Nanobody based approaches for the therapeutic targeting of conserved T cell subsets in cancer

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Chapter 1

Introduction

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1

General introduction

Tumors are often infiltrated with diverse immune cells such as T cells, dendritic cells (DC), myeloid derived suppressor cells (MDSC), macrophages and limited numbers of NK cells. These cells, and the cytokines they produce, play a crucial role in the development and control of cancer and can, individually and collectively, either promote tumor growth (immune regulatory) or hinder tumor progression (pro-inflammatory). However, the function of specific immune cells can be corrupted by the tumor cells and the tumor microenvironment.[1–4] The nature and balance between pro-inflammatory and immune regulatory immune cells in the tumor microenvironment is often related to clinical outcome.[5,6]

Conventionally, immune responses were strictly classified into two types; the innate immune response acting rapidly and specifically and the adaptive immune response requiring more time to develop into a more specialized immune response with pleiotropic effector functions and the ability to create memory. However, this strict distinction has become blurred with increasing knowledge and the identification of new immune subsets. Invariant natural killer T (iNKT) cells and Vγ9Vδ2-T cells are defined as "innate-like" lymphocytes as they combine the adaptive immune response of pro-inflammatory T cells with the rapid response of innate cells.[7,8] This makes them of particular interest for exploitation in tumor immune therapy. Both immune cell subtypes will be discussed in more detail below and an overview of their characteristics is given in Table 1.

Invariant natural killer T (iNKT) cells

iNKT cells are a conserved human T cell subset characterized by expression of the highly restricted Vα24Vβ11-T cell receptor (TCR) in humans and Vα14 preferentially paired with Vβ8.2 in mice and are involved in immune regulation, allergy, autoimmunity, antitumor immunity, a variety of inflammatory disorders and host defense to bacteria, fungi and viruses.[9–12] iNKT cells are reactive to glycolipid antigens presented by the conserved and monomorphic non-classic MHC-class I-like Ag presenting molecule CD1d, expressed on antigen-presenting cells and certain neoplastic cells of lymphoid and myeloid origin.[13–16] Though iNKT cells constitute only a small portion (0.01-0.1%) of the circulating pool of human T cells in healthy adults, they play a key role in initiating immune responses. This can either be an immune regulatory or a pro-inflammatory response, depending on the activation stimulus.[9,17,18] Human iNKT cells are generally $CD4^+$ or $CD4^-$ $CD8^-$ double negative (DN) but a small portion can be $CD8^+$. The $CD4^+$ subset secretes both Th1- and Th2-type cytokines, while the DN and $CD8^+$ subsets predominantly secrete Th1-type cytokines.[19,20] With the ability to rapidly produce a cytokine storm, iNKT cells trigger the activation of multiple immune cells and this is therefore referred to as the 'adjuvant' effect of iNKT cells.[21]

Table 1. Characteristics of iNKT and Vγ9Vδ2-T cells

	iNKT cells	Vγ9Vδ2-T cells
Frequency among human peripheral blood T-cells	0.01-0.1%	1-5%
T cell subtype	Mostly $CD4^+$ and DN but can be $CD8^+$	Mostly DN but can be $CD4^+$ or $CD8^+$
TCR	Vα24Vβ11	Vγ9Vδ2
Ligands	Endogenous ligand not yet identified, suggested are: -Isoglobotrihexosylceramide (iGb3)[66] -β-D-glucopyranosylceramide (β-GlcCer)[67] Exogenous ligand: α-GalCer	Non-peptide phosphoantigens
Antigen presentation	CD1d	Involves BTN3A1*
Functions upon activation	- Cytotoxic against target cells -Rapid activation of immune cell types; NK cells, $CD4^+$ and $CD8^+$ cells, B cells, neutrophils, macrophage, DC and Vγ9Vδ2-T cells (chapter 5)	-Cytotoxic against target cells -Stimulation of DC maturation -Antigen presentation to αβ-T cells

*Direct recognition remains currently elusive.[68]

Although the identity of endogenous iNKT cell ligands remains to some extent elusive, stimulation by the prototypic exogenous ligand α-galactosylceramide (α-GalCer) in the context of CD1d induces various antitumor immune responses. These result from direct cytotoxic effects of activated iNKT cells via the Fas-FasL and granule exocytosis pathways as well as via the production of large amounts of IFN-γ and TNF-α.[9,19] Reciprocal interactions between the activating CD1d-expressing DC and iNKT cell stimulate IFN-γ secretion by activated iNKT cells as well as DC maturation, upregulation of costimulatory molecules and IL-12 production. This together results in sustained IFN-γ production by iNKT cells and induces the activation and proliferation of NK cells and their prolonged secretion of IFN-γ. The latter is believed to have an antimetastatic effect, possibly through the inhibition of angiogenesis.[17,22] Furthermore, the combination of secreted cytokines (e.g. IFN-γ, IL-2 and IL-12) induces the antitumor activity of $CD8^+$ cells, activates B cells, induces the differentiation of $CD4^+$ cells to Th1 or Th2 cells and stimulates the migration of neutrophils and macrophages.[10,17]

Numerical and functional defects of iNKT cells have been reported in patients with various malignancies.[23–25] In addition, mouse studies have indicated enhanced tumor outgrowth and metastasis in the absence of iNKT cells which could be restored by the adoptive transfer

of iNKT cells.[26–28] Several clinical immunotherapy trials have been conducted aimed at the exploitation of iNKT cells and included e.g. administration of α-GalCer, adoptive transfer of α-GalCer pulsed DC or the adoptive transfer of iNKT cells. The treatments were overall well tolerated and multiple patients showed increases in peripheral iNKT cell levels, disease stabilization, and in some cases even partial responses were observed.[19,21,29]

Vγ9Vδ2-T cells

The majority of human T cells expresses an αβ-TCR, however, a small proportion of T cells instead expresses a γδ-TCR. Dependent on their specific chain composition, γδ-T cells are predominantly found in different locations; Vδ1-T cells are mostly located in the intestine and Vδ2-T cells are predominantly present in the peripheral blood, in the majority of cases paired with Vγ9.[30,31] In this manuscript, we focus on the latter subtype.

Vγ9Vδ2-T cells represent 1-5% of all T-cells in the peripheral blood of healthy adults and are mostly DN.[32] Vγ9Vδ2-T cells become activated by the recognition of cells with elevated levels of non-peptide phosphoantigens (pAg), in an MHC-independent manner. This may be the result of an enhanced mevalonate pathway activity which is instrumental in cholesterol synthesis, and is stimulated by cell stress or malignancy, or by the production of bacterial pAg in case of an infection.[33,34] Furthermore, aminobisphosphonate compounds (NBP, e.g. zoledronic acid) sensitize target cells to Vγ9Vδ2 T-cell killing by promoting the intracellular accumulation of endogenous pAg, through the inhibition of mevalonate metabolism.[35,36] In either case, elevated intracellular pAg levels promote translocation of the nuclear GTPase RhoB to the membrane anchored protein butyrophilin (BTN) 3A1 (also known as CD277) to induce a conformational change and membrane reorganization of BTN3A1. This in turn is sensed by the Vγ9Vδ2-T TCR, resulting in Vγ9Vδ2-T cell activation.[37–41]

Activated Vγ9Vδ2-T cells rapidly produce large amounts of pro-inflammatory cytokines (e.g. IFN-γ, TNF-α and chemokines MIP-1 and RANTES) in addition to cytolytic mediators (perforin, granzyme B) to induce specific lysis of cells with elevated pAg levels through the perforin pathway and Fas-induced apoptosis.[42,43] Activation of Vγ9Vδ2-T cells can be further enhanced by engagement of NKG2D which specifically binds ligands expressed by stressed cells (e.g. MICA, MICB and ULBP molecules).[44,45] The upregulation of pAg production in stressed/malignant cells allows Vγ9Vδ2-T cells to discriminate transformed cells from normal cells. Indeed, Vγ9Vδ2 T-cells have been shown to recognize and eliminate malignant cells from multiple tumor types, including multiple myeloma, non-Hodgkin lymphoma, prostate, breast, renal cell, liver and colon cancer.[34,44,46–48] Quantitative and qualitative defects in the Vγ9Vδ2 T-cell population have been found in various malignancies and have been reported to negatively correlate with disease-free survival and response to curative-

intent treatment.[36,49,50] Importantly, these functional Vγ9Vδ2 T-cell defects are reversible.[36] Besides obtaining cytotoxic function, Vγ9Vδ2-T cells can also stimulate the maturation of immature DC and can acquire antigen presenting capacities themselves, which can all contribute to their anti-tumor immune function.[34]

Several clinical trials have studied the use of Vγ9Vδ2-T cells for immunotherapy against both hematological and solid malignancies. These included administration of NBPs or synthetic pAgs alone or in combination with IL-2, and the adoptive transfer of autologous *ex vivo* expanded Vγ9Vδ2-T cells. In general, these approaches were well tolerated and induced clinically relevant antitumor responses in several cases. However, the to date obtained results have not been consistent.[51,52] A limitation of the mentioned Vγ9Vδ2-T cell based cancer immunotherapeutic strategies is that they are all aimed at systemic activation and not necessarily result in the preferential accumulation of these cells in the tumor microenvironment where they would be expected to exert their antitumor effects.

Interestingly, NBP's are already clinically registered for the treatment of patients with hypercalcemia, osteoporosis or metastatic bone disease. This is based on the fact that NBP exposure leads to the inhibition of a crucial step in the mevalonate pathway resulting in a (desired) defective formation, activity and survival of osteoclasts. However, as mentioned before, NBP exposure in this case also results in pAg accumulation and the subsequent (unintended) activation of Vγ9Vδ2-T cells. As a consequence, high levels of pro-inflammatory cytokines are produced leading to acute phase response (APR) symptoms (i.e. flu-like symptoms such as chills, fatigue, myalgia and elevated body temperature) in one third to half of all patients undergoing NBP treatment. Apart from being bothersome to patients, repeated NBP administration may result in Vγ9Vδ2-T cell unresponsiveness by the induction of anergy and exhaustion.[53] Though this will limit the severity of the APR, it might also reduce overall antitumor and antimicrobial immunity in these patients as this is in part controlled by a functional Vγ9Vδ2-T cell population. To date, no agents are available that can clinically inhibit Vγ9Vδ2-T cell activation.

Antibody based therapy using VHHs

The therapeutic use of various antibody (Ab) formats has substantially progressed in recent decades with demonstrated clinical efficacy in the treatment of cancer by e.g. mediating agonistic or antagonistic antigen or receptor functions, or by manipulating immune responses.[54] Generally, antibody platforms used and developed for cancer immunotherapy are based on conventional monoclonal Abs (Figure 1A-C). The family of Camelidea (i.e. llamas, camels and dromedaries) possess a unique antibody class consisting of heavy chain only antibodies which lack the light chain and the CH1 domain of the heavy chain (Figure 1D).[55] The variable antigen binding region of these antibodies consists of only a single

immunoglobulin domain and is called a VHH (or nanobody) (Figure 1E). VHHs share the same large, diversified and specific repertoire of antigen binding sites as conventional Abs but also allow specificity to unique epitopes, including cryptic and not otherwise easily accessible epitopes, due to their distinctive three-dimensional structure.[56,57] Because of their single domain nature, VHHs have unique characteristics such as high stability, solubility and ease of refolding. VHHs can easily be made into multispecificity molecules (Figure 1F) and have a low immunogenicity. Due to the absence of an Fc-domain, VHHs can be produced by Escherichia coli or yeast allowing time and cost reduction.[58,59] These properties render VHH with several unique advantageous characteristics for future clinical therapeutic applications.

Introduction to the chapters

In this thesis, we focus on the role that CD1d-restricted iNKT and BTN3A1-restricted Vγ9Vδ2-T cells can play in tumor immunotherapy and by what means specific VHHs that we have generated can modulate these conserved immune axes.

In Chapter 2, we report an updated analysis of the clinical outcome of head- and neck squamous cell carcinoma patients showing the prognostic relevance of peripheral blood iNKT cell levels as assessed before the start of curative-intent radiotherapy. In addition, the relation between HPV status and iNKT cell numbers in oropharyngeal cancer patients was investigated.

As iNKT cells have been shown to be important in regulating the outcome of various diseases, we additionally focused on exploiting the iNKT-CD1d axis for immunotherapy by generating anti-CD1d VHHs. In **Chapter 3**, the generation and characterization of a panel of CD1d specific VHHs is described which could be used for various applications. We have identified a diverse set of VHHs that could be used to trigger DC activation, block α-GalCer-CD1d induced iNKT cell activation and induce signs of early apoptosis in CD1d expressing tumor cells. These may be relevant for future clinical applications aimed at vaccination strategies, the control of iNKT cell regulated inflammatory disorders, and tumor immunotherapy.

As indicated before, activated iNKT cells can stimulate the antitumor function of conventional T, DC and NK cells. In **Chapter 4**, the stimulatory effect of activated iNKT cells on Vγ9Vδ2-T cell activation and cytotoxicity is explored, providing a rationale for combining iNKT and Vγ9Vδ2-T cell based approaches in cancer immunotherapy.

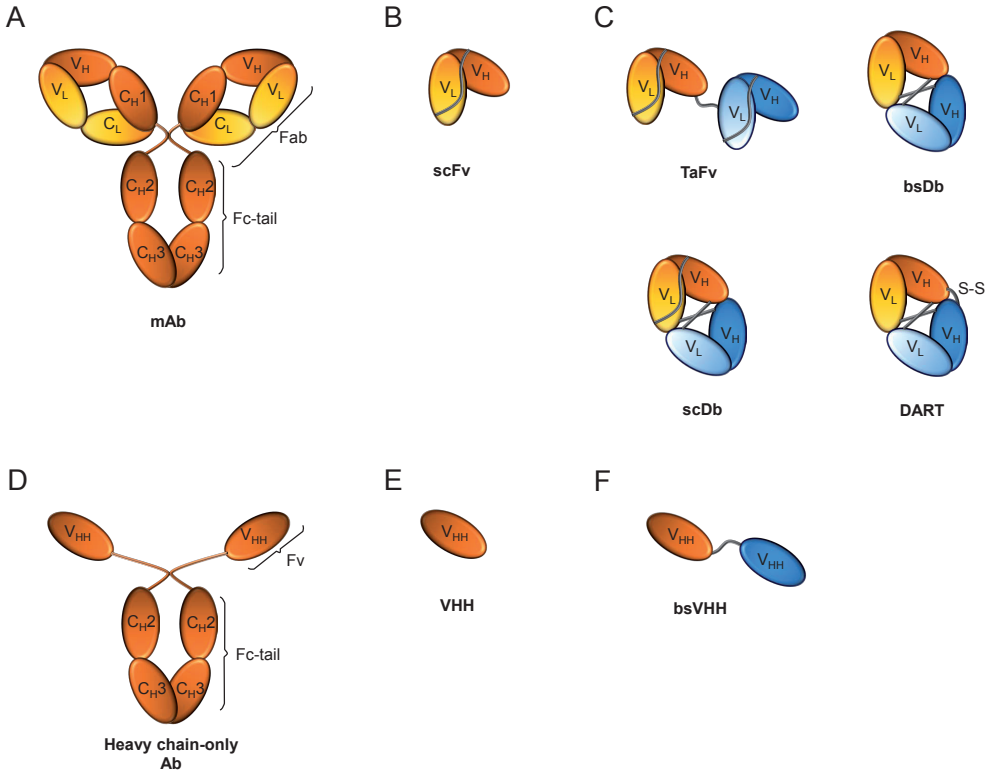

Figure 1. Naturally occurring antibodies and formats developed for immunotherapeutic approaches. Variable heavy chain domains (V_H) are depicted in dark blue and dark orange, variable light chain domains (V_L) are depicted in light blue and light orange. Orange and blue indicate arms with different specificities. Peptide linkers are shown as gray lines, S-S indicates a disulfide bond linker. (A) mAb, conventional monoclonal antibody (B) scFv, single chain variable fragment; (C) various scFv-based formats: TaFv, tandem single chain variable fragment; bsDb, bispecific diabody; scDb, single chain diabody; DART, dual affinity retargeting molecule; (D) heavy chain-only antibody; (E) VHH, variable domain of a heavy chain-only antibody and (F) bsVHH, bispecific VHH.

In **Chapter 5** we describe the generation and characterization of a novel set of Vγ9Vδ2-T cell specific VHH and demonstrate their use for various applications that can be useful for Vγ9Vδ2-T cell research and diagnostic purposes.

In **Chapter 6** the ability of the generated set of Vγ9Vδ2-TCR specific VHHs to inhibit Vγ9Vδ2-T cell activation was studied resulting in the identification of one particular Vγ9Vδ2-TCR specific VHH with the capacity to neutralize NBP-induced Vγ9Vδ2-T cell activation. Detailed cellular and *in silico* molecular modeling analyses were performed to further characterize the mechanism of action of this inhibitory VHH. The identified VHH might be useful as a future therapeutic agent to block the unintentional Vγ9Vδ2-T cell

activation that can occur as a consequence of NBP administration in case of e.g. osteoporosis, hypercalcemia or metastatic bone disease and in addition, may be able to prevent Vγ9Vδ2-T cell unresponsiveness in these patients as well as in patients with certain malignancies that are accompanied by continuous (low-level) Vγ9Vδ2-T cell activation as a result of increased mevalonate pathway activity.

In an effort to promote the preferential accumulation and activation of Vγ9Vδ2-T cells at the tumor site, an activating Vγ9Vδ2-TCR specific VHH was selected and coupled to an antagonistic VHH directed to the tumor antigen epidermal growth factor receptor (EGFR) to create a bispecific VHH. The bispecific VHH simultaneously bound Vγ9Vδ2-T cells and EGFR-overexpressing tumor cells and induced efficient lysis of the tumor cells, which was studied both *in vitro* and in *in vivo* mouse xenograft models in **Chapter 7.** The generated bispecific VHH represents a promising novel candidate for cancer immunotherapy for a large group of patients as this therapy was, in contrast to currently registered anti-EGFR-mAb therapies, also effective against *KRAS* and *BRAF* mutant EGFR-expressing tumors, and was not affected by alterations in the Vγ9Vδ2-TCR δ2-CDR3 region which are known to vary between individuals.[64,65]

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1