derived IL-15 DCs (32.30 ± 5.43 %) express CD56 as well. In view of the existence of several isoforms of CD56, generated by alternative splicing and posttranslational modifications, we identified the subsets expressed by the different immune cells on the molecular level by qPCR. Protein expression validations and specific amplification of CD56 isoforms by qPCR were performed on immune cells of the same donors (n = 3). Interestingly, apart from monocytes which favored no particular CD56 isoform for their expression, NK cells, γδ T cells, $CD8^{+}$ T cells and IL-15 DCs all strongly preferred the 120 kDa CD56 isoform, while still expressing a fair amount of the 140 kDa isoform. The proportion of the 180 kDa isoform, however, was considerably less to almost absent in all tested immune cell subtypes.

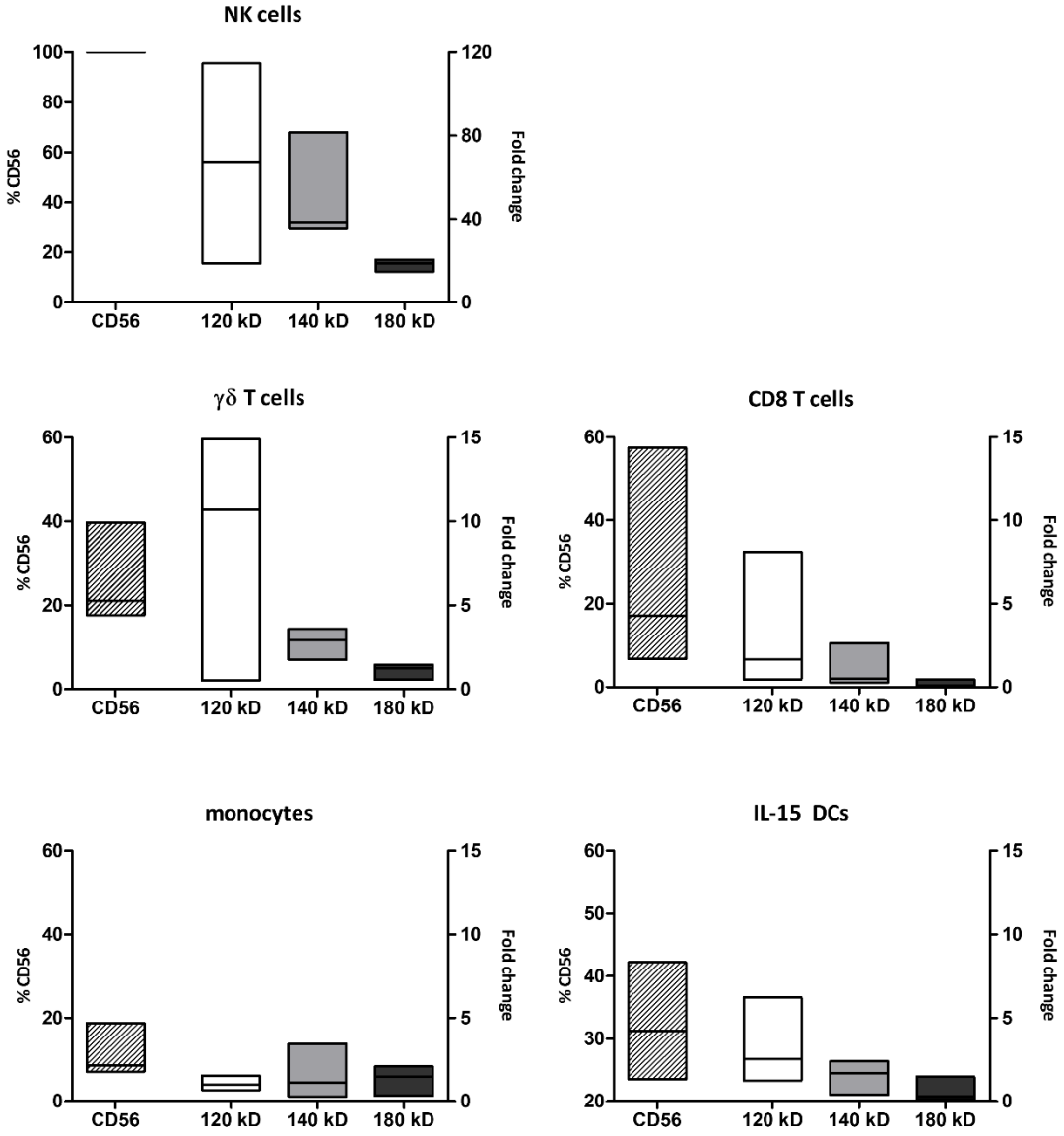


Figure 1. CD56 (isotype) expression by different immune cell subsets. Juxtaposition of the percentage CD56 expression on different immune cell subsets as determined by flow cytometry (left y-axis) and CD56 isoform (120-140-180 kD) mRNA expression (relative to $CD14^{+}$ cells) determined by qPCR (right y-axis). Data of three donors are represented as floating bars (min to max).

3.2. INVOLVEMENT OF CD56 IN IMMUNE EFFECTOR CELL ACTIVATION AND CD56+ TUMOR CELL KILLING

Next, we tested the cytotoxic capacity of the different CD56-expressing immune cell subsets against a panel of CD56$^+$ tumor cell lines (Figure 2). As members of the innate immune system, empowered with MHC-independent cytolytic capacity, unstimulated NK cells and γδ T cells were able to kill to a variable degree the CD56$^+$ tumor cell lines NB4, SH-SY5Y and U266 (Figure 3, left panels), while unstimulated CD56-enriched CD8 T cells only showed marginal killing. At an E:T ratio of 20:1, the IL-15 DC vaccine manifests its 'killer-like' DC profile as well, especially against SH-SY5Y (12.81 ± 4.65%) and U266 (12.72 ± 2.95). Importantly, direct cytotoxicity of IL-15 DCs, NK cells and γδ T cells is modulated by the addition of anti-CD56 blocking mAbs to varying degrees, depending on the target cell line used (Figure 3, right panels). This suggests at least in part an involvement of CD56 in the lysis of malignant CD56-expressing cells. Surprisingly, we observed a strong enhancement of the killing capacity of enriched CD56$^+$ CD8 T cells by IL-15 DCs. Tumor cell-killing by unprimed CD8 T cells co-cultured overnight with IL-15 DCs was 2- to 3-fold enhanced against NB4, SH-SY5Y and U266 cells, i.e. 17.43 ± 14.45 % → 43.32 ± 12.32 %, 8.46 ± 3.27 % → 23.87 ± 6.62 % and 8.82 ± 4.35 → 23.17 ± 10.61 %, respectively. Upon CD56 neutralization, the lytic activity of IL-15 DC-primed CD8 T cells was reduced to levels comparable to that of unstimulated CD8 T cells. Concerning NK cells and γδ T cells alike, a strong enhancement in tumor cell killing was seen after overnight co-culture with IL-15 DCs. The role of CD56 in innate effector cell activation by IL-15 DCs was, however, less pronounced as for the CD8 T cells.

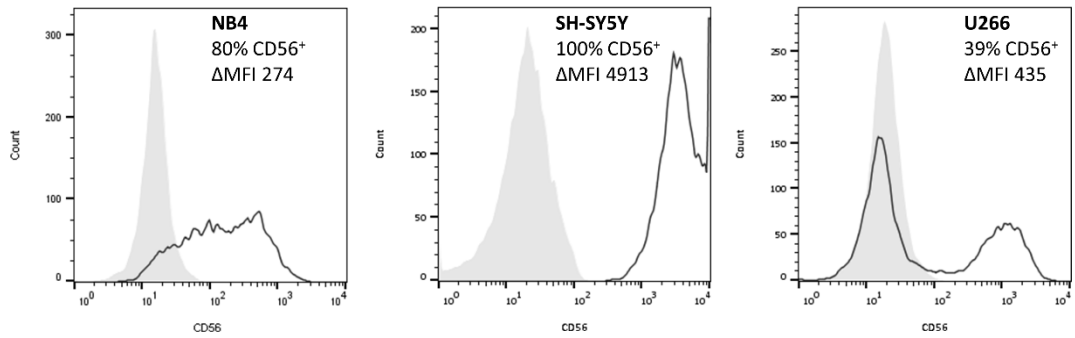
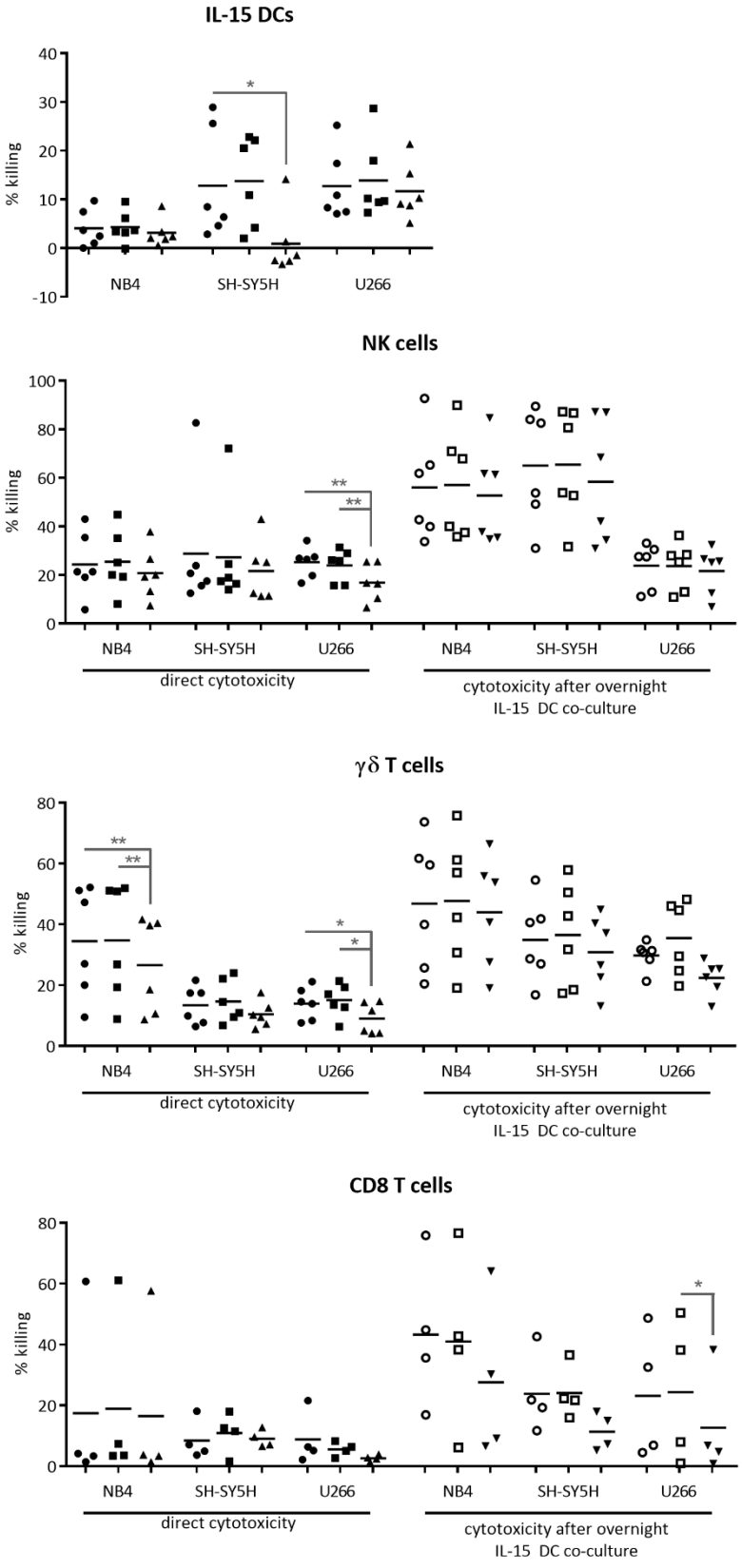


Figure 2. CD56 surface expression on human tumor cell lines. Flow cytometric analysis of tumor cells labelled with CD56-PE (BD, black line) or corresponding isotype control (filled grey), represented as histogram overlays.


IL-15 DCs
% killing
40
30
20
10
0
-10
*
NB4
SH-SY5H
U266
NK cells
% killing
100
80
60
40
20
0
**
**
NB4
SH-SY5H
U266
direct cytotoxicity
NB4
SH-SY5H
U266
cytotoxicity after overnight
IL-15 DC co-culture
γδ T cells
% killing
80
60
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NB4
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direct cytotoxicity
NB4
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cytotoxicity after overnight
IL-15 DC co-culture
CD8 T cells
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NB4
SH-SY5H
U266
direct cytotoxicity
NB4
SH-SY5H
U266
cytotoxicity after overnight
IL-15 DC co-culture

Figure 3. Involvement of CD56 in immune effector cell activation and tumor cell killing. Immune cell cytotoxicity was defined against the cell lines NB4, SH-SY5Y and U266, unstimulated and after overnight culture with IL-15 DCs. Immune cells were cultured in medium without neutralizing mAbs (circles), or in medium containing either CD56 neutralizing GPR165 mAbs (triangles) or its corresponding isotype control (squares) (n = 4-6). One-Way ANOVA with Bonferroni's Multiple Comparison test or Friedman test with Dunn's Multiple Comparison Test. **, $p < 0.01$; *, $p < 0.5$

3.3. PROXIMITY LIGATION ASSAY REVEALS HOMOPHILIC CD56 BINDING BETWEEN CD56-EXPRESSING IMMUNE AND TUMOR CELLS

Subsequently, we sought to identify whether a homophilic CD56-CD56 binding is formed between $CD56^+$ cells. To visualize this protein interaction, we performed a proximity ligation assay (PLA) analysis. From all the cultures tested, it was apparent that neuroblastoma cells, namely SH-SY5Y-eGFP cells, strongly interact with one another via CD56 homodimerization, not least in areas of cell confluency (Figure 4). $CD56^+$ immune cells (IL-15 DCs, NK cells and γδ T cells) were captured too interacting with $CD56^+$ tumor cells by means of a CD56 homophilic binding, whereby an accumulation of red signal (CD56-CD56 interaction) was observed (Figure 5). The interaction rate was lower as between neuroblastoma cells, likely due to $CD56^+$ immune cells' lower MFI of CD56 expression and their non-adherent nature.

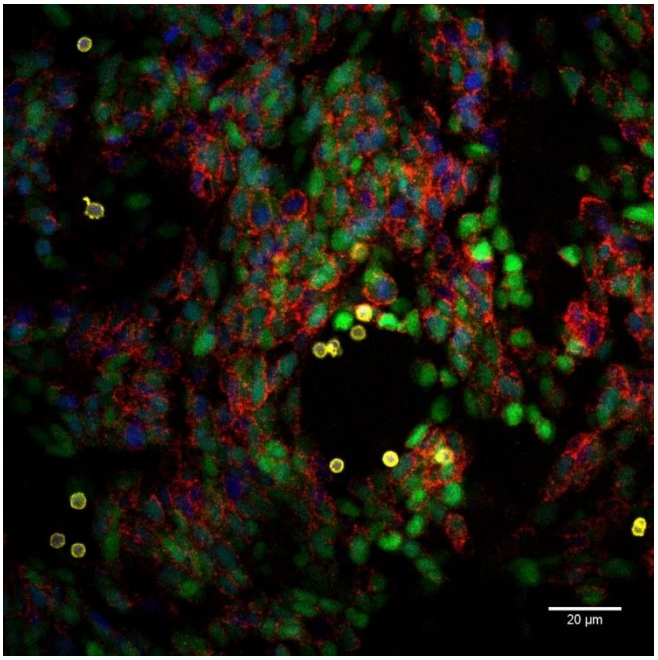
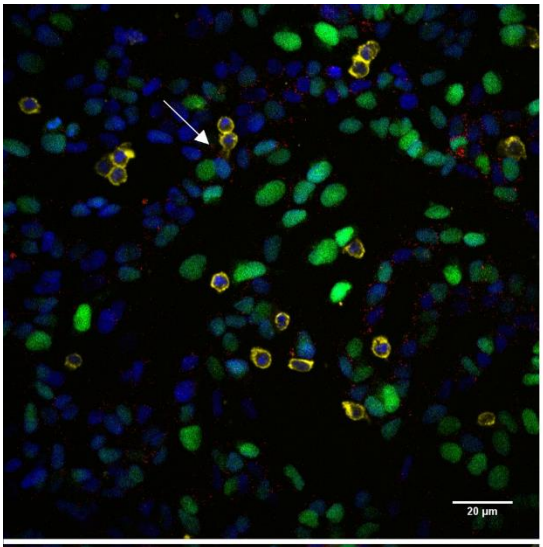
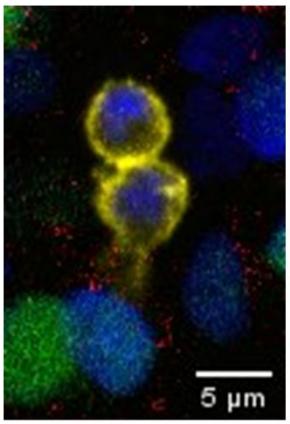
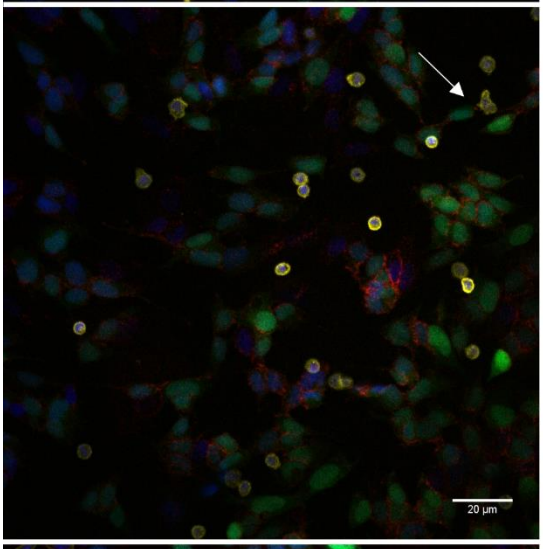


Figure 4. Image of a Duolink *in situ* proximity ligation assay (PLA) showing CD56 homodimer formation between tumor cells. Representative example of a SH-SY5Y-eGFP (green) culture [with NK cells (CD45; yellow)], mounted with DAPI (blue), whereby the CD56-CD56 interaction has been visualized as red dots.

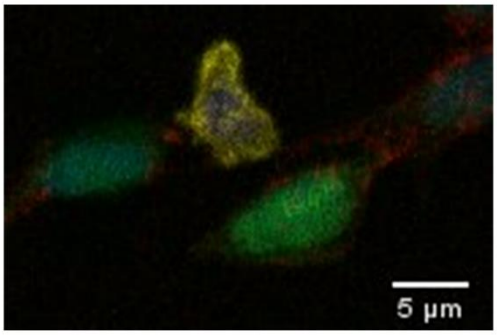


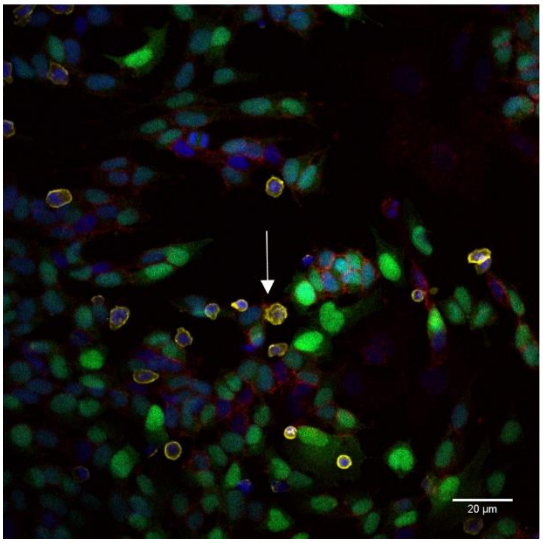
IL-15 DCs





NK cells





γδ T cells

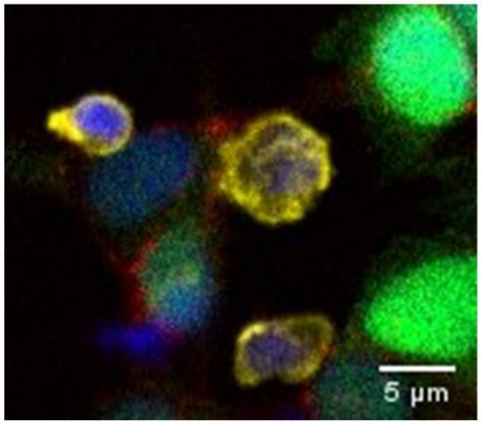


Figure 5. Association of CD56-CD56 interaction and the "kiss of death" – cytotoxic immunological synapse between immune cells and cancer cells. SH-SY5Y-eGFP cells (green) were co-cultured with immune effector cells (CD45; yellow), namely IL-15 DCs (upper row), NK cells (middle row) or γδ T cells (bottom row), and mounted with DAPI (blue). White arrows on the left overview images indicate a probable interface between immune cells and cancer cells – enlarged in the right column. The connection between both cell types is associated with an accumulation of red signal, being CD56 homodimerization. Scale bar; left column = 20 µm, right column = 5 µm

3.4. IL-15 STIMULATION DIRECTLY LEADS TO CD56 EXPRESSION

From our previously published work,[7,9,10] it became apparent that exposure to IL-15 might be linked to increased CD56 expression. In preliminary titration experiments, we established the optimum concentration of IL-15 to be 10 ng/mL for promoting CD56 expression by immune cells. Subsequently, the kinetics of the CD56 expression on the immune cell surface was assessed at different time points (Figure 6). Concerning NK cells, for which CD56 is a prototypic cell surface marker, we detected a steady increase over time in the number of CD56 molecules on the $CD56^{dim}$ NK cell surface following IL-15 stimulation (Figure 6B). $CD56^{bright}$ NK cells, on the other hand, initially seemed to downregulate their CD56 expression overnight, followed by an upregulation definitely visible after 48 hours of IL-15 stimulation (Figure 6B). After this time point, a clear distinction between the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets was no longer discernible (data not shown). Correspondingly, we also detected a clear rise in the percentage of CD56-expressing γδ T cells after an initial lag period of 24 hours after the addition of IL-15, and a concomitant increase in its density on the cell membrane (Figure 6C-D). This expansion of $CD56^{+}$ γδ T cells continued until at least one week post-stimulation. Concerning CD8 T cells, a more delayed yet rapid induction and upregulation in CD56 expression was evident after 72 hours of stimulation (Figure 6C-D). CD4 T cells, however, were found to be negative for CD56 and remained CD56-negative after IL-15 stimulation. A portion of $CD1c^{+}$ myeloid DC weakly expressed CD56 at baseline and there was only a limited, if any, effect of IL-15 after 48 hours of culture with IL-15. On the other hand, $CD14^{+}$ monocytes rapidly induced and/or upregulated their CD56 levels. However, $CD14^{+}$ cells were no longer detectable in PBMC exceeding 72 hours of culture with IL-15. Additionally, complementary analysis of cytotoxicity-related markers (perforin, granzyme B, TRAIL, FASL, NKG2D) as well as of some activation markers (CD69, HLA-DR) was performed (data not shown). However, no direct relationships between these markers and CD56 (following IL-15-mediated stimulation) could be drawn, except for a significant difference between granzyme B-positive cells amid the $CD56^{+}$ CD8 T cells (75.91 ± 7.81 %) as compared to $CD56^{-}$ CD8 T cells (14.33 ± 3.51 %) and $CD69^{+}$ cells amongst $CD56^{+}$ monocytes (10.59 ± 2.58 %) and $CD56^{-}$ monocytes (2.90 ± 0.85 %).

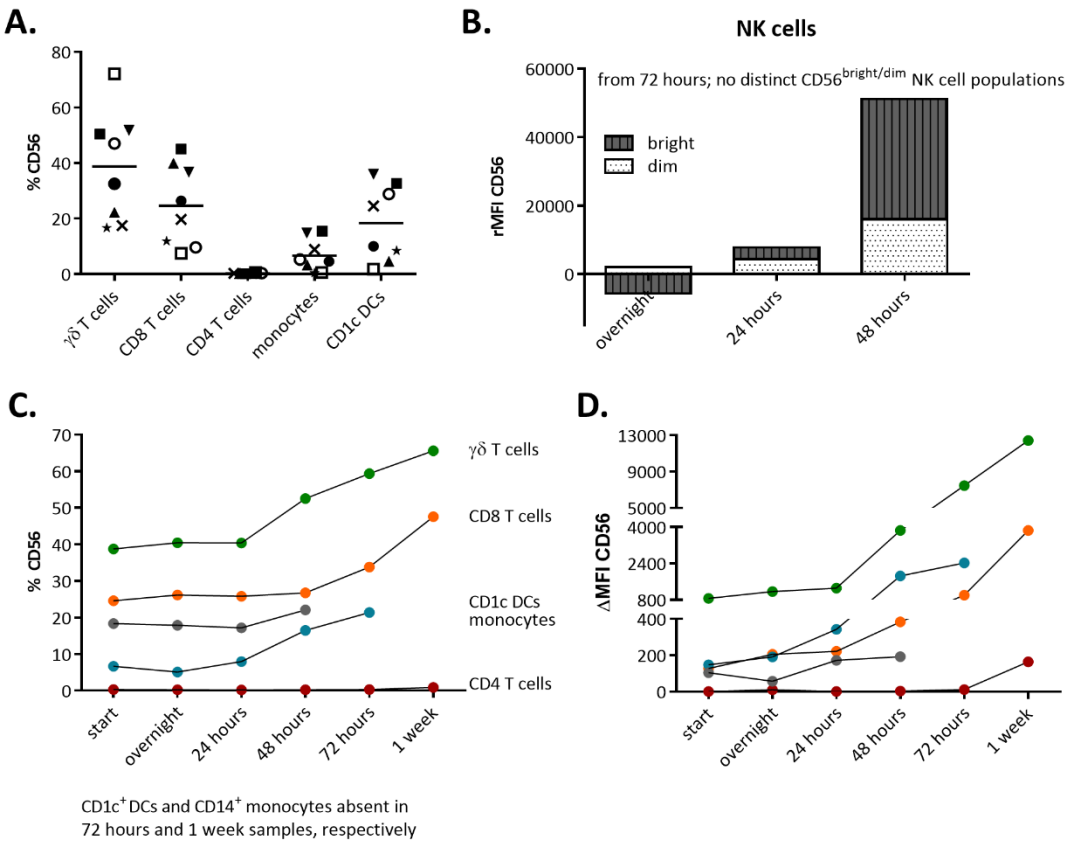


Figure 6. CD56 expression and kinetics following IL-15 stimulation. A. CD56 surface expression on different immune cell subsets in fresh PBMC as determined by flow cytometry. Donors are represented by unique symbols (n = 8). **B-D.** PBMC (1x10e^6 cells/mL) were stimulated with 10 ng/mL recombinant IL-15. At different time points the expression of CD56 was assessed flow cytometrically. **B**. CD56 expression levels in MFI, of CD56^{bright} and CD56^{dim} NK cells, were transformed to relative levels by the subtraction of the MFI of the fresh subset. n = 8 **C-D.** Monitoring of CD56 expression on the cell membrane of γδ T cells (green), CD8 T cells (orange), CD1c DCs (grey), monocytes (blue) and CD4 T cells (red), both in % (**C**) and ΔMFI (MFI condition – MFI isotype control; **D**). The evolution in CD56 expression is shown as a mean of 5 independent donors.

3.5. BLOCKING OF THE DIFFERENT IL-15 SIGNALING PATHWAYS

To assess the IL-15 signaling pathway(s) involved in the induction and/or upregulation of CD56 on immune cells, the selective inhibitors CAS 285986-31-4 (JAK/STAT pathway), Trametinib (MAPK pathway) and Afuresertib (PI3K pathway) were used (Figure 7).

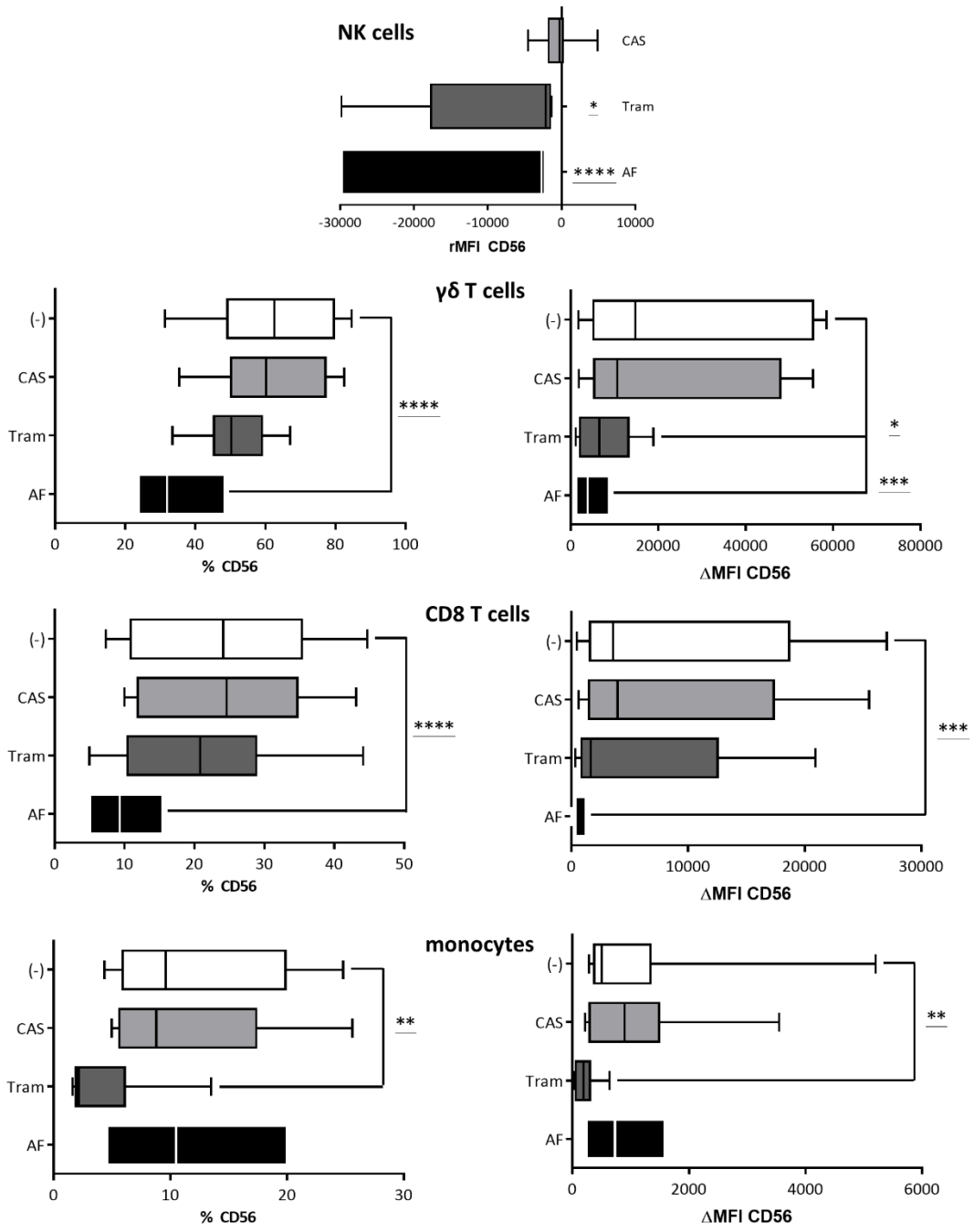


Figure 7. The effect of the selective inhibition of the IL-15 signaling pathways on CD56 expression. PBMC were cultured in IL-15 (10 ng/mL) containing medium (-) in the presence of CAS 285986-31-4 (1 µM; CAS), Trametinib (1µM; Tram) or Afuresertib (2 µM; AF) for 48 hours (NK cells, monocytes) or 1 week (γδ T cells, CD8 T cells). The percentage CD56-postive cells are shown, as determined by flow cytometry, as well as the ΔMFI of CD56 calculated by subtracting the 'background' MFI of the isotype control from the MFI of the sample.

Concerning NK cells, the MFI of the IL-15 medium (-) control was subtracted from the MFI of the sample (rMFI). Data rare represented as box and whiskers (10-90%) of 9 independent donors. One-Way ANOVA with Bonferroni's Multiple Comparison test or Friedman test with Dunn's Multiple Comparison Test. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.5$

Afuresertib drastically inhibited IL-15-mediated CD56 upregulation in NK cells, γδ T cells and CD8 T cells. Therefore, the PI3K pathway is likely to be involved in the IL-15 mediated upregulation of CD56 expression on effector lymphocytes. Additionally, albeit to a lesser extent, Trametinib significantly reduced the level of CD56 expression in NK cells and γδ T cells after IL-15 exposure (Figure 7), suggesting that also the MAPK pathway of IL-15 signaling might play a role in the upregulation of CD56 expression in NK and γδ T cells. In contrast, only Trametinib was able to inhibit IL-15-mediated upregulation of CD56 expression on the membrane of monocytes, suggesting distinct IL-15 signaling pathways are involved in the *de novo* and upregulated CD56 expression in different immune cell lineages.

3.6. IL-15 SHARES ITS CD56-BOOSTING EFFECT WITH IL-2

Given the fact that IL-15 is a cytokine of the common gamma-chain receptor family, the question arose whether the upregulation of CD56 by IL-15 was shared by other members of this family. IL-2 (200 IU/mL) and IL-21 (20 ng/mL) were tested, as these cytokines are known to bear the highest potential for the generation of anti-cancer immune effector cells.[21] From our results, it is evident that IL-2, but not IL-21, shares the CD56-enhancing feature of IL-15, although to a lesser extent (Figure 8). Next, we assessed the contribution of the different downstream cytokine signaling pathways of IL-2 on the expression of CD56 (Figure 9). Like for IL-15-mediated CD56 induction on monocytes, primarily the MAPK pathway is involved in the IL-2 induced CD56 expression. Thereto, as compared to IL-15, this pathway is also more involved in the upregulation of CD56 by IL-2 on lymphocytes. For CD8 T cells for example, the MAPK pathway and PI3K pathway are equally important and regarding NK cells, the MAPK pathway even gets the better of the PI3K pathway. Concerning γδ T cells, no effect is actually seen of the addition of Afuresertib, whereas it is manifest with the addition of Trametinib. Therefore, despite the strong similarities between IL-2 and IL-15, (important) differences are seen between their signaling and effects on immune cells.

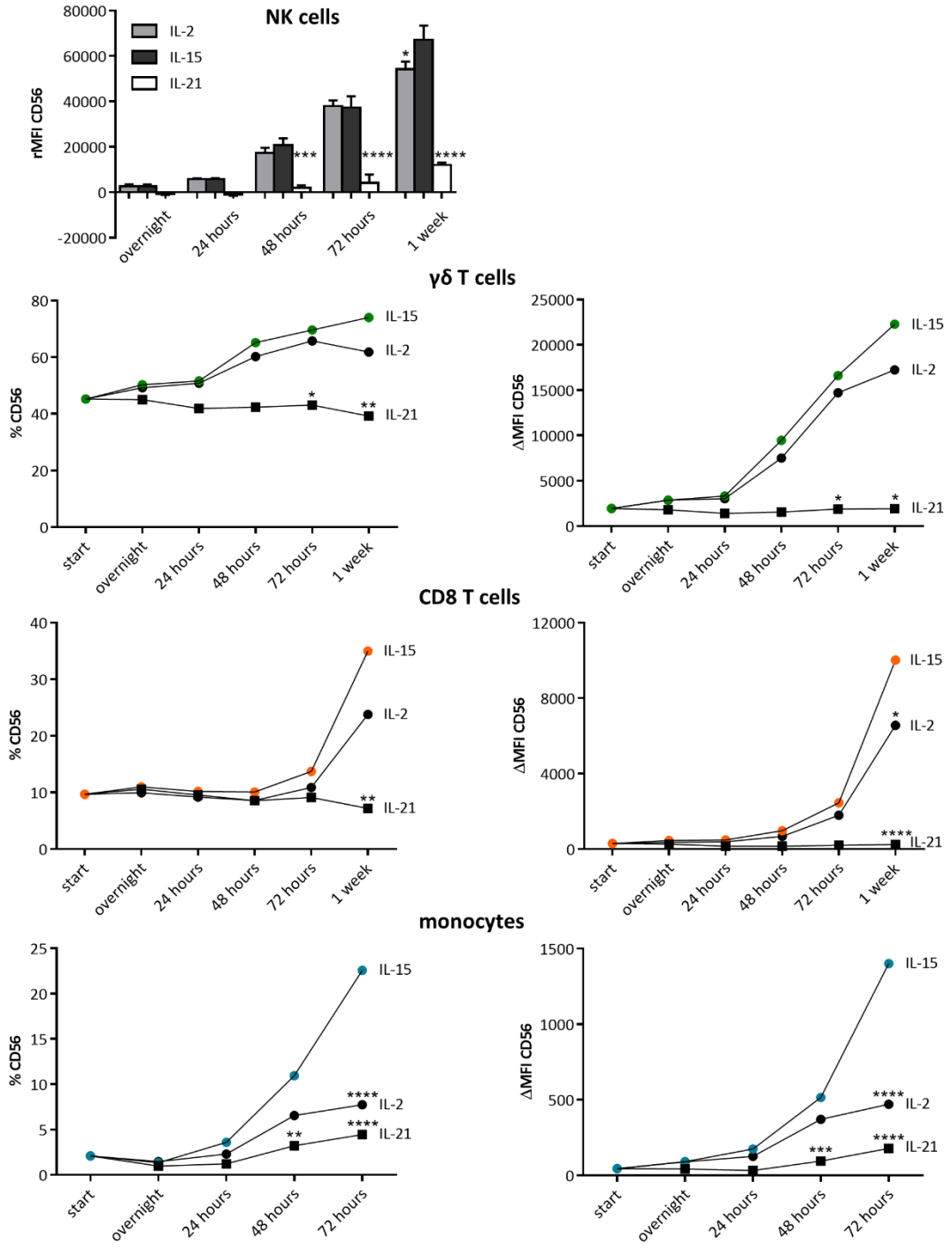


Figure 8. Kinetics of CD56 expression following IL-2, IL-15 and IL-21 stimulation. PBMC were stimulated with IL-2 (200 IU/mL), IL-15 (10 ng/mL) or IL-21 (20 ng/mL) for different periods of time (n = 3). Statistical significance is shown as compared to IL-15 stimulated immune cells. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.5$

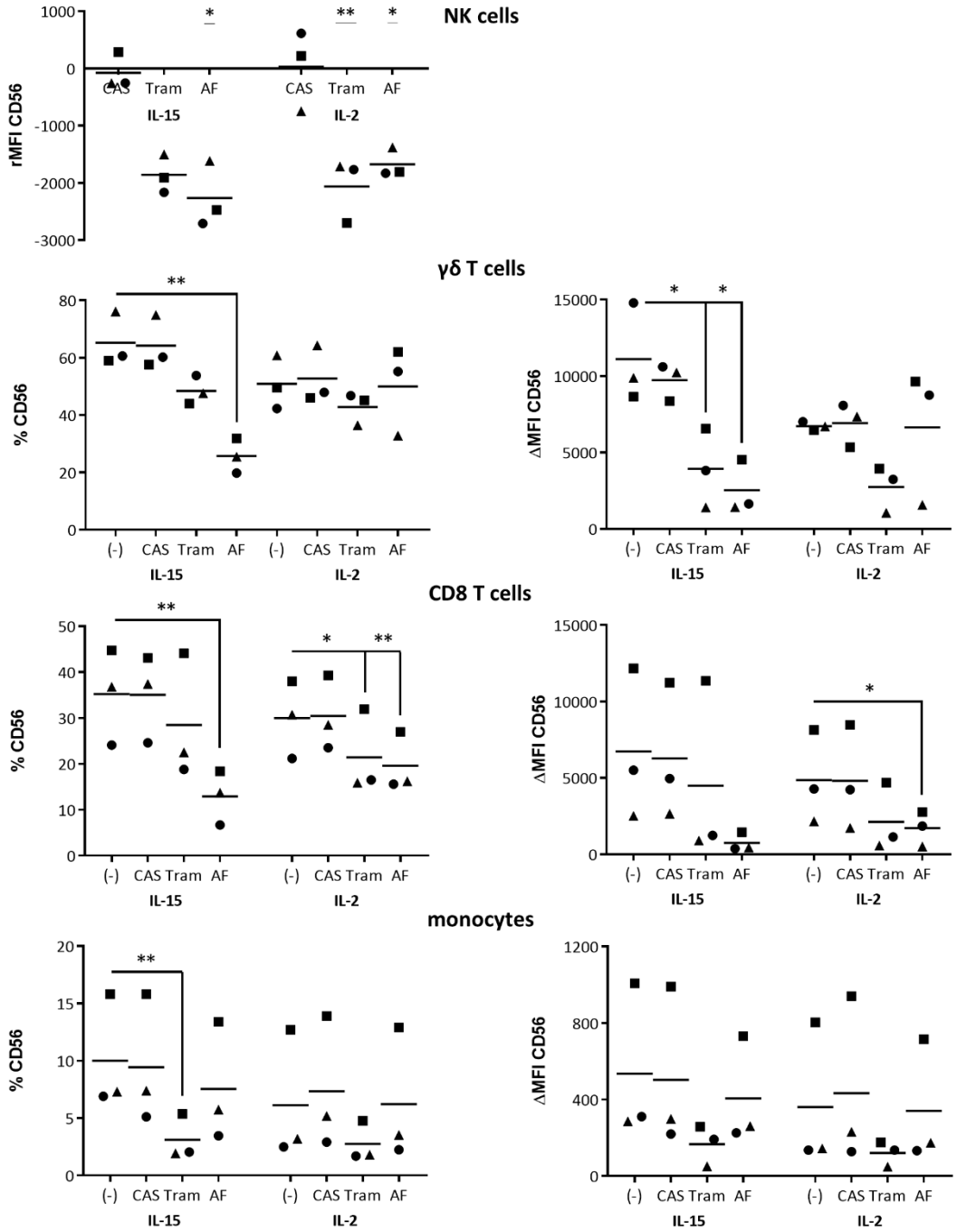


Figure 9. Comparison between blocking the IL-2 and IL-15 signaling pathways. By analogy with Figure 6, the different signaling pathways of IL-2 and IL-15 were blocked separately and assessed for their involvement in the CD56 upregulation on immune cells by their respective cytokine. Abbreviations: CAS, CAS 285986-31-4; Tram, Trametinib; AF, Afuresertib. One-Way ANOVA with Bonferroni's Multiple Comparison test or Friedman test with Dunn's Multiple Comparison Test. **, $p < 0.01$; *, $p < 0.5$

4. DISCUSSION

New determinants of anti-cancer immune effector cells are warranted, enabling tumor immunologists to select and expand those cells with superior tumoricidal capacity. We have here demonstrated that CD56 expression on immune cells seems to be a common denominator of potent immune effector cells, endowed with cytotoxic activity against cancer cells. Moreover, CD56 itself contributes to the killing capacity of immune cells, including through the formation of homodimers. In order to induce and/or boost the expression of CD56, IL-15 stimulation can be employed, pointing to the importance of this cytokine in cellular therapy manufacturing processes.

CD56 is a known cell adhesion molecule in the neuronal system, of which the three major isoforms are called NCAM-120, NCAM-140, and NCAM-180. While the 140 and 180 kD isoforms are predominantly present in the central nervous system, extraneural human tissues mainly express NCAM-120 and NCAM-140. Although some early reports of Lanier *et al.* identified NCAM-140 to be the principal isoform expressed by NK cells,[22,23] we predominantly found the 120 kD isoform to be expressed by lymphocytes (120 kD > 140 kD > 180 kD). The difference between those findings could be attributed to the use of a human peripheral blood NK cell clone by Lanier *et al.* and freshly sorted immune cells in our case. This underscores the hypothesis that the expression of the different isoforms is dependent on the cellular state and environment. In line with this, and as opposed to fresh immune cells, CIK cells were shown to express the 140 kD isoform as well.[19] Other examples include the shift observed in neoplasia from NCAM-120 to NCAM-140 (and -180)[24,25] and the exclusive upregulation of NCAM-140 on cardiomyocytes in the context of ischemic cardiomyopathy.[26]

Accordingly, functional differences can be expected from the expression of different CD56 isoforms. Whereas the transmembrane proteins NCAM-140 and NCAM-180 are known for their binding of various signaling proteins, activating a range of intracellular pathways, the GPI-anchored NCAM-120 preferentially associates with lipid rafts. As such, NCAM-120 is characterized by a high motility and a dual role as a cell-surface molecule and extracellular protein after secretion. The physiological role of shedded CD56, as well as its pros and cons in the context of immunotherapy, remain to be discovered.[27] However, the higher motility of NCAM-120, could be biologically important for immune cells, facilitating cellular communication and a rapid response to external stimuli.[28]

Looking further into the role of CD56 in immune effector functions, we were able to establish a direct involvement of CD56 in the cytotoxic capacity of IL-15 DCs, NK cells and γδ T cells against $CD56^+$ tumor cells. Our data corroborate earlier findings of a direct role of CD56 in CIK-mediated cytotoxicity against $CD56^+$ hematopoietic tumor cell targets.[19] In addition, from the anti-CD56 blocking experiments shown in Figure 3, the enhanced cytotoxic capacity of CD8 T cells after co-culture with IL-15 DCs seems to be, at least in part, regulated by CD56 engagement. This suggests a potential crosstalk mechanism, where CD56 could function as a co-stimulatory molecule between IL-15 DCs and CD8 T cells. The specific mode of action is, however, still unknown. Indeed, besides facilitating and strengthening cell-to-cell contact, adhesion molecules like CD56 could also play a role in transmitting molecular signals across the cell membrane, regulating in turn a pleiotropy of cellular functions. Other indications of a functional role of CD56 in the immune system include; the CD56-mediated inhibition of tumor cell growth by IL-2 activated NK cells,[29] the induction of NK cell maturation by CD56 forming a developmental synapse[30] and the role of CD56 as a pathogen recognition receptor.[20]

Of note, although innate/unlicensed responses are generally not attributed to CD8 T cells, under certain conditions CD8 T cells can acquire these characteristics.[31-33] By way of example, innate CD8 T cell activation is associated with increased immunopathology in Leishmania infection[34] and more related to our current research, Sosinowski *et al.* demonstrated the generation of functional antigen-inexperienced T cells through IL-15 trans-presentation by DCs.[35] Interestingly, pro-inflammatory cytokine receptor signals have further been shown to use mechanisms exclusive to the TCR/CD3 signalosome to mediate bystander effector/memory CD8 T cell responses.[36]

These findings raise intriguing questions regarding the nature and extent of the role of CD56 in cancer and immune cell functioning. As shown here, CD56 homodimerization is an important interaction between neuroblastoma cells. It is therefore likely that such connections exist between more types of CD56-expressing malignancies, including ovarian carcinomas,[34] renal neoplasms[35] and small cell lung cancer.[36] Conceivably, CD56 homodimers might be involved in the adhesion between cancer cells, and implicated in the communication and organization within the tumor micro-environment.[37] The latter could explain why CD56 expression is associated with advanced stages or dismal prognosis in different cancers.[38-40] On the other hand, we showed a role for CD56 in effector cell cytotoxicity and were able to provide evidence for interaction between immune cells and cancer cells via CD56-CD56 adhesion. This could account for the reports indicating a positive effect of the presence of CD56 on survival.[4, 41] Altogether, CD56 expression seems to be confined to either potent immune effector cells or to malignant cancer cells, resulting

in a difficult balance to manipulate in view of cancer treatment. Therapeutically eradicating $CD56^+$ cancer cells could therefore imply incapacitating $CD56^+$ cytotoxic effector cells by obstructing their connection with malignant cells.

Finally, the question arose as to how CD56 expression is regulated and induced. There is a body of evidence showing a direct or indirect role of IL-15 signaling for upregulation of CD56: *(1)* culturing NK cells with IL-15 leads to an upregulation of CD56 on both $CD56^{bright}$ and $CD56^{dim}$ NK cells,[42] *(2)* stimulation of isolated γδ T cells with isopentenyl pyrophosphate and IL-15 significantly upregulates CD56 levels,[10] *(3)* purified $CD8^+CD56^-$ T cells express *de novo* CD56 after 12 days of culture with IL-15,[14] *(4)* umbilical cord blood T cells acquire CD56 after culture in IL-15,[43] and *(5)* monocyte-derived DCs which are differentiated with IL-15 also have a CD56-expressing subset.[7] In this study we provide evidence for a direct upregulation of CD56 by IL-15, potentially through the recruitment of shc, binding to a phosphotyrosine residue on the IL-2/15Rβ chain, activating the MAPK and/or the PI3K-Akt pathway.[15] This could explain why IL-2 stimulation also leads, although at a lower level, to CD56 expression and why no such effect is observed with IL-21. Namely, the IL-2 and IL-15 receptor not only share the common gamma-chain, but also the IL-2/15Rβ chain, contrary to for example IL-21.[16] From this it can be deduced that IL-15 can induce CD56 expression on immune cells bearing the IL-2/IL-15Rβ subunit. However, it is unlikely that IL-15 (and IL-2) are the sole triggers for CD56 upregulation,[44] but nonetheless are an attractive and straightforward approach to induce immune cell activation and CD56 expression.

In conclusion, this study has identified a variable expression profile of CD56 on fresh and cultured human lymphocytes and monocytes, whereby the NCAM-120 isoform is more commonly expressed than previously presumed. Secondly, the presence of CD56 on the surface of effector immune cells is more than a mere activation marker, being directly linked to their cytotoxic capacity and immunostimulatory effect. Furthermore, we described and visualized a CD56 homophilic interaction between cancer cells among themselves as well as between immune cells and malignant cells. Finally, in order to induce and upregulate CD56 expression on immune cells, we discovered the potency of the pleiotropic cytokine IL-15, which occurs mainly through the PI3K pathway.

5. REFERENCES

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ADDENDUM:

BISPHOSPHONATES FOR CANCER TREATMENT: MECHANISMS OF ACTION AND LESSONS FROM CLINICAL TRIALS

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ABSTRACT

A growing body of evidence points towards an important anti-cancer effect of bisphosphonates, a group of inexpensive, safe, potent and long-term stable pharmacologicals that are widely used as osteoporosis drugs. To date, they are already used in the prevention of complications of bone metastases. Because the bisphosphonates can also reduce mortality in among other MM, breast and prostate cancer patients, they are now thoroughly studied in oncology. Especially the more potent nitrogen-containing bisphosphonates have the potential to improve prognosis. The first part of this review will elaborate on the direct and indirect anti-tumoral effects of bisphosphonates, including induction of tumor cell apoptosis, inhibition of tumor cell adhesion and invasion, anti-angiogenesis, synergism with anti-neoplastic drugs, and enhancement of immune surveillance (e.g. through activation of γδ T cells and targeting macrophages). In the second part we shed light on the current clinical position of bisphosphonates in the treatment of hematological and solid malignancies, as well as on ongoing and completed clinical trials investigating the therapeutic effect of bisphosphonates in cancer. Based on these recent data, the role of bisphosphonates is expected to further expand in the near future outside the field of osteoporosis and to open up new avenues in the treatment of malignancies.

1. INTRODUCTION

Bisphosphonates (also called diphosphonates) are inhibitors of osteoclastic bone resorption and are therefore used in the treatment of a variety of skeletal disorders such as Paget's disease of the bone, osteoporosis (including postmenopausal and glucocorticoid-induced forms) and heritable skeletal disorders in children (for example osteogenesis imperfecta). Additionally, bisphosphonates are used in the management of (tumor-induced) hypercalcemia and to reduce skeletal-related events resulting from bone involvement in hematological or solid malignancies. Since the first clinical trial to assess the effectiveness of the bisphosphonate clodronate in suppressing the excessive mobilization of skeletal calcium in patients with MM[1], various clinical trials have followed across a range of malignancies. In the first part of this review, we will discuss the rationale to use bisphosphonates in cancer patients. In the second part, we will review the current clinical position of bisphosphonates in the treatment of hematological and solid malignancies.

2. MECHANISMS OF ACTION

Structurally, the bisphosphonates are synthetic analogues of the naturally occurring pyrophosphates of the bone matrix and are consequently incorporated into active bone remodeling sites. In this manner they suppress bone turnover by inhibiting bone mineral breakdown. The bisphosphonates can be divided into 2 subclasses based on their structure and molecular mechanism of action: *(I)* the simple and *(II)* the nitrogen-containing bisphosphonates.[2] The first group consists of the following clinically available molecules: clodronate, etidronate and tiludronate. Alendronate, ibandronate, pamidronate, risedronate and zoledronate are examples of bisphosphonates that belong to the second category (Figure 1). Two routes of administration are used for treatment with bisphosphonates, intravenously (i.v.) and *per os*. Under ideal conditions, less than 1% of an orally administered dose is absorbed, due to the negative charge hampering transport across the lipophilic cell membrane. Bisphosphonates exhibit a short plasma half-life, are not metabolized and are excreted unchanged by the kidneys. Roughly 50% of the absorbed dose will bind to bone, mostly avidly at sites of active remodeling.[3] For example, when the standard clinical dose of 4 mg zoledronate i.v. is being administered, a plasma half-life of 105 minutes and a peak plasma concentration (CMax) of 1-2 µM are recorded.[4]

2.1. EFFECT ON BONE DENSITY

The primary pharmacological action of the bisphosphonates involves the inhibition of osteoclastic bone resorption. The simple bisphosphonates are metabolized by osteoclasts into methylene-containing analogues of adenosine triphosphate (ATP), resulting in a deficiency of usable ATP and induction of osteoclast apoptosis.[5] The nitrogen-containing bisphosphonates, which are more potent inhibitors of bone resorption, act primarily by inhibiting FPP synthase, a key enzyme of the mevalonate pathway. This results in a depletion of the isoprenoid lipid FPP and of geranylgeranyl diphosphate in the osteoclast, which, in turn, causes osteoclast dysfunction and reduced bone resorption.[5] In the context of cancer, this may indirectly reduce the development and progression of bone metastases as well as the overall skeletal tumor burden.

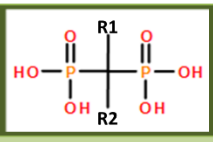
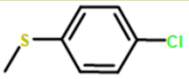
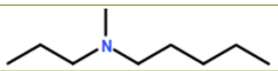
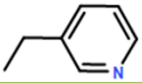
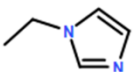
common bisphosphonate skeleton: 		
simple bisphosphonates	R1-group	R2-group
clodronate	-Cl	-Cl
etidronate	$-CH_3$	-OH
tiludronate	-H	
nitrogen-containing bisphosphonates	R1-group	R2-group
alendronate	-OH	$-(CH_2)_3-NH_2$
ibandronate	-OH	
pamidronate	-OH	$-(CH_2)_2-NH_2$
risedronate	-OH	
zoledronate	-OH	

Figure 1. Chemical structure of the bisphosphonates.

2.2. ANTI-CANCER PROPERTIES

In addition to their positive effect on bone density, a growing body of evidence indicates that bisphosphonates exert a range of direct and indirect anti-tumor effects,[6] including inhibition of tumor cell proliferation and induction of tumor cell apoptosis,[7] inhibition of tumor cell adhesion and invasion,[8] anti-angiogenesis,[9] synergism with anti-neoplastic drugs,[10] and enhancement of immune surveillance (e.g. through activation of γδ T cells) (Figure 2).[11,12]

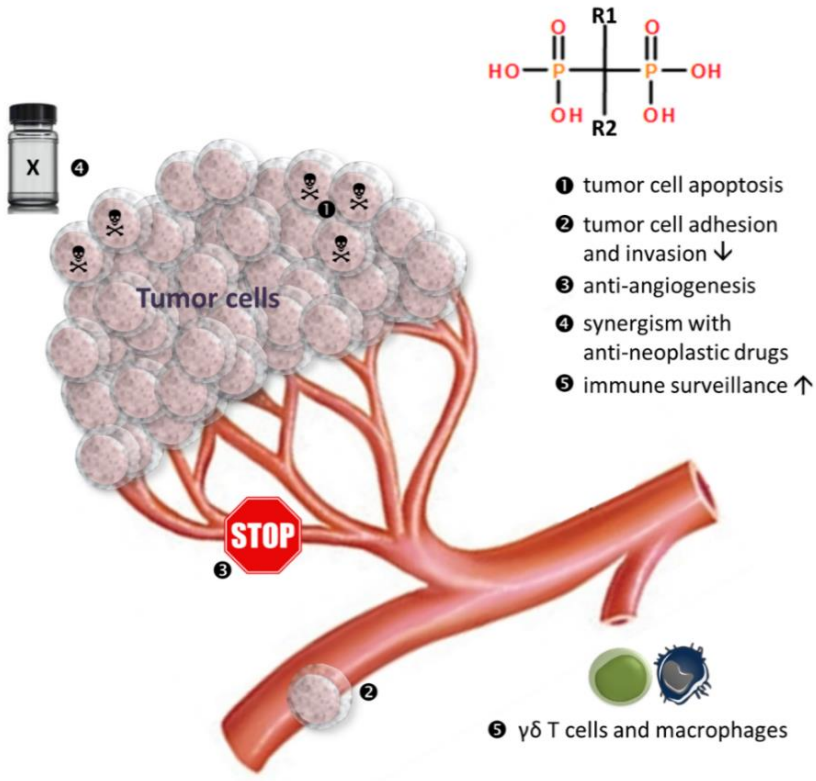


Figure 2. Anti-cancer properties of the bisphosphonates in addition to their effect on bone.

Inhibition of tumor cell proliferation and induction of tumor cell apoptosis – Strikingly, bisphosphonates have a direct anti-cancer effect. There are several *in vitro* studies on nitrogen-containing bisphosphonates inhibiting cellular growth and inducing apoptosis of human MM cell lines and primary cells from patients with MM. The molecular mechanism underlying this direct anti-neoplastic effect also involves inhibition of the mevalonate pathway, and this in a dose-dependent manner.[13-15] These *in vitro* observations have been confirmed in *in vivo* murine models.[16] The induction of apoptosis through the inhibition of FPP synthase is the result of the accumulation of the pathway intermediate, IPP, which in turn becomes conjugated to adenosine monophosphate to form a cytotoxic ATP analog. The latter inhibits mitochondrial adenine nucleotide translocase, leading to apoptosis. Another path leading to apoptosis is the inhibition of protein prenylation as mentioned before.[13] A similar direct anti-tumor effect has moreover been observed in cancer types other than MM. For example, zoledronate had anti-proliferative and anti-apoptotic effects caused by disruption of the prenylation of Ras proteins in a murine model of prostatic neuroendocrine carcinoma.[17] Similar findings were observed in a lung cancer mouse model where a lipophilic bisphosphonate effectively blocked prenylation of KRAS and other small G proteins critical for tumor growth and cell survival.[18] In breast cancer patients,

zoledronate used in monotherapy has been shown to increase the level of tumor cell apoptosis.[19] Furthermore, zoledronate combined with systemic therapy results in an improved clearance of disseminated tumor cells in breast cancer patients, confirming that bisphosphonates exert beneficial effects that reach beyond their well-known effects on bone density.[20-22] In addition to the above mechanisms, it was recently shown that bisphosphonates mediate a direct anti-proliferative effect on tumor cells by inhibiting the human epidermal growth factor receptor (human EGFR or HER) tyrosine kinase.[23] These drugs bind directly to the HER1/2 kinase domain and, by inhibiting downstream signaling, reduce the cell viability in HER-driven lung, breast, and colon cancers.[23] Therewithal, bisphosphonates enhanced the effects of tyrosine kinase inhibitors and overcame erlotinib resistance, offering an option for tyrosine kinase inhibitor-resistant cancers.[24]

Inhibition of tumor cell adhesion and invasion - Tumor cell adhesion and invasion are essential steps in the metastatic process and can be counteracted by low doses of bisphosphonates.[25-27] Changes in fibronectin and actin expression in tumor cells through bisphosphonate exposure might underlie this effect,[25] as could be the inhibition of geranylgeranylation and the inhibition of Ras prenylation.[8,26] At the level of extracellular matrix interacting molecules, zoledronate induces diverse changes in tumor cells, for instance with regard to specific heparan sulfate proteoglycans, integrins, hyaluronan and its receptor CD44.[27] Also, bone marrow stromal cells of MM patients showed a significant reduced expression of the adhesion molecules CD106, CD54, CD49d, and CD40 after exposure to zoledronate.[14]

Anti-angiogenesis - Neo-angiogenesis plays in general a critical role in the growth and spread of tumor cells. Numerous studies on solid tumors demonstrated manifest inhibition of (1) tumor angiogenesis, (2) differentiation and recruitment of endothelial progenitor cells, and (3) myeloid cell differentiation into tumor-associated macrophages by zoledronate.[28-31] Additionally, plasma cells secrete angiogenic factors such as vascular endothelial growth factor (VEGF), making pathologic angiogenesis a hallmark of the bone marrow microenvironment in MM.[32] Zoledronate may counter pathologic angiogenesis by inhibition of VEGF-dependent proliferation, chemotaxis and angiogenesis, and blockade of the VEGF/VEGF receptor 2 autocrine loop, which is under control of the mevalonate pathway.[32] This inhibition of neo-angiogenesis is durable through a long-lasting reduction in serum VEGF levels. Thirty patients with bone metastatic breast cancer were treated with a single infusion of 4 mg of zoledronate prior to start of chemotherapy, which led to a sustained decrease in serum VEGF levels for up to 3 weeks after infusion.[33] Moreover, components of the immune system, more specifically the macrophages, contribute to the generation of cancer-supporting vasculature, plasma cell growth and invasion. Exposure of

macrophages to zoledronate, either used in monotherapy or in combination with bortezomib (synergistic effect), results in the inhibition of nuclear factor kappa beta activity and in phosphoactivation of VEGF receptor 2 and the extracellular signal-regulated kinases 1 and 2, thereby reducing the pro-angiogenic and vasculogenic properties of these macrophages.[34]

Synergism with anti-neoplastic drugs - As introduced in the previous paragraph, combination of a bisphosphonate with other treatments can improve their efficacy, as shown for numerous chemotherapy agents (reviewed elsewhere).[35]

Targeting macrophages – Tumor-associated macrophages (TAMs) are a potential target of the bisphosphonates, which has been excellently reviewed by Rogers and Holen.[12] They display multiple phenotypes that are classified − for simplicity reasons − into two major principal states of activation: the pro-inflammatory (M1, killing/inhibitory capacity) and the anti-inflammatory (M2, heal/growth promoting) subtype.[36,37] It is widely acknowledged that TAMs (generally M2 phenotype) provide an immunosuppressive microenvironment for tumor growth and play a significant role in several stages of tumor progression.[37] With this, consideration should be given to the fact that TAMs are important components of the cancer stem cell niche, sustaining cancer and metastasis.[38,39]

In view of this, bisphosphonates could impair the function of TAMs, inducing macrophage apoptosis and inhibiting among other the release of pro-angiogenic factors, as discussed above.[12] Injection with clodronate-containing liposomes led to a marked reduced tumor growth in a NSG mouse model with xenografted human cutaneous T-cell lymphoma cells as compared to controls. This inhibition in tumor progression was attributed to macrophage depletion, along with a drop in macrophage downstream pro-tumor effects including (lymph)angiogenesis and signal transducer and activator of transcription (STAT)3 activation.[40] The same strategy also dramatically decreased Forkhead box Q1 (master regulator of tumor metastasis)-induced hepatocellular carcinoma spreading in a mouse model.[41] Positive effects of clodronate-induced macrophage apoptosis on tumor progression has also been described in various other preclinical studies as well,[42-44] making it an interesting path to ascertain in clinical trials. Moreover, macrophage depletion has been described to improve efficacy of radiotherapy, since induction of VEGF by TAMs was shown to mediate rapid tumor regrowth following irradiation.[45] Liposomes are readily taken up by macrophages, but as recently reported, nitrogen-containing bisphosphonates are rapidly and efficiently internalized by tumor-associated macrophages as well.[46] The latter is associated with their capacity to engulf calcium-containing complexes (including microcalcifications). The benefits of macrophage apoptosis is therefore not only seen with

clodronate, but also with other bisphosphonates, of which zoledronate has been shown to be the most active.[47,48] Lastly, evidence indicates that bisphosphonates are also able to reverse polarity of macrophages into a tumoricidal phenotype (M1 phenotype).[12,49] Notwithstanding a clear body of preclinical data indicating an indirect anti-tumor effect of the bisphosphonates through interaction with macrophages, clinical data, with the exception of data on VEGF levels (*vide supra*), are lacking.

Immune surveillance by γδ T cells - Bisphosphonates are also able to activate γδ T cells. Human γδ T cells are a group of unconventional T cells that can be subdivided based on their δ T-cell receptor chain into Vδ1 and Vδ2 T cells.[50] The most abundant subset of circulating γδ T cells, Vγ9Vδ2 T cells, is mainly activated by phosphoantigens, including IPP.[51] IPP concentrations in healthy somatic mammalian cells are too low for γδ T cell activation. However, many types of tumor cells have a deregulated mevalonate pathway, leading to accumulation of IPP and subsequent γδ T cell activation.[52] Inhibiting FPP synthase by bisphosphonates results in IPP accumulation as well, as mentioned before.[51] Subsequently, γδ T cells are able to exert their killing capacity and secrete pro-inflammatory cytokines.[50] In this regard, γδ T cells have been shown to exert natural cytotoxicity towards myeloma cell lines and primary myeloma cells,[53,54] via a mechanism that involves IPP.[55] Vγ9Vδ2 T cells activated *ex vivo* with zoledronate have been used in myeloma patients and have led to objective anti-tumor responses.[56] A relation between the efficacy of zoledronate and Vγ9Vδ2 T cells is currently being investigated in other cancer types, including breast cancer,[57] hormone-refractory prostate cancer[58] and advanced malignancies, namely renal cell carcinoma, malignant melanoma and AML.[59] The combination of zoledronate with low-dose IL-2 has been shown to result in Vγ9Vδ2 T cell activation in patients with terminally advanced metastatic breast cancer. This was evidenced by an up-regulation of the activation markers CD69 and HLA-DR with a peak in expression three months after treatment. Moreover, Vγ9Vδ2 T cells were converted to an effector memory state.[60] Similar observations were made in hormone-refractory prostate cancer patients[58] and in patients with other advanced malignancies (renal cell carcinoma, malignant melanoma, and AML), concomitant with improved γδ T cell effector functions (in terms of enhanced IFN-γ serum levels).[59] Here, the clinical benefit rate was 40%, whereby most benefits were observed in the AML patient cohort.[59] However it should be noted that the phenotype and functional properties of the Vγ9Vδ2 T cells did not predict patient outcome. Nevertheless, the latter could be forecasted by their total number. In the above-mentioned breast cancer patient trial, which included a total of 10 patients, three out of four patients surviving more than twelve months exhibited strikingly robust Vγ9Vδ2 T cell numbers. The other patients died sooner after precipitously progressive declines in the total number of Vγ9Vδ2 T cells. This study, albeit small, strongly emphasizes a correlation of peripheral blood Vγ9Vδ2 T cell numbers and status with arrested disease

progression.[60] Of eighteen patients with prostate cancer with an expected life span of ±3 months treated with zoledronate and IL-2, 67% achieved an objective tumor response. The response at 12 months showed a correlation with the absolute total γδ T cell number.[58] Moreover, TNF-related apoptosis-inducing ligand production by zoledronate/IL-2-activated γδ T cells was significantly enhanced and was related to patient survival.[58]

In conclusion, γδ T cells are an important immune component on which in any event a part of the anti-cancer effect of the bisphosphonates is based. The activation of γδ T cells by the bisphosphonates occurs by accumulation of the phosphoantigen IPP, resulting in the up-regulation of activation markers and the manifestation of γδ T cell cytotoxicity towards tumor cells and the secretion of pro-inflammatory cytokines. Clinical trial data suggest that the total number of γδ T cells following zoledronate treatment predicts patient outcome.

2.3. COMMON SIDE EFFECTS OF BISPHOSPHONATE TREATMENT

Finally, common side effects of treatment with bisphosphonates should be mentioned. No adverse effect of the bisphosphonates has received greater attention than osteonecrosis of the jaw, primarily associated with the high doses used in cancer patients (extensively reviewed elsewhere).[61-63] It is a condition characterized by necrosis of the maxillofacial skeleton, in particular the tooth-bearing areas, which become exposed to the oral cavity. Despite the still existing controversy on the underlying pathophysiology, damage to the bone through tooth extraction, dental surgery, or other traumas are believed to be main risk factors. Other reported side effects include hypocalcemia,[64] the development of acute phase reaction after i.v. administration of bisphosphonates, possibly associated with γδ T cell activation,[65] and some renal complications which are predictable and reversible. Yet, bisphosphonates should be delivered with caution to patients with impaired renal function,[66] and are not recommended for patients with creatinine clearance below 30 (risedronate, ibandronate) to 35 (alendronate, zoledronate) mL/min.[67] Despite the theoretical concern that long-term treatment with bisphosphonates may cause over-suppression of bone turnover ("frozen bone") and increased skeletal fragility, fragility fractures or atypical fractures are rare side effects.[68-70] To conclude, bisphosphonate treatment is in general well tolerated and the benefits of treatment almost always outweigh the risks.[71,72]

3. CURRENT POSITION OF BISPHOSPHONATES IN THE TREATMENT OF CANCER

3.1. HEMATOLOGICAL MALIGNANCIES

3.1.1. Multiple myeloma

Among the hematological malignancies, bisphosphonates have been most extensively studied in patients with MM. The therapeutic role of bisphosphonates in improving bone strength and preventing skeletal-related events of bone involvement in MM is well established. Bisphosphonates are therefore an essential part of the management of MM, especially in patients with bone lesions.[73] The use of bisphosphonates for their anti-cancer properties is a relatively new topic that is gaining increasing attention (Table 1). For example, in a large multi-centric clinical trial involving 1960 newly diagnosed myeloma patients receiving induction chemotherapy, addition of zoledronate (4 mg as an infusion every 3-4 weeks) reduced mortality by 16% and extended median overall survival by 5.5 months as compared to clodronate (1600mg oral daily).[74] This survival benefit was independent of the presence of skeletal-related events, indicating that bisphosphonates have intrinsic anti-myeloma properties.[74] The mutual anti-cancer benefits of zoledronate and its capacity to reduce skeletal-related events also emerges from other clinical trials[75-77] and a recent Cochrane meta-analysis confirmed that treatment with zoledronate results in superior overall survival as compared with placebo.[78] Pamidronate is also approved by the food and drug administration along with zoledronate to reduce skeletal complications of MM. Treatment with either of both drugs is advised for all patients with active MM as primary treatment in combination with other myeloma therapy.[73] However, a recent comparison between adjuvant zoledronate and pamidronate in 1018 newly diagnosed MM patients, shows a significant advantage of zoledronate over pamidronate. Patients receiving zoledronate had a 22% and 25% reduction in risk of death and risk of skeletal-related events compared to pamidronate, respectively.[79] Therefore zoledronate is likely to become the preferred bisphosphonate for most patients with MM.

3.1.2. Other hematological malignancies

The effect of the bisphosphonates on bone density has also been studied in other hematological conditions, e.g. the management of pain and motor function recovery in osteonecrosis as a consequence of acute lymphoblastic leukemia treatment,[80,81] the prophylactic use of zoledronate to prevent osteopenia and osteoporosis in patients with AML undergoing HSCT,[82] and the treatment of low bone mineral density in patients

diagnosed with lymphoma.[83] In addition, Pavlu *et al.* investigated the addition of zoledronate (4-8 mg monthly i.v.) to imatinib in patients with chronic myeloid leukemia with a suboptimal response to imatinib alone.[84] Imatinib is a protein tyrosine kinase inhibitor which powerfully inhibits the Bcr-Abl tyrosine kinase, an oncoprotein activating the ras signaling pathway.[85] The combination of imatinib with a nitrogen-containing bisphosphonate, such as zoledronate, might be synergistic, since inhibition of FPP synthase by zoledronate leads to a reduced availability of FPP and consequently disrupts necessary prenylation steps of the ras signaling pathway.[84] However, the investigators did not find an improved response in patients upon addition of zoledronate to imatinib. A possible explanation for the absence of increased response rates *in vivo* is the relatively low plasma levels of zoledronate achieved in this study.[84] To date, the potential benefits of treatment with bisphosphonates are still being investigated in clinical trials and are therefore not yet included in standard care of patients with other hematological malignancies.

3.2. SOLID MALIGNANCIES

3.2.1. Breast cancer

The use of a bisphosphonate is generally the preferred intervention to improve bone mineral density in breast cancer patients.[86] In these patients, bone loss can occur naturally as a consequence of aging, after menopause or as a side effect of therapy, including radiation, chemotherapy (i.e. cyclophosphamide and methotrexate), selective estrogen receptor modulators (tamoxifen and fulvestrant) or aromatase inhibitors (anastrozol, exemestan and letrozol). Moreover, the additional anti-cancer effect of the bisphosphonates (Table 2) next to their positive influence on bone density is most intensively studied in the setting of breast cancer. This has been especially demonstrated for zoledronate,[87] as will be discussed in detail below. Of note, bisphosphonates have also been tested in a large clinical trial for the prevention of postmenopausal breast cancer, but treatment with alendronate or zoledronate over a period of three to four years did not reduce the risk of breast cancer in postmenopausal women.[88] Results of the different bisphosphonates in breast cancer are further summarized per type of bisphosphonate.

Clodronate trials - Paterson *et al.* conducted a large 1:1 randomized controlled trial in 3323 women with stage 1-3 breast cancer, comparing the effect of daily oral clodronate (1600 mg for 3 years) with placebo.[89] Overall, no differences were observed between both groups with respect to disease-free survival, overall survival, recurrence-free survival or bone metastasis-free survival.

Table 1. Clinical trials investigating treatment with bisphosphonates in hematological malignancies; anti-cancer properties

	phase (n)	condition	setting	results (reference [status])
clodronate	III (535)	MM	+ chemotherapy (+prednisolone)	↓ new skeletal disease, ↑ survival in patients without overt skeletal disease at diagnosis[91]
ibandronate	n.a. (198)	MM	+ chemotherapy	(-) bone morbidity reduction and prolonged survival , ↓ bone morbidity in patients with strongly suppressed bone-turnover markers[92]
pamidronate	III (504)	MM	+ induction therapy	30mg = 90 mg pamidronate for prevention of bone disease (NCT00376883 [completed])[93]
	III (177)	MM	untreated patients	↓ bone involvement at progression in asymptomatic MM, (-) risk of transformation and time to progression into overt MM[94]
	n.a. (90)	MM	untreated patients	'prophylactic'; ↓ skeletal event development, (-) time and rate to disease progression in early-stage MM[95]
	n.a. (44)	MM	+ chemotherapy	90 mg pamidronate > 4 mg ibandronate; ↓ osteoclast activity, bone resorption, IL-6 levels, and tumor burden[96]
	n.a. (13)	MM	+ thalidomide	↑ responses ≥ 50% of patients (treatment-resistant MM with advanced osteolytic lesions)[97]
	n.a. (62)	MM	+ chemotherapy	synergistic effect; ↓ osteoclastic and myeloma activity, ↓ pain, ↑ quality of life[98]
zoledronate	II (17)	CML	+ imatinib	n.a. (NCT00210119 [terminated])
	I/II (10)	CML	+ imatinib	(-) possibly due to relatively low plasma concentrations[84]
	II (56)	MM	+ dexamethasone + thalidomide	n.a. (NCT00263484 [completed])
	I/II (15)	MM	+ chemotherapy + simvastatin	n.a. (NCT01772719 [recruiting]) *note: Statins inhibit HHMG-CoA reductase, depleting the starting products of IPP.*
	III (143)	MM	+ calcium, + Vitamin D	↑ progression free survival, ↓ occurrence of skeletal-related events (NCT00171925 [terminated])
	III (320)	MM	+ cytoreductive therapy (+ HSCT)	n.a. (NCT01234129 [completed])
	IV (103)	MM	no treatment	n.a. (NCT01087008 [not recruiting])
	IV (179)	MM	n.a.	15-minute and 30-minute infusion time → 33% and 24% of patients showed disease progression after 24 months, respectively (NCT00104104 [completed])
	III (1018)	MM	CO	zoledronate > pamidronate: 22% ↓ risk of death and 25% ↓ risk skeletal-related events[79]
	III (1111)	MM	+ chemotherapy + HSCT	response category posttransplant may influence the impact of bisphosphonate therapy, ↓ skeletal-related events, ↑ overall survival (patients with a partial response)[76]

	III (2464)	MM	meta-analysis	↑ overall survival, (-) progression free survival[78]
	III (1960)	MM	(non)intensive treatment	zoledronate > clodronate; ↑ overall survival[99]
	III (1970)	MM	+ chemotherapy	zoledronate > clodronate ↑ overall survival independent of skeletal-related events[74]
	III (163)	MM	untreated patients	↓ skeletal-related events at progression, (-) natural history of the disease[100]
	III (94)	MM	+ chemotherapy	↓ skeletal events,↑ event-free survival and overall survival[101]
	II (17)	MMM	n.a.	n.a. (NCT00287261 [completed])

Abbreviations: (-), no effect; CML, chronic myeloid leukemia; CO, conventional treatment; HSCT, hematopoietic stem cell transplantation; IL, interleukin; MM, multiple myeloma; MMM, myelofibrosis with myeloid metaplasia; n.a., not available

Table 2. Clinical trials investigating treatment with bisphosphonates in breast cancer patients; anti-cancer properties

	phase (n)	subgroup	setting	results (reference [status])
clodronate	III (3323)	36% < 49 years 64% > 50 years	following surgery	(-) disease-free survival, ↑ anti-cancer benefits for post women (NCT00009945 [completed])[89]
	III (851)	53 years	+ chemotherapy	↓ bone turnover and ↑ protection against bone metastases[102]
	n.a. (290)	> 18 years	+ CO	↑ overall / disease free survival, ↓ bony and visceral metastases (8.5 years, long-term)[90]
	III (1069)	50% pre	+ CO	↑ 5-year bone relapse free survival [103]
	n.a. (252)	50% pre	+ CO	outcome patients low ↓ and high (-) serum matrix metalloproteinase-2 and -9 levels[104]
	n.a. (299)	> 18 years	+ CO	10-year follow up; (-) spreading to the skeleton, ↓ disease free / (-) overall survival[105]
	3 (1079)	> 18 years	+ CO	↓ mortality, ↓ risk of death over 5.5 years by 23%[106]
ibandronate	III (3000)	18-65 years	(+) CO	n.a. (NCT00196872 [completed])
	II (71)	> 18 years	+ CO	low-risk patients; 3-4 weekly = 12 weekly therapy, (-) serum C-telopeptide levels and skeletal-related events[107]
	III (3023)	< 65 years	+ dose-dense chemotherapy	(-) disease free / overall survival, ↑ disease free survival for patients with low estrogen levels[108]
	n.a. (18)	39-85 years	no treatment	disease free with disseminated tumor cells → ↓ or absence of disseminated tumor cells[109]
	III (150)	58 years	+ CO	↓ skeletal-related events, ↑ time to first skeletal-related event[110]

	III (435)	> 18 years	+ CO	↓ skeletal morbidity period rate (20 or 50 mg), ↓ need for radiotherapy (50 mg)[111]
	n.a. (564)	> 18 years	+ CO	↓ skeletal morbidity period rate, ↓ radiotherapy and surgery, ↓ skeletal events[112]
	III (466)	> 18 years	+ CO	6 mg > 2mg > placebo; ↓ skeletal morbidity period rate, ↓ new bone events, ↑ time to first new bone events[113]
pamidronate	IV (93)	> 18 years	n.a.	n.a. (NCT01907880 [(not recruiting])
	IV (120)	> 18 years	n.a.	n.a. (NCT00128297 [completed])
	III (953)	> 18 years	+ CO (no endocrine therapy)	(-) occurrence of bone metastases and fractures, (-) overall survival[114]
	I/II (33)	42-67 years	+ local radiotherapy	88% complete and 12% partial responses[115]
	III (751)	> 18 year	+ CO	↓ skeletal complications and palliating symptoms[116]
zoledronate	III (250)	> 18 years	+ CO	n.a. (NCT01099436 [completed])
	II (60)	33-73 years	one month no other treatments	low > conventional dose, ↓ VEGF and N-telopeptide of type I collagen (> 18 nM BCE = benefit most) and stbilizatuon of CA 15-3[117,118]
	III (654)	> 18 years	no treatment	n.a. (NCT00512993 [completed])
	IV (168)	70.9 years	+ letrozole	tumor response rate based on MRI- or mammography and/or sonography at 6 months: control = 54.5%, + zoledronate = 69.2% (NCT00375752 [completed])
	III (430)	> 18 years	previous treatment with zoledronate	annual overall skeletal morbidity rate: 3-monthly = 0.26 and 4-weekly = 0.22 (NCT00375427 [completed])
	III (416)	> 18 years	previous treatment with zoledronate	proportion of patients who experienced at least one skeletal-related event: 4-weekly = 22 and 12-weekly = 23.2 (NCT00320710 [completed])
	III (1803)	pre	+ anastrozole or tamoxifen	↑ disease-free survival, persistent benefits with zoledronate (median follow-up of 62 months)[119-121]
	II (190)	> 65 years	+ letrozole (surgery)	n.a. (NCT00247650 [completed])
	II (120)	> 18 years	+ epirubicin, docetaxel	↓ detectable disseminated tumor, ↓ rate development micrometastatic disease, ↓ treatment-related bone-mineral density loss, irrespective of menopausal status[21]
	II (86)	> 18 years	+ CO	↑ elimination disseminated tumor cells, overall survival (NCT00172068 [completed])[20]
	III (5400)	> 18 years	+ CO	n.a. (NCT00127205 [not recruiting])
	III (3360)	51 years	+ CO	(-) disease-free survival, ↓ development of bone metastases, ↑ disease outcomes for women with established menopause[122]

	III (1803)	pre	+ CO	↑ efficacy of adjuvant endocrine treatment (long-term)[123]
	III (250)	49 years	+ CO	(-) pathological or clinical response to chemotherapy[124]
	II (168)	post	+ letrozole	↑ clinical response rate (trend)[125]
	n.a. (53)	34-81	4 weeks no therapy before onset	↓ tumor growth markers, ↓ circulating tumor cells (rebound after 14 days), critical role in anti-tumor proliferation and dissemination[19]
	III (1326)	61 years (♂/♀)	+ CO	zoledronate > ibandronate; ↓ skeletal-related events[126]
	III (425)	60 years	+ CO	12- > 4-weekly regimen: maintaining therapeutic effects after one year[127]
	II (40)	50 years	+ chemotherapy	↑ recovery cell growth, ↓ serum VEGF, ↓ follistatin levels in post women[128]
	II (119)	50 years	+ CO	after 61.9 months' median follow-up (-) recurrence or survival, but differed between subgroups defined by estrogen receptor status (- > +)[129]
	n.a. (96)	54 years [27-77]	+ CO	↑ elimination of disseminated tumor cells[22]
	III (7354)	> 18 years	+ CO	(-) overall / disease free/ bone metastasis free / distant and locoregional recurrence free survival; in post women ↑ disease free survival, distant and locoregional recurrence[130]
	III (3360)	> 18 years	+ CO	(-) disease free survival, disease recurrence and death[131]
	II (172)	n.a.	+ only endocrine treatment allowed	↓ isolated tumor cells in bone marrow, ↑ recurrence free survival[132]
	I (10)	63 years [55-70]	+ low-dose IL-2	↑ effector maturation of Vγ9Vδ2 T cells in all patients, clinical outcome was correlated with peripheral Vγ9Vδ2 T cell numbers[60]
	n.a. (205)	47 years	+ chemotherapy	↓ tumor size, ↑ pathological complete responses[133]
	n.a. (23)	66 years [47-85]	no treatment	long-lasting activation of effector γδ T cells after a single-dose of zoledronate[134]
	III (1130)	56 years	+ CO	4 mg zoledronate > 90 mg pamidronate; ↓ skeletal complications in a subset of patients with at least 1 osteolytic lesion at study entry[135]
	III (1648)	> 18 years	+ CO	4 mg zoledronate > pamidronate; ↓ risk of developing skeletal complications[136]

Abbreviations: (-), no effect; BCE, bone collagen equivalents; CO, conventional treatment; IL, interleukin; n.a., not available; post, postmenopausal; pre, premenopausal; VEGF, vascular endothelial growth factor

However, in the subpopulation of postmenopausal women, clodronate was found to result in an improvement of recurrence-free interval, bone metastasis-free interval and non-bone metastasis-free interval.[89] This finding is in line with a study of Diel *et al.* (n=290) who reported a significant improvement in overall survival in patients with primary breast cancer receiving clodronate,[90] 60% of whom were postmenopausal.

Ibandronate trials - The GAIN (German Adjuvant Intergroup Node-Positive) study enrolled 3023 patients with node-positive early breast cancer, randomly assigned to ibandronate 50 mg per day orally for 2 years or observation only. Ibandronate-treated patients displayed no superior disease-free survival or overall survival compared with the observation group. In analogy with clodronate, postmenopausal women seem to benefit more from treatment with ibandronate. The same applied to premenopausal breast cancer patients who develop amenorrhea following chemotherapy or luteinizing hormone-releasing hormone treatment. Hence, the overall negative study results could result from an underrepresentation of patients older than 60 years and the fact that study participants were at a considerable risk of relapse. Moreover, the results would have been more solid if compliance to treatment had been better documented.[108] Another phase III study (n=466) compared the efficacy of 2 mg or 6 mg ibandronate i.v. (every 3–4 weeks) with placebo in breast cancer patients with metastatic bone disease. The skeletal morbidity period rate was lower in both ibandronate groups compared with the placebo group, whereby the difference with placebo was statistically significant for the 6 mg i.v. ibandronate group.[113] The route of administration is however not determinative, since ibandronate (50 mg) orally demonstrated similar efficacy in reducing the risk of skeletal complications in breast cancer patients with metastatic bone disease.[112]

Pamidronate trials - Only little experience has been obtained with the use of pamidronate in breast cancer patients. In a randomized trial of 953 patients with lymph node-negative primary breast cancer, 150 mg oral pamidronate twice daily was compared with no adjuvant pamidronate. No significant advantage in terms of bone metastasis-free survival and overall survival was demonstrated for the pamidronate-treated group.[114]

Zoledronate trials - Opposed to the aforementioned bisphosphonates, more research has been performed with zoledronate, investigating its anti-cancer effect and underlying mechanisms (Table 2). In the AZURE trial – which included 3360 women with stage II or III breast cancer – patients were randomized to receive either standard adjuvant systemic treatment alone or in conjunction with 4 mg i.v. zoledronate for five years.[122] Treatment with zoledronate protected against fractures and reduced bone metastases, which in the

majority of patients were reported before or coincidence with fracture. Overall no improvement was noted concerning disease-free survival or overall survival. However, invasive disease-free survival improved significantly for postmenopausal women, regardless of the estrogen-receptor status of the primary tumor. The heterogeneity in disease recurrence outside the bone depended on menopausal status , with an apparent increase in extraskeletal metastases in premenopausal women, a neutral effect in perimenopausal women, and a beneficial effect postmenopausal women.[122] The ABCSG-12 trial (median follow-up of almost 8 years) included 1803 premenopausal women with hormone-responsive early-stage breast cancer who underwent ovarian suppression with goserelin. Zoledronate (4 mg every 6 months) was added to the treatment for 3 years in half of the patients. This led to the conclusion that twice-yearly zoledronate administration improves the effectiveness of adjuvant anti-hormonal treatment, a benefit that is maintained long-term.[123]

In summary, studies investigating the effect of bisphosphonates on bone metastasis-free survival, disease-free survival, and overall survival in breast cancer patients have yielded inconsistent results. In general, benefits are mostly suggested for women with low concentrations of reproductive hormones or older women. A recent meta-analysis scrutinized all non-confounded randomized trials in early breast cancer that randomized between bisphosphonate and control and found a decidedly significant reduction in bone recurrence after treatment with bisphosphonates in all patients. Furthermore, in the subgroup of postmenopausal women, recurrence, breast cancer mortality, and overall mortality were all highly significantly reduced.[137] This is in line with preclinical research, indicating an effect of reproductive hormones on bisphosphonate efficacy against cancer cells. In a mouse model of breast cancer, treatment with zoledronate was only effective in ovariectomized mice, but not in sham-operated animals.[138] Generally it can be concluded that treatment with bisphosphonates offers oncological benefit and should be considered for routine use in the treatment of early breast cancer in women with either a natural or medically induced menopause to extend survival and improve bone health.[137] Ongoing clinical trials will provide better insight on the best choice of bisphosphonate (e.g. SWOG0307) and its regimen (e.g. SUCCESS), and anti-cancer effects in pre- (e.g. HOBOE-premenopausal and TEAM-IIb) and postmenopausal women (e.g. IBIS 3).

3.2.2. Prostate cancer

Maintaining bone health is essential in the management of prostate cancer, since bone is the most common site of metastasis and the development of metastasis in essence implies the patient is incurable.[139] Given this importance, much research is being done to preserve bone function, such as, inter alia, with the bisphosphonates. An important cause of the loss

of bone mineral density in prostate cancer patients is androgen deprivation therapy. Herewith, the bisphosphonates are the most commonly used drugs in the management of bone complications in bone-metastasizing cancers including prostate cancer.[139] Similar to what has been discussed above, the question arises whether the bisphosphonates also play a direct anti-tumoral role in prostate cancer (Table 3).

Two clinical trials have tested the effect of the simple bisphosphonate clodronate on overall survival of prostate cancer, one trial involving 311 prostate cancer patients with bone metastasis and another involving 508 men with non-metastatic prostate cancer.[140] Patients were given oral clodronate (2080 mg/day) or placebo, next to standard treatment. The addition of clodronate resulted in an overall survival benefit for patients with bone metastases (exemplified by a 5-year survival of 21% with placebo versus 30% with clodronate). No difference was observed in the group of non-metastatic prostate cancer patients.[140] Remarkably, the Netherlands Prostate Study did not find any benefit of adding the nitrogen-containing bisphosphonate risedronate to treatment with docetaxel in patients with castration-resistant prostate cancer.[142] Zoledronate is, also in this disease setting, the most intensively studied bisphosphonate, although there are only a limited number of advanced clinical trials. A phase III trial involving 1071 locally advanced prostate cancer patients investigated the effectiveness of eighteen months of androgen suppression plus radiotherapy or six months of neoadjuvant androgen suppression plus radiotherapy, both with or without zoledronate (4 mg every 3 months for 18 months, i.v.). Notably the registered regimen of zoledronate is 4 mg one-monthly instead of every 3 months. After a median follow-up of 7.4 years, there was no difference between the various groups with regard to mortality.[158] On the other hand, zoledronate was found to improve clinical outcome in patients with bone-metastatic hormone-naïve prostate cancer. 205 patients were treated with a combined androgen blockade with or without monthly zoledronate infusion. Patients in the zoledronate group demonstrated improved prostate-specific antigen progression-free survival time.[146] While encouraging, further studies are needed to fully address the clinical benefit of adding zoledronate (or other bisphosphonates) to standard therapy in prostate cancer. The results of the STAMPEDE (Systemic Therapy in Advancing or Metastatic Prostate Cancer: Evaluation of Drug Efficacy) trial are anticipated, which will provide further insight into the potential roles of zoledronate in patients with prostate cancer.[159]

3.2.3. Primary bone tumors

Only a few reports have been published to date on the use of bisphosphonates in the treatment of bone tumors (Table 4). It mostly concerns studies with very small patient populations, making it impossible to draw general conclusions on treatment efficacy.

Table 3. Clinical trials investigating treatment with bisphosphonates in prostate cancer patients; anti-cancer properties

	Phase (n)	setting	results (reference [status])
alendronate	I/II (28)	restriction of therapies	n.a. (NCT01595087 [completed])
clodronate	III (819)	+ CO	↑ overall survival metastatic disease, (-) overall survival non-metastatic disease[140]
pamidronate	n.a. (21)	+ CO	↓ accelerated turnover of bone metabolism caused by metastases[141]
risedronate	II/III (592)	+ docetaxel and prednisone	(-) time to progression, overall survival, prostate specific antigen levels and pain[142]
zoledronate	n.a. (11)	only LHRH agonist allowed	proportion of participants with PET response = 0.091 (NCT01205646 [not recruiting])
	II/III (100)	+ ADT	n.a. (NCT01006395 [recruiting])
	II (300)	only LHRH agonist allowed	n.a. (NCT00554918 [completed])
	II/III (8100)	+ hormone treatment	n.a. (NCT00268476 [recruiting])
	III (522)	+ ADT	skeletal-related event-free survival: early group = 77.6% and delayed group = 81.4% (NCT00242567 [completed])
	IV (284)	+ hormone treatment	n.a. (NCT00237159 [completed])
	IV (38)	no hormonal treatment before	n.a. (NCT00237146 [completed])
	IV (60)	restriction of therapies	n.a. (NCT00219271 [completed])
	III (1071)	+ Androgen suppression	(-) zoledronate every 3 months for 18 months on patient-reported-outcome ([143,144], NCT00193856 [completed])
	IV (60)	restriction of therapies	n.a. (NCT00172016 [completed])
	II (28)	+ estramustine and docetaxel	n.a. (NCT00151073 [completed])
	III (645)	+ ADT	(-) time to first skeletal-related event and overall survival (NCT00079001 [completed])([145]
	II (50)	+ rebimastat	n.a. (NCT00039104 [completed])
	n.a. (205)	+ LHRH agonist + bicalutamide	↑ time to prostate-specific antigen failure[146]
	n.a. (60)	+ combined androgen blockade	(-) progression-free survival, but ↑ progression-free survival patients with Gleason score ≥ 8, ↓ skeletal-related events and bone pain[147]
	n.a. (137)	+ hormone therapy	zoledronate > clodronate; ↑ bone progression-free survival, ↓ bone pain, ↑ lumbar spine bone mineral density, (-) overall survival and skeletal-related events[148]
	III (636)	+ no cytotoxic chemotherapy, but estramustine	↓ risk of bone disease progression per unit prostate specific antigen increase, suggesting protection against bone lesion progression [149]

	n.a. (47)	+ LHRH agonist + anti-androgen	(-) prostate-specific antigen levels, ↑ time to progression/overall survival (in advanced disease) [150]
	n.a. (65)	+ androgen blockade	↑ prostate-specific antigen and bone turnover markers response [151]
	n.a. (422)	restriction of therapies	↑ delayed onset of first skeletal-related event, ↓ risk of developing one, ↓ skeletal morbidity rate (65%) and ↓ overall risk of developing a skeletal-related event (40%) [152]
	I (18)	+ low-dose IL-2	↑ immunologic and clinical responses in metastatic patients, γδ T cell phenotype is possibly novel biomarkers of prognosis [58]
	n.a. (122)	+ CO	↓ risk of skeletal-related events (36%), long-term treatment with zoledronate provides sustained clinical benefits [153]

abbreviations: (-), no effect; ADT; androgen deprivation therapy, CO, conventional treatment; LHRH; luteinizing hormone-releasing hormone, IL, interleukin; n.a., not available

Table 4. Clinical trials investigating treatment with bisphosphonates in patients with bone neoplasms; anti-cancer properties

	Phase (n)	tumor type	setting	results (reference [status])
pamidronate	n.a. (40)	osteosarcoma	+ cisplatin, doxorubicin, and methotrexate	safe combined with chemotherapy, does not impair its efficacy and possibly improves durability of limb reconstruction[154]
zoledronate	I/II (24)	giant cell tumors of bone	no aminoglycosides	n.a. (NCT01564121 [terminated])
	III (1383)	Ewing's sarcoma	+ CO	n.a. (NCT00987636 [recruiting])
	II (15)	osteoclastoma	+ CO	n.a. (NCT00889590 [terminated])
	I (24)	osteosarcoma	+ surgery + chemotherapy	safe, maximum tolerated dose is 2.3 mg/m(2) (max 4 mg) for patients with metastatic osteosarcoma (NCT00742924 [completed])[155]
	II/III (60)	osteoclastoma	+ CO + no treatment	n.a. (NCT00691236 [unknown])
	III (440)	sarcoma	+ surgery + chemotherapy	n.a. (NCT00470223 [unknown])
	n.a. (8)	benign bone tumors	no treatment	bisphosphonates appear to be an effective option without adverse effects for the non-operative management of symptomatic benign bone tumors[156]
	n.a. (25)	giant cell tumors of bone	+ adjuvant therapy possible	zoledronate may be useful in controlling disease progression, directly inhibition giant cell tumor of bone - derived osteoclast resorption[157]

Abbreviations: CO, conventional treatment; n.a., not available

A first study indicates that bisphosphonates could be a potential therapeutic option in the management of unresectable symptomatic benign bone tumors whatever their origin or location.[156] Adult patients were given one cycle of 4 mg zoledronate, whereas minors were treated with 1 mg/kg of pamidronate. All treated lesions exhibited a significant increase in density, with a complete and partial vanishing of osteolysis in two and five patients out of eight, respectively. Notably, a drastic decline in inflammatory response was seen. Concurrently, pain, which can be caused by osteoclastic activity or production of growth factors from the bone, was quickly reduced.[156] A second study investigated the effect of treatment with bisphosphonates on 25 cases of aggressive primary, recurrent and metastatic giant cell tumor of the bone.[157] Most tumors did not increase in size after treatment. However, results should be interpreted cautiously given no control group was included and different bisphosphonates were administered on an individual ad hoc basis often in combination with other therapies.[157] The third study included 40 patients with osteosarcoma who were given cisplatin, doxorubicin, and methotrexate in combination with a 2 mg/kg dose pamidronate monthly for one year.[154] The combination proved to be feasible and safe. Despite being a pilot study, treatment with pamidronate seemed to improve patient outcome for osteosarcoma by direct anti-tumor effects, reducing the risk of metastasis, and improving the durability of reconstruction following tumor resection.[154] This observation, taken together with preclinical data suggesting benefit from treatment with bisphosphonates in many types of primary bone tumors, i.e. chondrosarcoma,[160,161] Ewing's sarcoma[162,163] and osteosarcoma,[164,165] warrants further clinical studies to determine the efficacy of bisphosphonates in patients with primary bone tumors.

3.2.4. Other solid malignancies (with bone metastasis)

Many types of cancer have a tendency to metastasize to the skeleton, causing osteolysis or abnormal bone formation. Metastatic bone disease is a potentially debilitating or life-limiting complication of cancer. Bisphosphonate therapy is already included in current guidelines to prevent skeletal-related events or palliate bone pain resulting from bone metastasis in breast cancer, prostate cancer, and lung cancer.[166] Despite established recommendations,[167,168] many physicians wait until the development of symptomatic bone disease before initiating therapy. However, patients may benefit from early treatment with zoledronate before onset of bone pain.[166]

Other types of malignancies could benefit as well from treatment with bisphosphonates, apart from their effect on bone (Table 5). A retrospective study involving 208 bone metastatic gastric cancer patients found a substantial extension of time to first skeletal-related events as well as an increase in the median survival when patients were additionally treated with zoledronate 4 mg every 4 weeks.[169]

Table 5. Clinical trials investigating treatment with bisphosphonates in patients with other solid malignancies; anti-cancer properties

	Phase (n)	tumor type	setting	results (reference [status])
ibandronate	n.a. (73)	colorectal cancer	n.a.	↓ skeletal events/morbidity ↑ time to first event by at least 6 months, ↑ time to progression of bone lesions[171]
zoledronate	I (4)	neuroblastoma	+ IL-2	n.a. (NCT01404702 [terminated])
	II (50)	hepatocellular carcinoma	+ soranefib	n.a. (NCT01259193 [unknown])
	II (8)	mesothelioma	no treatment	n.a. (NCT01204203 [not recruiting])
	II (3)	lung cancer	+ chemotherapy	n.a. (NCT01004510 [terminated])
	I (24)	adenocarcinoma	+ pancreatic resection	n.a. (NCT00892242 [terminated])
	IV (156)	lung cancer	no treatment	n.a.(NCT00762346 [completed])
	I/II (20)	kidney cancer	+ IL-2 + autologous lymphocytes	n.a. (NCT00588913 [completed])
	II (12)	kidney cancer	+ IL-2	patients with anti-tumor response with low-dose IL-2 and zoledronate is 5 out of 8 (NCT00582790 [completed])
	II (12)	cervical cancer	restriction of therapies	n.a. (NCT00278434 [terminated])
	I (21)	neuroblastoma	+ cyclophosphamide	n.a. (NCT00206388 [completed)
	I (34)	lung cancer	+ vitamine D and calcium	(-) progression free survival or overall survival, trend toward worsening progression free survival in longer-term follow-up (NCT00172042 [completed])[172]
	IV (300)	lung cancer	restriction	n.a. (NCT00099541 [completed])
	III (250)	lung cancer	+ docetaxel + carboplatin	n.a. (NCT00086268 [completed])
	II (100)	lung cancer	+ docetaxel	(-) progression-free survival and overall survival[170]
	n.a. (208)	gastric cancer	+ CO	↑ time to first skeletal-related event and ↑ median survival time after diagnosis of bone metastasis[169]
	I/II (21)	renal cancer, melanoma, AML	+ low-dose IL-2	(-) objective responses in solid tumors, ↑ objective responses in AML (25% partial remission), significant *in vivo* activation and expansion of γδ T cells in all evaluable patients[59]

	I (21)	neuroblastoma	+ metronomic cyclophosphamide	↑ clinical and biologic responses[173]
	n.a. (12)	renal cancer	+ IL-2 (repeatedly)	(-) objective clinical responses, inhibits the proliferative capacity of Vγ9 Vδ2 T cells[174]
	II (60)	nasopharyngeal cancer	+ cisplatin, 5-fluorouracil	↓ N-telopeptide of type I collagen[175]
	n.a. (55)	lung cancer	+ chemotherapy	zoledronate = ibandronate; ↑ efficacy[176]
	n.a. (40)	bladder cancer	+ palliative radiotherapy	↓ incidence of skeletal-related events and ↑ 1-year survival rate[177]

Abbreviations: (-), no effect; acute myeloid leukemia, AML; CO, conventional treatment; n.a., not available

For non-small-cell lung cancer patients with bone metastases, however, no advantage concerning progression-free survival was found of adding zoledronate to treatment with docetaxel.[170] A small (n=60) trial with newly diagnosed nasopharyngeal cancer patients with bone metastasis investigated the addition of i.v. zoledronate to cisplatin/5-fluorouracil-based chemotherapy. The addition of zoledronate decreased the urinary level of N-telopeptide of type I collagen, a sensitive biomarker of bone resorption, more rapidly and successfully than chemotherapy alone.[175] Finally, a group of 21 patients (≤25 years) with bone-metastasized neuroblastoma was treated with an escalating dose of i.v. zoledronate every 28 days in combination with oral metronomic cyclophosphamide. This particular combination resulted in prolonged disease stability and reduced serum IL-6 levels.[173] The latter might be beneficial given the knowledge that high IL-6 levels tend to be linked with advanced disease stage and poor outcome.[178]

4. FUTURE PERSPECTIVES

The use of bisphosphonates in cancer therapy definitely warrants further investigation to establish their role as a potential (adjuvant) cancer treatment. Current research efforts strive to improve their function, modify them to have multi-functions, perfect their bioavailability and reduce side effects. Given their unique ability to target the bone, bisphosphonates can be used as a bone-targeting anti-cancer agent. Bisphosphonate-conjugated nanoparticles are currently being explored to deliver chemotherapeutic agents to the bone microenvironment. For example alendronate-conjugated nanoparticles containing curcumin and bortezomib highly localized to the bone and significantly deducted tumor growth rate along with protection of bone resorption in an intraosseous mouse model of bone metastasis.[179] Docetaxel-encapsulated nanoparticles with zoledronate, not only displayed a prolonged blood circulation half-life and a higher retention at the bone site, but also brought about enhanced tumor control.[180] As discussed previously in this review, liposome-encapsulated bisphosphonates are also used to deplete (tumor-associated) macrophages in preclinical settings. Encapsulation in a lipid bilayer prevents the dissemination of these drugs to the bone matrix, increases their plasma half-life and allows for systemic distribution.[12,46,181] These novel formulations not only permit to change the pharmacokinetic profiles of the bisphosphonates, which at the same time will need attention concerning (cyto)toxicity, but also leaves the possibility to target more cancer cells.[181,182] Of note, erythrocytes are explored as well as carrier system for bisphosphonates.[47] Conjugation to other drugs is another line of attack that can pave the way to an innovative approach. Cleavage rate is here essential for the achievement of successful dual therapy. Examples in this group are the combination of the

radiopharmaceutical rhenium-188 with alendronate,[183] cytarabine with zoledronate[184] and doxorubicin with alendronate.[185]

5. DISCUSSION AND CONCLUSION

Due to the exciting but sometimes inconsistent results of trials with (adjuvant) bisphosphonates, the ability of these drugs to improve the outcome for patients with other tumor types remains in most cases unsettled and further clinical trials are therefore warranted. Foremost it remains to be established which subgroup of patients benefits most of treatment and what the optimal treatment protocol/combination scheme is.

On the other hand, there is enough evidence that the bisphosphonates, and more specifically the nitrogen-containing subgroup, have clear anti-cancer properties in different tumor types next to their positive effect on bone lesions. However, it must not be overlooked that improvement in clinical outcomes (e.g. overall survival, disease-free survival) does not necessarily reflect a direct anti-tumor effect. For example, patients with among other prostate cancer or myeloma indeed often die of what is described as "a skeletal death," meaning that reduced survival is directly related to skeletal complications. In such cases, a pure bone effect could potentially lead to improvements in overall survival.[186,187] However, notwithstanding the fact that tracing the origin of an effect can be challenging, demonstrable non-bone related-effects were observed in patients. For example in the MRC Myeloma IX trial, overall survival improved independently of prevention of skeletal-related events, this being specifically analyzed as variable in a Cox model, and thus was consistent with clinically meaningful anti-cancer activity.[74] The ABSCG-12 trail included 1803 premenopausal women with endocrine-receptor-positive early-stage (stage I-II) breast cancer, meaning breast cancer is still contained in the breast or growth has only extended to the nearby lymph nodes. Here as well, addition of zoledronate, to adjuvant endocrine therapy, significantly improved disease-free survival. Moreover, fewer patients receiving zoledronate had to deal with locoregional recurrence and contralateral breast cancer.[119] These data are consistent with the idea that bisphosphonates (zoledronate) have as well a direct anti-cancer effect, counteracting cancer cell survival and seeding of disease recurrence. However, regarding prostate cancer, improvement in patient outcome is foremost seen in metastatic patients, as a consequence of effects on bone.[140,146,149] In line with this, the results of the Zometa European Study (ZEUS) (n=1393) were published this year, indicating no effect of 3-monthly zoledronate administrations on the prevention of bone metastases in patients with high-risk localised prostate cancer.[188]

As discussed in the first part of this review, bisphosphonate-activated γδ T cells are important players in the elimination of cancer cells. Since patient outcome can possibly be predicted by the number of γδ T cells, the latter should receive attention in therapy monitoring. Measuring more parameters than only the number of cells will give more insight if certain other characteristics of γδ T cells might have predictive value. Also, their effect on TAMs, another pivotal cellular immune component in the tumor milieu, might potentially be worthwhile to monitor.

Due to the relative safety, potency and long-term stability of nitrogen-containing bisphosphonates, these agents represent ideal candidates to be further tested in a clinical setting, particularly when anti-resorptive effects are beneficial.

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FUTURE PERSPECTIVES

Now what do we do …

… concerning γδ T cells?

There are still many secrets to be unveiled concerning γδ T cells. For example, what is the steady state function of γδ T cells in tissues? Which are the molecular mechanisms of tissue surveillance? How does the role of the butyrophilins in sensing stress compare to the role of NK cell receptors? Is there something as an antigen-specific γδ T cell response and/or γδ TCR-mediated memory formation?

From a clinical point of view, alternative therapeutic opportunities for immune interventions against malignant cells are urgently needed. One interesting approach arises from recent insight that daily cancer immune surveillance is executed not only by αβ T cells, but by multiple layers of the immune system. One such layer consists of γδ T cells, which appear to be more potent than many other lymphocyte subpopulations.[1,2] A key power of γδ T cells is their capacity to sense alterations in cellular lipid pathways selectively active in cancer cells. As such, they provide an alternate pathway to detect and eliminate cancer; seeing cancer as a metabolic disease.[3] While the pleiotropic anti-tumor characteristics of γδ T cells are highly attractive, only phase 1 and small phase 2 clinical trials have been performed so far. Therefore, although γδ T cell-based therapies have been proven safe and feasible, the γδ T cell field craves for large phase 3 studies with the primary objective to look at the effectivity of the therapy, indicating the way forward.

Either way it will be interesting to incorporate complementary strategies. Namely, potential synergistic interactions could take place with; for example, cytokines (i.e. IL-15),[4] immune checkpoint blockade (i.e. anti-PD1, anti-TIM-3),[5] chemotherapy,[6] vaccines made from neoantigens[7] or oncolytic viruses.[8] In this way we could hope to enhance or broaden the benefits of γδ T cells. Cellular engineering of γδ T cells is another interesting avenue. Preliminary evidence already indicates that CAR-modified γδ T cells are superior to their αβ T cell counterparts[9,10] and ongoing research in our lab has shown that mRNA electroporation of γδ T cells allows for high transgene expression without affecting the cytotoxic functioning of γδ T cells.

… concerning the IL-15 DC vaccine?

The past decade the Laboratory of Experimental Hematology has intensively focused on developing a novel DC vaccine with improved potency, resulting in the IL-15 DCs.[11] We established that, at the pre-clinical level, IL-15 DCs hold immunogenic assets upstaging the gold-standard IL-4 DCs. Namely, in addition to *bona fide* DC properties, IL-15 DCs possess (I) a direct cytotoxic capacity, linked to their expression of CD56,[12] (II) provide a favorable chemokine milieu for recruiting CD8 T cells, γδ T cells and NK cells,[13] and (III) are able to

harness γδ T cells and NK cells, representing the innate immune system, in their fight against cancer.[14,15] Given these results, favorable outcomes can be expected when applied in a clinical setting. Therefore, we believe that the momentum has come to bring the IL-15 DC vaccine into the clinic. Certainly in view of the added benefits regarding the manufacturing process as IL-15 DCs have the logistic advantage over IL-4 DCs to be ready after only 48 hours of culture, significantly shortening the time-frame of production, and reducing the risk of contamination and the costs.

Although it should not prevent or stall the translation of IL-15 DC vaccines into clinical trials, DC vaccination could benefit from the combination with other therapies to strengthen the overall anti-cancer effect, similar to γδ T cell-based therapy.[16] Certainly when looking into possibilities of implementing DC vaccination outside the context of remission therapy in AML, where the tumor microenvironment is known to suppress elimination of cancer cells effectively, additional treatments will probably be needed.

… concerning IL-15?

First and foremost, the results obtained in this thesis regarding the immunostimulatory effect of IL-15 underscore its position as top-ranked molecular agent for cancer immunotherapy.[17] Still, things are seldom black or white in life, and hence, in the immunology field as well. For instance, MM cells are also sensitive to IL-15 signaling, expressing all three components of the IL-15R heterotrimer.[18] In general, neoplastic counterparts of hematopoietic cells, of which the growth and survival is stimulated by IL-15, may experience the same inducing effects by IL-15.[19-21] On the other hand, IL-15 signaling may lead to progression in solid tumors too. Human renal cancer cells constitutively express membrane-bound IL-15. Stimulation by soluble IL-15Rα leads to epithelial-to-mesenchymal transition, favoring tumor invasiveness.[22,23] To conclude, when using IL-15 to battle malignancy, one should be aware of a potential dual face of the molecule. However, unintended support of cancer by IL-15 therapy can be minimized by using IL-15 in *ex vivo* cell culture protocols instead of using IL-15 for general systemic administration.

… concerning the expression of CD56 by immune cells?

To start with, it would be interesting to investigate the binding partners of CD56 and further develop our understanding on how the expression of CD56 on the immune cell surface can contribute to their cytotoxicity and interaction with other immune cells/cancer cells. One could think of the fact that aberrant CD56 expression is seen in a range of hematological malignancies as well as solid tumors, ambiguously correlated with both a positive and negative prognosis.[24] Could this be attributed to different molecules binding CD56; similar to the co-stimulatory interaction between CD80/86 with CD28 as opposed to the co-

inhibitory signals following CD80/86 binding with CTLA-4? Or does the specific NCAM isoform determine the outcome of the interaction? For instance, NCAM-120 is a GPI-anchored protein, with the possibility to be easily shed from the cell surface,[25] whereas NCAM-140 and NCAM-180 are transmembrane proteins. Lung epithelial cells have already been shown to reduce tissue damage during inflammatory processes by shedding of CD56.[26] Additional research into this area will be needed to further elucidate the role of CD56 and its putative binding partners.

Moreover, further investigation into the differences between $CD56^{+}$ and $CD56^{-}$ immune cells is warranted. In analogy with $CD56^{bright}$ and $CD56^{dim}$ NK cells, which are developmentally related but functionally distinct subsets, the question arises if, for example, $CD56^{+}$ γδ T cells are a different subset with respect to the $CD56^{-}$ γδ T cells? One potential avenue to study any difference (in effector functions) between both "groups", would be the performance of a micro-array analysis of freshly isolated immune cells, comparing both the positive and negative fractions. Another question that could be raised is; are $CD56^{+}$ cells precursors of $CD56^{-}$ cells, is it the other way around or none of the previous? The latter is based on the notion of $CD56^{bright}$ NK cells being predecessor of $CD56^{dim}$ NK cells.[27] To tackle this question, a flow cytometric measurement of telomere length, with telomere-specific (C3TA2)3 fluorescently labeled peptide nucleic acid probes might be undertaken. Of note, subsets could switch between one another, depending on the microenvironment, as being insinuated by the results of the addition of IL-15. *In vivo,* the relationship between $CD56^{+}$ and $CD56^{-}$ cells could be examined by the injection of CFSE-labeled $CD56^{+/-}$ immune cell subsets into mice, and monitor their development and/or persistence over time. Concomitantly, it will be possible to explore the localization and migration of the different immune cell fractions. Furthermore, so far, we have been working with immune cells isolated from the blood. It is, however, conceivable that other organs display a different immune cell milieu, leaving room for deepened research into the occurrence of CD56 on immune cells in different tissues.

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List of abbreviations

A

Aβ	alpha beta
ADCC	antibody-dependent cellular cytotoxicity
AML	acute myeloid leukemia
APC	antigen-presenting cell
ATP	adenosine triphosphate

B

BCG	*Bacillus Calmette-Guérin*
BrHPP	bromohydrin pyrophosphate
BTLA	B and T lymphocyte attenuator
BTN	butyrophilin

C

CAR	chimeric antigen receptor
CCL	chemokine (C-C motif) ligand
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
CIK	cytokine-induced killer
CTL	cytotoxic T lymphocytes
CXCL	chemokine (C-X-C motif) ligand

D

DC	dendritic cell

E

ECLIA	electrochemiluminescence immunoassay
ELISA	enzyme-linked immunosorbent assay
E:T	effector-to-target

F

FBS	fetal bovine serum
FPP	farnesyl pyrophosphate

G

γδ	gamma delta
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol

H

hAB	heat-inactivated human AB serum
HER	human epidermal growth factor receptor (EGFR)
HIV	human immunodeficiency virus

HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HSCT	hematopoietic stem cell transplantation

I

iDC	immature DC
IFN	interferon
IL	interleukin
ILC	innate lymphoid cell
IMDM	Iscove's Modified Dulbecco's Medium
IPP	isopentenyl pyrophosphate
i.v.	intravenous

J

JAK/STAT	Janus kinase-signal transducer and activator of transcription

K

KIR	killer-cell immunoglobulin-like receptor

L

lnc	long non-coding

M

mAbs	monoclonal antibodies
MAPK	mitogen-activated protein kinase
mDC	myeloid DC
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MM	multiple myeloma
mo-DC	monocyte-derived DC
MRD	minimal residual disease

N

NCAM	neural cell adhesion molecule
NFAT	nuclear factor of activated T cells
NK	natural killer
NKG2D	natural killer group 2D

P

PAMP	pathogen-associated molecular pattern
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PD	programmed cell death
pDC	plasmacytoid DC
PG	prostaglandin
PI	propidium iodide

PI3K	phosphoinositide 3-kinase
PLA	proximity ligation assay

Q

qPCR	quantitative-PCR

R

RPMI	Roswell Park Memorial Institute

S

SD	standard deviation

T

TAM	Tumor-associated macrophage
TCR	T cell receptor
TEMRA	terminally differentiated CD45RA ($CD8^+$) T cells
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper type
TIL	tumor-infiltrating lymphocytes
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cell

V

V	variable
VEGF	vascular endothelial growth factor

U

UPN	unique patient number
UZA	Antwerp University Hospital

Dankwoord

"It is good to have an end to journey toward;
but it is the journey that matters, in the end"
- Ernest Hemingway

… en of ik genoten heb van dit doctoraatsavontuur, het was een onvergetelijke ervaring. Dit kan ik nu gemakkelijk zeggen, mijn doctoraatsthesis is afgerond en ik kijk vol trots terug op de afgelopen vijf jaar. Een doctoraat afleggen is echter niet alleen rozengeur en maneschijn. Zonder de steun, kennis, vriendschap, het vertrouwen en relativerings-vermogen van vele anderen zou ik hier vandaag niet staan. Via deze weg wil ik dan ook een welgemeende 'dankjewel' zeggen aan al de mensen die ik heb mogen leren kennen en iedereen die, elk op zijn/haar eigen manier, zijn steentje heeft bijgedragen.

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Verder wil ik graag het Fonds Wetenschappelijk Onderzoek – Vlaanderen bedanken, zonder hun financiële steun was dit onderzoek niet mogelijk geweest.

In het rijtje belangrijke mensen die hebben bijgedragen aan dit doctoraat vermeld ik graag nog mijn begeleider Dr. Sébastien Anguille. **Séba**, bedankt om mij, ondertussen bijna zes jaar geleden, te introduceren in de wondere wereld van het wetenschappelijk onderzoek en tumorimmunologie. Het is altijd makkelijk om reeds succesvolle mensen te helpen, het is veel minder vanzelfsprekend om tijd te steken in een masterstudent farmacie zonder lab skills en specifieke kennis van het onderwerp. MERCI! Excuses ook aanvaard om mij te doen geloven dat ik aan Hairy cell leukemie leed 😉. De afgelopen jaren kon ik ook altijd op jou rekenen voor het kritisch nalezen van mijn artikels en reviews, tabellen op te stellen met patiëntgegevens of bloedstalen te gaan halen in de "middle of nowhere". Nu alleen nog die Win for Life winnen zodat we een legertje DC-oogsters kunnen aannemen.

"If you want to go far go together"

I look back on my London adventure with great fondness. **Prof. John Anderson**, thank you for your warm welcome and making me part of your great team. Expanding my horizon, being exposed to a new research environment and methods, revived my passion for science. Anna, Aysha, Christine, Jack, Jenny, Jon, Lea, Luciana, Pierre, Roberto, Theo and Tom; you are an amazing group of people. I still have my Great Ormond Street Hospital mug standing on my desk for needed coffee breaks. **Jon** special thanks to you for showing me the best spot In London to eat mac and cheese. **Anna** thank you for letting me be your side-kick. You are a wonderful person and I am sure you will finish that PhD soon with great success.

"Always look on the bright side of life"
– Monty Python

Vervolgens zou ik graag de mensen willen bedanken die elke dag opnieuw zorgden voor een gezellige en leuke werksfeer, waarmee ik samen successen kon vieren en bij wie ik tijdens slechte momenten even kon ventileren … a.k.a. de container collega's en sympathisanten; Amaryllis, Amber, Ann, Barbara, Bert, Bram, Brice, Chantal, Charlotte, Diana, Donovan, Dries, Eline, Elly, Eva, Geert, George, Gils, Griet, Hans, Ho-Wa, Ibo, Ilse, Inez, Isabelle, Jessica, Jinthe, Johan, Jolien, Jonas, Jorrit, Joséphine, Joyce, Judith, Kaat, Katrijn, Kelly, Kim C., Kim DR, Maarten, Marjolein, Maxime, Megha, Michel, Nadia, Naomi, Nathalie C., Nathalie D., Rachid, Sam, Sandy, Sanne, Steffi, Stephanie, Steven, Tahnee, Tim, Tine en Wai-Ping. Kerstbomen versieren, bike to work deelnames (en overwinningen!), boeiende conversaties tijdens de vele uren labwerk, quizzen, een kroegentocht Antwerpen, de flash mob repetities voor de trouw van Tine & Rolf, de mannequin challenge, plezier op congressen of gewoon 's middag even uitwaaien in het zonnetje achter het UZA (met special thanx to de huidige Lab-Lunchers crew) … het maakt allemaal dat ik 's morgens met de glimlach kwam werken. Er zijn echter een aantal mensen die ik specifiek wens te vermelden.

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Bij de rechtstreekse collega's reken ik ook het IRIS team van Prof. Nathalie Cools. **Nathalie**, je bent een duizendpoot en straffe madam. Indrukwekkend hoe jij, altijd met de glimlach, 1001 zaken weet te combineren; waaronder Anicells uit de grond stampen. Daarnaast behoud je aandacht voor de mensen rondom jou, ben je down-to-earth, een levensgenieter en gewoonweg een toffe persoon. **Wai-Ping**, bedankt voor alle cheer-up en vrolijke momenten tijdens de eerste jaren; memes, ecards, etentjes, balletvoorstellingen of zotte 'toerten' bakken in je kriekenhuisje. *Have a coffee; do stupid things faster with more energy. Cheers.* **Judith**, je straalt professionaliteit en rust uit, houden zo. Het was altijd leuk om jou tegen te komen in het lab en even bij te babbelen. **Ibo**, work hard play hard. Je kijkt kritisch naar je eigen werk, stelt veel vragen en bent duidelijk gedreven om je project tot een goed einde te brengen. Om het met de wijze woorden van Stephen Hawking te zeggen "Inteligence is the ability to adapt to change" en dat doe je goed. Je enthousiaste en spontane persoonlijkheid zorgde voor een frisse wind door het lab.

"Be yourself; everyone else is already taken"
— Oscar Wilde

Team ophtalmo, onze directe buren en een essentiële waarde in de U-blok symbiose, allemaal een dikke merci. **Nadia**, we miss your bubbly personality. Hope you are having a blast in Boston. **Michel**, mijn zaag en klaag partner in crime, bedankt voor al de opkrikkende

babbels, tips and tricks (verhuizen, reizen, doctoraat finaliseren …) en gezellige onderonsjes. Met jou erbij is het nooit saai. Je hebt meer dan één keer een lach op mijn gezicht getoverd. Niet vergeten, ik verwacht binnen afzienbare tijd een uitnodiging voor de uitreiking van de nobelprijs voor de geneeskunde/vrede 😉. **Steffi**, je staat meestal op de eerste rij om dingen mee te organiseren en coördineren of voor wat welverdiende ontspanning. Bedankt voor de fijne babbels over koetjes en kalfjes. **Bert**, je bent een klasbak wetenschapper, gemotiveerd met een duidelijke visie. Nog eens proficiat met je recente Van den Bogerd *et al*. NEJM 2018! Daarnaast heb je een goede smaak in films en series, en was je altijd wel te vinden voor Friday drinks. Bedankt om mij te introduceren in de wondere wereld van het bierflesjes openen zonder flesopener.

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"When the snows fall and the white winds blow, the lone wolf dies but the pack survives"
— George R.R. Martin

Last but not least zijn de **vrienden** en **familie** aan de beurt. "Hoe vlot het doctoraat?", "waar ben je nu mee bezig?", ook al was het niet altijd even eenvoudig om hier een duidelijk antwoord op te geven, de interesse op zich betekende veel. Sorry dat jullie mij – vaker dan mij lief was – hebben moeten missen omdat ik afspraakjes afzegde wegens een te halen deadline of de onverwachte beschikbaarheid van een (patiënten)staal waarmee ik een experiment wou inzetten. Carlijn, Christine, Daisy, Dounia, Eline, Géraldine, Isa, Isabelle, Katrien, Liesbeth, Shanna, Stefanie, Veerle … merci voor alle leuke momenten tot nu toe: badmintonnen, brunch, farmaweekendjes, restaurantbezoekjes, spelletjesavonden, trouwfeesten en vrijgezellen, het vieren van verjaardagen …, elke keer opnieuw kon ik zo de batterijen weer even opladen.

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Curriculum Vitae

Heleen Van Acker

CAREER OVERVIEW

Oct 2015 - Current	PhD fellow of the Research Foundation - Flanders (FWO)
Jan 2014 - Sep 2015	PhD fellow of the agency for Innovation by Science and Technology (IWT)
Jul 2013 - Dec 2013	PhD in medical sciences Laboratory of Experimental Hematology (LEH), Tumor Immunology Group (TIGR), University of Antwerp Promotors: Prof. Viggo Van Tendeloo and Prof. Evelien Smits Supervisor: Dr. Sébastien Anguille

INTERNATIONAL RESEARCH STAYS

07/01/2019-06/05/2019
I am invited for a research visit to the laboratory of prof. Bruno Silva-Santos in Instituto de Medicina Molecular, the University of Lisbon, Portugal. I will join a sub-team that explores the potential of the patented technology, Delta One T (DOT) cells, in cancer immunotherapy. For this scientific stay I received an FWO Grant for a long stay abroad.

05/02/2017-06/05/2017
I joined John Anderson's Research group at University College London (United Kingdom) from the 5th of February till the 6th of May of 2017. For this visit I received an FWO Grant for a long stay abroad. The work focused on the (antigen-presentation) properties of chimeric antigen receptor engineered γδ T cells (CAR γδ T cells).

HIGHER EDUCATION

Degree	Master of Science in Drug Development: pharmacist
Obtained	July 3, 2013 (with great distinction; 2nd place)
Institute	University of Antwerp
Period	2011-2013
Internship	Pharmacy internship 6 months (Multipharma Heistraat 8, 2610 Wilrijk)
Elective course	Advanced research skills

Thesis	Interleukin-15 dendritic cells induce potent activation of natural killer cells: towards the design of an improved dendritic cell vaccine to treat acute myeloid leukemia Laboratory of Experimental Hematology, Tumor Immunology Group, University of Antwerp / Antwerp University Hospital Faculty of Medicine and Health Sciences Promotor: Dr. Eva Lion, Co-promotor: Prof. Dr. Ingrid De Meester, Mentor: Dr. Sébastien Anguille
Degree	Bachelor of Science in Pharmaceutical Sciences
Obtained	July 6, 2011 (with distinction)
Institute	University of Antwerp
Period	2008-2011

CERTIFICATES

2014 'Laboratory Animal Science for Scientists cat. C' certificate (FELASA accredited) University of Antwerp, prof dr. D. De Deyn and prof dr. C. Van Ginneken

2013 Certificate University of Antwerp/Antwerp University Hospital: taking venous blood with vacutainer system/ placing peripheral venous catheter

DISTINCTIONS / PRIZES

2018 Grant for a long stay abroad (travel expenses and 5385.60 EUR)
| awarded by: Research Foundation - Flanders (FWO)

2018 The AAI Young Investigator Award, 1st Prize poster presentation Gamma delta T cell conference 2018, Bordeaux, France (150 EUR)
| awarded by: the American Association of Immunologists

2018 EHA23 Travel Grant 2018 (free registration and 500 EUR)
| awarded by: the European Hematology Association

2017 Grant for a long stay abroad (travel expenses and 4950 EUR)
| awarded by: Research Foundation - Flanders (FWO)

2016 CIMT Annual Meeting Travel Award 2016 (free registration and 300 EUR)
| awarded by: CIMT (Association for Cancer Immunotherapy)

2014 CIMT Annual Meeting Travel Award 2014 (free registration and 300 EUR)
| awarded by: CIMT (Association for Cancer Immunotherapy)

2013 Immunotools Special Award 2013 (25 reagents)

| awarded by: Immunotools

2013 Qualiphar prize, best master thesis Drug Development UA (500 EUR)
| awarded by: University of Antwerp

PRESENTATIONS

2018 γδ T cells, interleukin-15 and CD56 are up-and-coming players in the fight against leukemia. UZA Research Club, Antwerp University Hospital, Edegem, Belgium

2016 Superior cytolytic immune effector cell recruitment by interleukin-15 dendritic cells. Tumor Immunology and Immunotherapy Symposium, Leuven 2016, Blitz Talk, Campus Gasthuisberg, Leuven, Belgium

2015 Dendritic cell vaccination: the NK cell chapter (Interleukin-15 dendritic cells harness NK cell cytotoxic effector function in a contact- and IL-15-dependent manner.) FNRS contact group on immune microenvironment meeting, GSK venue, Rixensart, Belgium

2014 Contest masterclass scientific communication University of Antwerp
Middelheim Campus, Antwerp, Belgium

POSTERS

- **Heleen H. Van Acker**, Maarten Versteven, Hans De Reu, Peter Ponsaerts, Zwi N. Berneman, Viggo F. Van Tendeloo and Evelien L. Smits. CD56 participation in immune effector cell activation and tumor cell eradication; a role for interleukin-15. Abstract B192, Fourth CRI-CIMT-EATI-AACR International Cancer Immunotherapy Conference: Translating Science into Survival, September, 2018, New York, USA
- **Heleen H. Van Acker**, Sébastien Anguille, Hans De Reu, Zwi N. Berneman, Evelien L. Smits, and Viggo F. Van Tendeloo. *Interleukin-15-cultured dendritic cells enhance anti-tumor gamma delta T cell functions through IL-15 secretion.*
  - Abstract PS1146, 23rd Congress of EHA, June, 2018, Stockholm, Sweden
  - Abstract 005, Gamma delta T cell conference 2018, June, 2018, Bordeaux, France
- **Heleen H. Van Acker**, Ottavio Beretta, Sébastien Anguille, Lien De Caluwé, Angela Papagna, Johan M. Van den Bergh, Yannick Willemen, Herman Goossens, Zwi N. Berneman, Viggo F. Van Tendeloo, Evelien L. Smits, Maria Foti and Eva Lion. *Superior innate immune effector cell recruitment by interleukin-15 dendritic cells*.
  - Abstract C-27, Gamma Delta Conference 2016, June, 2016, London, United-Kingdom
  - Abstract 069, 14th CIMT Annual Meeting, May, 2016, Mainz, Germany
  - Abstract 36, Tumor Immunology and Immunotherapy Symposium, September 2016, Leuven, Belgium

- **Heleen H. Van Acker**, Sébastien Anguille, Johan M. Van den Bergh, Yannick Willemen, Viggo F. Van Tendeloo, Evelien L. Smits, Zwi N. Berneman, and Eva Lion. *Natural killer cells are potently activated by interleukin-15 dendritic cells*. Abstract 316, 12th CIMT Annual Meeting, May, 2014, Mainz, Germany

CONGRESSES and SYMPOSIA

2018 Fourth CRI-CIMT-EATI-AACR International Cancer Immunotherapy Conference: Translating Science into Survival (New York, USA)
2018 23rd Congress of EHA (Stockholm, Sweden)
2018 Gamma delta T cell conference 2018 (Bordeaux, France)
2016 Tumor Immunology and Immunotherapy Symposium 2016 (Leuven, Belgium)
2016 Gamma Delta Conference 2016 (London, United Kingdom)
2016 CIMT annual meeting 2016 (Mainz, Germany)
2016 Kick-off symposium Immuno-Oncology Network Ghent (Ghent, Belgium)
2014 CIMT annual meeting 2014 (Mainz, Germany)
2014 7th Conference on WT1 in Human Neoplasia (Barcelona, Spain)
2014 BACR annual meeting 2014: Immunity and inflammation in cancer (Ghent, Belgium)

EDUCATIONAL ACTIVITIES

Supervision of dissertations

- Co-reader master thesis Charlotte Donders (master in biochemistry and biotechnology 2017-2018): 'Targeting EGFR as therapeutic strategy in HNSCC and CRC'
- Supervision bachelor thesis Jolien De Schrijver (bachelor of biomedical sciences 2016-2017): 'Cellulaire interacties tussen γδ T cellen en dendritische cellen'
- Supervision bachelor thesis Bart Stuer (bachelor of biomedical sciences 2015-2016): 'Gamma/delta T cellen in de strijd tegen leukemie: van bench tot bed'
- Supervision internship and master thesis Lien De Caluwé (master in biochemistry and biotechnology 2014-2015): 'How can IL-15 DC recruit immune cells for subsequent interaction'

Educational guidance - popularization of science

2018 Take part in the "Biotechdag 2018: Geneeskunde op maat": Giving tours to the general public on the subject of harnessing our own immune system in the fight against disease

2015/16/18 Presentation: Vaccinatie als kankerbehandeling, bestaat dat? (in the context of higher education study choice guidance), Sint-Lievenscollege, Antwerp

2014 Interview *DWARS* (90), the student newspaper of the University of Antwerp, 'MICROSCOOP OP WETENSCHAP: ons immuunsysteem in de strijd tegen kanker'

PUBLICATIONS (h-index = 9)

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