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

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Chapter 8

Summarizing discussion and future perspectives

8

Both iNKT and Vγ9Vδ2-T cells are exceptional T cell subsets as they combine the adaptive immune characteristics of pro-inflammatory T cells with the rapid response of innate cells. Furthermore, and in contrast with conventional T cells, ligand recognition and subsequent cellular activation via their highly restricted and conserved T cell receptor (TCR) is MHC-independent and circumvents the need for therapy personalization.[1–3]

iNKT cells are reactive to glycolipid antigens presented by the MHC-class I-like CD1d molecule and, depending in part on co-stimulatory signals and the cytokine microenvironment, can mediate and promote either an anti-inflammatory or pro-inflammatory immune response. The prototypic ligand α-galactosylceramide (α-GalCer) has been widely studied for its ability to induce a pro-inflammatory iNKT cell mediated immune response in which activated iNKT become cytotoxic against target cells and in addition stimulate the activation of other immune effector cells via the predominant production of IFN-γ.[4–6]

Vγ9Vδ2-T cells are the major γδ-T cell subset in human peripheral blood and become activated by the recognition of non-peptide phosphoantigens (pAg) upregulated by "altered-self" cells as a consequence of bacterial infection, stress or malignancy. Vγ9Vδ2-T cell activation can be further enhanced by the engagement of NKG2D with stress-related ligands. Upon activation, activated Vγ9Vδ2-T cells can kill solid and hematological tumor cells of various origins, stimulate the maturation of immature dendritic cells (DC) and present antigens to αβ-T cells.[7–9]

This thesis

In this thesis, we have addressed the role that CD1d-restricted iNKT and BTN3A1-restricted Vγ9Vδ2-T cells can play in tumor immunotherapy and by what means these conserved immune axes could be modulated through the use of variable domains of heavy chain-only Abs (VHH or Nanobodies). The single-domain nature of VHHs provides multiple advantages compared to conventional antibodies e.g. enhanced tissue/tumor penetration, increased stability, solubility, a strong ability to easily refold, low immunogenicity, easy construction into multimeric molecules and the production of functional molecules in *E.coli* or yeast allowing cost- and time reduction.[10–12]

For both Vγ9Vδ2-T and iNKT cells, functional and numerical defects have been observed in cancer patients.[8,13] In **Chapter 2**, we report an updated analysis on the clinical outcome of 47 head- and neck squamous cell carcinoma (HNSCC) patients in whom the peripheral iNKT cell levels before the start of curative-intent radiotherapy were assessed. Patients were stratified according to their iNKT cell levels one day before the start of radiotherapy. Subsequently, overall survival (OS), disease-specific survival (DSS), locoregional control

(LRC) and the development of distant metastasis (DM) were recorded from the start of the therapy until the time of first failure or the most recent follow-up if no relapse was detected, with a median follow-up time of 8.7 years. It was found that patients with low iNKT cell levels had a strikingly unfavorable clinical outcome as determined by OS, DSS and LRC as compared with patients without this deficiency. Since human papillomavirus (HPV) status is a strong independent prognostic factor of survival in oropharyngeal cancer[14], a possible correlation between HPV status and iNKT cell levels was investigated. However, all the oropharyngeal cancer patients with intermediate/high iNKT levels were found to be negative for HPV infection which implicated that the survival benefit found in the intermediate/high iNKT level group was not correlated to HPV status. As a direct relation between low intratumoral iNKT numbers and poor prognosis was found in neuroblastoma and colorectal cancer[15,16], the predictive value of iNKT cells could be a more general phenomenon and underscores the relevance of this small immune cell subset in antitumor immunity.

Cognate interactions between iNKT and CD1d expressing DC are important for reciprocal activation of both iNKT and DC. This cognate help to DC can, at least in part, be mimicked by direct ligation of CD1d by mAbs.[17] In addition, mAb ligation of CD1d expressed by tumor cells induced apoptosis in several malignancies, including B-lymphoblastic and MM cell lines, as well as in MM patient samples.[18] In **Chapter 3** we set out to generate anti-CD1d VHHs with similar functional features as described for the CD1d mAbs**.** From a panel of 21 specific anti-CD1d clones, select VHH clones could be linked to three specific functions. First, two clones were found to induce the maturation and cytokine production of monocyte-derived DC potentially skewing immune responses towards a pro-inflammatory Th1-type response in the absence of other co-stimulatory signals (e.g. TLR- and CD40-signaling). Since CD40 ligation further stimulates DC activation[19], one could envision that a bispecific VHH targeting both CD1d and CD40, whether or not fused to a tumor-associated antigen (TAA), could be a powerful tool for vaccination purposes. Importantly, the size restriction of the lymphoid compartment of lymph nodes (<70 kDa) prevents pathogens from entering[20] though should facilitate easy access of (bi-)specific VHH (~30 kDa) to this conduit system to subsequently ligate DC. This surrogate "cognate" signal could possibly mimic iNKT help and permit DC to prime $CD4^+$ and $CD8^+$ T-cells.[19] Second, one CD1d-specific VHH clone was found to induce increased binding of annexin V on CD1d expressing tumor cells such as B-lymphoblast cells and MM cells, indicative of early apoptosis. Third, one CD1d-specific VHH was identified that could effectively inhibit recognition of the CD1d-α-GalCer complex by iNKT cells thereby inhibiting iNKT cell activation and cytokine production. This VHH could be useful in iNKT cell mediated auto-immune and pulmonary inflammatory disorders. The small size and stability of VHHs make them of particular interest to evaluate their therapeutic effect after local aerosol delivery. Together these anti-CD1d VHHs provide

novel tools that can be useful when considering immunotherapeutic approaches that focus on either blocking CD1d, targeting DC for vaccination, or the induction of apoptosis in CD1d-expressing tumor cells.

Activation of iNKT cells via α-GalCer in the context of CD1d induces a rapid cytokine storm that promotes the activation of NK cells, $CD4^+$ and $CD8^+$ cells, B cell, neutrophils, macrophages and the DC with which they interact.[21–23] In **Chapter 4**, the stimulatory effect of activated iNKT cells on Vγ9Vδ2-T cell activation and cytotoxicity was investigated. Indeed, activated iNKT could induce and enhance Vγ9Vδ2-T cell activation, IFN-γ production and the lysis of tumor cells. This effect was cell-contact independent and co-culture with neutralizing antibodies against several cytokines revealed that this was regulated by TNF-α. The stimulatory effect of iNKT cells on the effector function of Vγ9Vδ2-T cells can be used to strengthen future Vγ9Vδ2-T cell based immunotherapeutic approaches, e.g. by co-loading of monocyte-derived DC with α-GalCer and pAg in order to potentiate anti-tumor immune responses.

The combination of MHC-independent recognition of ligands predominantly exposed by stressed or malignant cells and powerful antitumor effector functions make the Vγ9Vδ2-T cell subset of major interest for exploitation in cancer immunotherapy.[2,24] In **Chapter 5** we have described the generation and characterization of a novel set of 20 different anti-Vγ9Vδ2-TCR VHHs through the specific immunization of llamas. We have shown that the generated VHHs were highly specific for the Vγ9Vδ2-TCR and display variable but overall high affinity to predominantly either the Vγ9- or Vδ2-TCR. The Vγ9- and Vδ2-TCR specific VHHs with the highest affinity were further explored and found to be useful as tools for various research applications e.g. flow cytometric and immunocytochemical detection of Vγ9Vδ2-T cells and efficient Vγ9Vδ2-T cell enrichment from PBMC by magnetic-activated cell sorting (MACS). Importantly, we found both activating and non-activating Vγ9Vδ2-T cell VHHs among the panel of Vγ9Vδ2-TCR VHHs, which are of interest for the further development of Vγ9Vδ2-T cell based immunotherapeutic approaches.

Although Vγ9Vδ2-T cells play an important role in antitumor and antimicrobial defense, there are circumstances in which Vγ9Vδ2-T cell activation can be considered detrimental to the host. Patients given aminobisphosphonates (NBP) for the treatment of hypercalcemia, osteoporosis or metastatic bone disease often experience bothersome side effects resembling an acute phase response (APR).[25–27] This is believed to be caused by the high level of pro-inflammatory cytokine production by activated Vγ9Vδ2-T cells as a consequence of mevalonate pathway inhibition and pAg accumulation. To date, no agents are available that can clinically inhibit Vγ9Vδ2-T cell activation.[24,27–30] In **Chapter 6**, the non-activating Vγ9Vδ2-TCR specific VHHs were investigated for their ability to block the Vγ9Vδ2-TCR.

The VHH with the best Vγ9Vδ2-TCR neutralizing properties was VHH clone 5E7. When stimulated by human NBP-exposed cells, this VHH could significantly inhibit the activation and production of pro-inflammatory cytokines by both human Vγ9Vδ2-T cell lines and *ex vivo* PBMC of various healthy adult volunteers. VHH 5E7 could also neutralize Vγ9Vδ2-T cell activation when exposed to tumor cells that are known to naturally activate Vγ9Vδ2-T cells as a result of an overactive mevalonate pathway. It has been reported that such tumor cells thereby can produce long-lasting supraphysiological pAg levels *in vivo* which in combination with an absence of adequate IL-2 co-stimulation can result in Vγ9Vδ2-T cell unresponsiveness in e.g. chronic lymphocytic leukemia (CLL) and perhaps in relapsed/refractory low-grade non-Hodgkin lymphoma (NHL) and multiple myeloma (MM). Abrogation of such continuous Vγ9Vδ2-T cell activation, e.g. by VHH 5E7, could possibly prevent Vγ9Vδ2-T cell anergy and exhaustion and thereby improve antitumor responses; however, this will need further investigation.[30–32] Moreover, VHH 5E7 can block Vγ9Vδ2-T cell activation by the activating anti-BTN3A1 monoclonal antibody (mAb) 20.1. So far, it remains unclear how the Vγ9Vδ2-TCR exactly senses alterations in BTN3A1 conformation and/or membrane distribution upon elevated levels of pAg leading to its activation.[33] *In vitro* and *in silico* binding analyses in our study indicate that VHH 5E7 predominantly binds to the Vδ2-chain of the Vγ9Vδ2-TCR, and that its predicted binding regions show substantial overlap with the binding regions predicted in our study for the interaction between the Vγ9Vδ2-TCR and BTN3A1, which together is strongly suggestive of a direct interaction of BTN3A1 with the Vγ9Vδ2-TCR. In summary, this reported Vγ9Vδ2-T cell specific VHH that binds with high affinity and stability and that can block pAg-dependent as well as pAg-independent activation of Vγ9Vδ2-T cells, holds promise for the development of an immunotherapeutic strategy designed to prevent unintended Vγ9Vδ2-T cell activation in patients treated with NBP's and possibly in patients that suffer from an unresponsive Vγ9Vδ2-T cell population as a result of continued low-level stimulation by tumor cells.

Multiple clinical studies have explored Vγ9Vδ2-T cells for tumor immunotherapy e.g. by adoptive transfer of *ex vivo* expanded Vγ9Vδ2-T cells or the *in vivo* activation of Vγ9Vδ2-T cells through the administration of NBP's or synthetic pAg, alone, or in combination with low-dose IL-2.[34,35] Though these approaches were well tolerated and capable of inducing clinically relevant anti-tumor responses in several cases, overall results lack consistency. This is probably caused by the fact that all these therapies resulted in a systemic Vγ9Vδ2-T cell activation and not necessarily in the preferential accumulation of these cells in the tumor microenvironment. In **Chapter 7** the generation of a bispecific VHH was described. In this bispecific VHH an agonistic anti-Vγ9Vδ2-TCR VHH was fused to an antagonistic anti-EGFR VHH aiming for a preferential accumulation and activation of Vγ9Vδ2-T cells specifically at the site of the EGFR-expressing tumor, resulting in their specific lysis. As a Vδ2-TCR specific VHH is more specific for Vγ9Vδ2-T cells than a Vγ9-TCR specific VHH,

and the Vδ2-specific VHH 6H4 consistently activated Vγ9Vδ2-T cells of multiple donors, this VHH was selected as the anti-Vγ9Vδ2-TCR VHH unit to be fused to the anti-EGFR VHH unit. The previously reported highly specific antagonistic anti-EGFR VHH 7D12 was selected as a suitable targeting candidate for integration in the bispecific VHH.[36] EGFR was selected as a tumor target as this is a proven clinically relevant tumor target and agents directed at EGFR inhibition (e.g. anti-EGFR mAbs competing for ligand binding, such as cetuximab and panitumumab, and EGFR-specific small molecule kinase inhibitors, such as erlotinib or gefitinib) are relevant treatment options for several advanced-stage epithelial cancers including non-small-cell lung cancer, colorectal cancer, pancreatic cancer, and head and neck squamous cell carcinoma. Though treatment with these agents is related to improved progression free and overall survival, this only applies to a minority of the cancer patients. Furthermore, *RAS* and *BRAF* mutation related unresponsiveness together with frequently occurring skin toxicities are limiting factors that indicate there is room for improvement in EGFR directed therapy.[37,38] The bispecific VHH facilitated simultaneous binding of Vγ9Vδ2-T cells and EGFR-overexpressing tumor cells and thereby stimulated strong Vγ9Vδ2-T cell activation and degranulation resulting in potent lysis of EGFR expressing tumor cells both *in vitro* and in *in vivo* mouse xenograft models. In addition, this novel bispecific VHH would be applicable for a large patient group as induced tumor cell lysis was independent of *KRAS* or *BRAF* mutations and was not affected by variations in the Vγ9Vδ2-TCR δ2-CDR3 region known to vary between individuals.[39] As the anti-EGFR VHH can easily be exchanged for VHHs directed to various other tumor-associated epitopes[40], this novel Vγ9Vδ2-T cell targeted therapy can be applied to a broad range of tumor types.

Future perspectives

Reduced levels and/or an impaired function of iNKT cells have been found in the peripheral blood of patients with multiple malignancies and have been correlated with poor clinical outcome but not with disease stage, nor were their numbers or functionality restored after surgical removal of the tumor.[41,42] The latter observation suggests this iNKT impairment to be an intrinsic cause rather than an effect of cancer and stipulates the importance of this small subset in cancer control. Table 1 provides an overview of published data reporting on a relation between iNKT cell levels and iNKT cell functions in the tumor microenvironment and clinical impact. Varying findings have been reported on γδ-T cell levels in cancer patients, which vary in relation to the γδ-T cell subset and the type of malignancy. However, an impaired function and reduced level of peripheral blood Vδ2$^+$ (most often paired with Vγ9) and Vγ9Vδ2-T cells is a common event in cancer patients and moreover, reduced Vγ9Vδ2-T cell levels in the tumor microenvironment have also been related to clinical stage as summarized in Table 2.[43,44]

Table 1. Study results for iNKT cell levels and function in the peripheral blood and tumor microenvironment of cancer patients.

Study	Study population	Findings
		Levels in the peripheral blood compared to healthy controls
		Low
Yoneda et al.[51]	Hematopoietic malignancy patients (*n*=70)	- Absolute numbers of CD161$^+$ Vα24$^+$-T cells and CD4$^-$CD8$^-$ CD161$^+$ Vα24$^+$-T cells were significantly decreased in patients with hematopoietic malignancies compared to healthy donors. - As individual groups, acute myelogenous leukemia (AML) with no-remission (NR)(*n*=15), malignant lymphoma (ML) with NR (*n*=8) and myelodysplastic syndrome (MDS) with NR (*n*=4) patients had significantly decreased CD161$^+$ Vα24$^+$-T cells numbers, but not chronic myelogenous leukemia (CML)(*n*=7) or ML with complete remission (CR)(*n*=8) patients compared with healthy donors. - Absolute numbers of CD4$^-$CD8$^-$ CD161$^+$ Vα24$^+$-T cells in deceased patients was significantly lower compared to surviving patients.
Azakami et al.[52]	HTLV-1 infected leukemia patients (*n*=11)	- Percentage of Vα24$^+$-T cells in PBMCs was significantly decreased in HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP, *n*=13) and adult T-cell leukemia (ATL, *n*=11) patients compared with that in healthy donors. The percentage of Vα24$^+$-T cells in HTLV-1infected asymptomatic carriers (ACs, *n*=12) was not significantly decreased compared to healthy donors.
Kawano et al.[53]	Melanoma patients (*n*=13)	- Absolute numbers of Vα24Vβ11-T cells were significantly decreased in melanoma patients compared to healthy donors.
Molling et al.[54]	HNSCC patients (*n*=33)	- Absolute numbers of Vα24Vβ11-T cells in PBMC were significantly reduced in HNSCC patients compared to age-matched controls. - Reduced absolute numbers of Vα24Vβ11-T cells in the CD3$^+$ population in HNSCC patients compared to age-matched controls did not reach significance, however a trend was observed. - Lowered % Vα24Vβ11-T cells in the CD3$^+$ population of HNSCC patients correlated to poor disease outcome.
Motohashi et al.[41]	Primary lung cancer patients (*n*=60)	- Percentage and absolute numbers of Vα24Vβ11-T cells in PBMC in primary lung cancer patients (*n*=60) are significantly reduced compared to healthy controls. However, no significant correlation was found between clinical stage of the cancer patients and % Vα24Vβ11-T cells or numbers in PBMC.
Tahir et al.[55]	Advanced prostate cancer patients (*n*=6)	- Percentage of Vα24Vβ11-T cells in PBMC was diminished in advanced prostate cancer patients compared to healthy controls.
Giaccone et al.[56]	Advanced solid malignancy patients (*n*=21)	- Absolute numbers of Vα24Vβ11-T cells were significantly decreased in cancer patients compared to healthy donors.
Molling et al.[42]	Advanced cancer patients (*n*=120)	- Absolute numbers of Vα24Vβ11-T cells are approximately 50% lower in cancer patients compared to age- and sex matched controls. The decrease in Vα24Vβ11-T cell number was independent of tumor type or tumor load and was not restored after surgery in breast cancer patients (*n*=18) or radiotherapy of head and neck cancer patients (*n*=14).
		Similar
Dhodapkar et al.[57]	Myeloma patients (*n*=21)	- No significant difference between absolute numbers of Vα24Vβ11-T cells in PBMC of patients with progressive (*n*=7) or nonprogressive myeloma (*n*=4), MGUS (*n*=10), or healthy controls (*n*=13)
Crough et al.[58]	Solid malignancy patients (*n*=109)	- No significant difference between % or number of Vα24Vβ11-T cells in cancer patients and age- and sex matched controls. However, melanoma patients (*n*=17) had a significantly lower % Vα24Vβ11-T cells and numbers, and breast cancer patients (*n*=10) had a significantly lower % Vα24Vβ11-T cells but not numbers compared to controls.
Yanagisawa et al.[59]	Advanced malignancy patients (*n*=21)	- No significant difference between % Vα24$^+$-T cells in PBMC of patients with advanced malignancy (*n*=21) compared to healthy controls.

		Ex vivo functionality in the peripheral blood compared to healthy controls
		Impaired
Azakami et al.[52]	HTLV-1 infected leukemia patients (*n*=5)	- *In vitro* culture of PBMC with GalCer plus IL-2; Vα24$^+$ cells in the PBMCs from most of the HAM/TSP (n=11) and ATL (n=5) patients showed little or no expansion in contrast to Vα24$^+$ cells in the PBMCs of most of the ACs (n=6). - *In vitro* culture of PBMC with GalCer plus IL-2; perforin expression levels indicative for cytotoxicity were lower in Vα24$^+$ cells from ACs (*n*=4) and HAM/TSP (*n*=4) patients than in those from healthy donors.
Dhodapkar et al.[57]	Myeloma patients (*n*=23)	- *In vitro* culture of PBMC with GalCer loaded DC plus IL-2; impaired IFN-γ production by Vα24Vβ11-T cells in PBMC of patients with progressive (*n*=9) myeloma compared to nonprogressive myeloma (*n*=4), MGUS (*n*=10) or healthy controls (*n*=8). Impaired IFN-γ production by Vα24Vβ11-T cells in PBMC of patients with progressive (*n*=9) myeloma was not related to enhanced IL-4 production.
Tahir et al.[55]	Advanced prostate cancer patients (*n*=6)	- *In vitro* culture of isolated Vα24$^+$ cells with irradiated PBMC, α-GalCer and IL-2; reduced expansion of iNKT cells in advanced prostate cancer patients (*n*=6) compared to prostate cancer patients in remission (*n*=3) and healthy donors (*n*=7). - *In vitro* culture of isolated Vα24$^+$ cells with CD1d-transfected cells; reduced IFN-γ production by iNKT cells of advanced prostate cancer patients (*n*=6) compared to healthy donors (*n*=7).
Crough et al.[58]	Solid malignancy patients (*n*=28)	- *In vitro* culture of Vα24Vβ11-T cells with α-GalCer and IL-2; significant reduced expansion of iNKT cells in cancer patients (*n*=28) compared to healthy donors (*n*=37).
Yanagisawa et al.[59]	Advanced malignancy patients (*n*=21)	- *In vitro* culture of PBMC with α-GalCer and IL-2; significant reduced expansion and cytokine production of Vα24$^+$ cells in cancer patients (*n*=21) compared to healthy donors. However, upon Vα24$^+$ cell sorting and *in vitro* culturing with APC, α-GalCer and IL-2 no significant difference was observed between iNKT cells expansion or cytotoxicity against cell line U937 of cancer patients compared to healthy donors.
		Similar
Kawano et al.[53]	Melanoma patients (*n*=13)	- *In vitro* culture of isolated Vα24$^+$ cells α-GalCer and IL-2; fold expansion and cytotoxicity against the cell lines Daudi lymphoma and SK-Mel-28 melanoma of iNKT cells of melanoma patients were comparable to iNKT cells of healthy donors.
Motohashi et al.[41]	Primary lung cancer patients (*n*=10)	- *In vitro* stimulation isolated Vα24Vβ11-T cells with α-CD3 mAb; no difference observed in IFN-γ production between primary lung cancer patients (n=10) and controls.
Molling et al.[42]	Advanced cancer patients (*n*=11)	- *In vitro* stimulation isolated Vα24Vβ11-T cells with α-GalCer; no difference observed in IFN-γ production between cancer patients (*n*=11) and controls.
		Levels of tumor infiltrating cells in relation to clinical outcome
		Relation
Motohashi et al.[41]	Primary lung cancer patients (*n*=10)	- Median Vα24Vβ11-T cell numbers per gram lung tissue were 2.5 times higher in the lungs of cancer patients (*n*=10) compared to healthy controls.
		No relation
Dhodapkar et al.[57]	Myeloma patients (*n*=4)	- Vα24Vβ11-T cells in tumor bed (bone marrow) were comparable between patients with progressive (*n*=2) and nonprogressive myeloma (*n*=2).
		Ex vivo functionality of tumor infiltrating cells
Dhodapkar et al.[57]	Myeloma patients (*n*=4)	- *In vitro* culture of PBMC with GalCer loaded DC plus IL-2; impaired IFN-γ production by Vα24Vβ11-T cells in tumor bed (bone marrow) of patients with progressive (*n*=2) myeloma compared to nonprogressive myeloma (*n*=2). - *In vitro* culture of PBMC with GalCer loaded autologous DC plus IL-2 for 2-3 weeks; no differences were found in fold expansion of Vα24Vβ11-T cells in PBMC of patients with progressive (*n*=5) and nonprogressive myeloma (*n*=3), MGUS (*n*=4) or healthy donors (*n*=5).

Table 2. Study results for Vδ2$^+$ and Vγ9Vδ2-T cell levels and function in the peripheral blood and tumor microenvironment of cancer.

Study	Study population	Findings
		Levels in the peripheral blood compared to healthy controls
		Low
Argentati et al.[60]	Primary melanoma patients (*n*=23)	-Patients had a significantly lower proportion of Vδ2$^+$-T cells compared to age-matched healthy controls.
Nicol et al.[61]	Advanced solid tumor patients (n=45)	-Patients had significantly lower Vγ9Vδ2-T cells compared to healthy controls. -As a group, patients with melanoma had lower initial Vγ9Vδ2-T cells compared to cancer patients with other malignancies.
		Ex vivo functionality in the peripheral blood compared to healthy controls
		Impaired
Argentati et al.[60]	Primary melanoma patients (*n*=23)	- *In vitro* culture of isolated PBMC; % of Vδ2$^+$-T cells producing IFN-γ and TNF-α before and after stimulation with the pAg isopentenyl pyrophosphate plus IL-2, and expansion, was significantly reduced in melanoma patients in comparison with healthy controls. No difference was determined in cytotoxicity.
Thedrez et al.[62]	Epithelial ovarian carcinoma (EOC) patients (*n*=60)	- *In vitro* culture of isolated PBMC; % and absolute number of Vδ2$^+$-T cells after stimulation with the pAg BrHPP or the NBP zoledronate both plus IL-2, was significantly less expanded in EOC patients in comparison with healthy controls.
Nicol et al.[61]	Advanced solid tumor patients (n=45)	-*In vitro* culture of isolated PBMC with the NBP zoledronate plus IL-2; the majority of cancer patients had a reduced expansion capacity of Vγ9Vδ2-T cells however, as a group, expansion capacity was similar for cancer patients as compared with healthy controls.
		Levels of tumor infiltrating cells in relation to clinical outcome
		Relation
Cordova et al.[43]	Primary melanoma patients(*n*=46)	- % δ2$^+$-T cells of tumor-infiltrated mononuclear cells is inversely correlated with clinical stage in primary melanoma (*n*=46).

Because of the apparent relation between numerical and functional defects in iNKT and Vγ9Vδ2-T cells and clinical outcome of cancer patients, as well as the strong anti-tumor functions that these cell types can effectuate, these immune subsets are very promising for cancer immunotherapy. We have aimed to enhance and leverage the antitumor functions of Vγ9Vδ2-T cells through the development of a bispecific VHH designed to specifically retain and activate Vγ9Vδ2-T cells at the tumor site. A similar approach has recently been reported in which a tribody was constructed from two mouse antibodies, one directed to the γ9-chain of the Vγ9Vδ2-TCR and the other directed to the tumor associated antigen Her2/neu. This (Her2)2xVγ9 tribody efficiently lysed Her2/neu overexpressing pancreatic cells both in *in vitro* and in *in vivo* mouse xenograft experiments[45], as did our bispecific VHH. However, the use of the nearly full-sized (~100kD) tribody has several limitations related to e.g. the mispairing of heavy and light chains, limited stability and the risk of developing human-anti-mouse antibodies (HAMA) in patients leading to antibody neutralization and adverse events in the form of a cytokine release syndrome[46,10] which is expected to be circumvented by the use of VHHs. In addition, due to their small size, VHHs have enhanced tissue/ tumor penetration and can also be cost- and time-effectively produced in bacteria and yeast.[10,47] For our work on the bispecific Vγ9Vδ2-T cell activating VHH, we used EGFR as a model tumor antigen. As mentioned before, this tumor target specific VHH can be easily replaced by a different tumor antigen specific VHH in order to make the bispecific VHH applicable for a larger and variable set of tumors and malignancies. Indeed, VHH specific for various other tumor antigens have already been developed including Her2/neu, VEGFR2, c-Met, CXCR7[40] -and CD1d as described in chapter 7. CD1d can be an interesting tumor target as CD1d has been reported to be expressed by several malignancies e.g. MM, B- and T-acute lymphoblastic leukemia, and colorectal cancer.[6,48,49] Among the CD1d-specific VHHs that we have developed, the CD1d-specific VHH that induced signs of early apoptosis in CD1d transfected B-lymphoblast and multiple myeloma cells would probably be most suitable for incorporation into a bispecific VHH fused to the Vγ9Vδ2-TCR specific VHH, as such a bispecific VHH would on the one hand induce apoptosis via the CD1d-specific VHH and on the other hand simultaneously trigger Vγ9Vδ2-T cell activation and subsequent lysis of the CD1d-expressing tumor cells to which they bind. iNKT cells exert their effects in part by stimulating different immune cells, among which Vγ9Vδ2-T cells, and in this way regulate immune responses. Recently, an iNKT cell tumor targeting construct was described that was generated by fusing a β2-microglobulin (β2m)-CD1d molecule loaded with α-GalCer to a scFv of a humanized anti-HER2 mAb that induced iNKT and NK cell activation, IFN-γ secretion, DC maturation and the inhibition of growth of HER2/neu-expressing lung metastasis in mice.[50] Also in this construct, the tumor antigen Her2-targeting scFv portion could conceivably be replaced by a VHH directed to other tumor antigens making this construct interesting for tumor immunotherapy aimed at iNKT activation. However, it will need to be investigated whether the mentioned iNKT and Vγ9Vδ2-T cell targeting

constructs are able to activate the functionally impaired iNKT and Vγ9Vδ2-T cells in cancer patients and stimulate their proliferation, especially given the fact that iNKT and Vγ9Vδ2-T cell numbers are often reduced. Importantly, iNKT and Vγ9Vδ2-T cells of patients with various advanced-stage malignancies could still be activated upon α-GalCer pulsed autologous mature DC or NBP administration, respectively, indicating that iNKT and Vγ9Vδ2-T cell reactivity in these patients was not completely abolished.[63,] and Schneiders FL et al., submitted. These are promising findings suggesting that an impaired iNKT and Vγ9Vδ2-T cell function can be restored and offer opportunities for cancer immunotherapies aimed at iNKT and/or Vγ9Vδ2-T cell activation.

To date, multiple efforts have been made to redirect immune effector cells to the tumor microenvironment e.g. by using bispecific antibody-based constructs and CAR-T cells. Pronounced effects have been observed in clinical trials, especially for various B-cell malignancies targeting both CD19 and CD3 (e.g. the BiTE blinatumomab and anti-CD19 CAR)[46,64], though there is still room for improvement, in particular for solid malignancies. A major limitation in the targeting of CD3 is that it is expressed by all T cells including immunosuppressive T cells such as regulatory T cells (Tregs) that predominate in the tumor microenvironment and are related to unfavorable prognosis.[65] It has been shown that by using CD3-based targeting approaches, Tregs are target-specifically activated alongside T effector cells and actively suppress the activation and proliferation of effector T cells.[66] For this reason, antibody-based constructs designed to exclusively trigger immune cells with a pro-inflammatory function, such as Vγ9Vδ2-T cells, may be preferable over the targeting of CD3. Of note, the use of these pro-inflammatory VHH constructs may increase immune infiltration of tumors and consequently make them more responsive to currently clinically applied immunotherapies such as immune checkpoint inhibitors, e.g. anti-CTLA-4 or anti-PD-(L)1. In addition and as mentioned before, the use of antibody fragments derived from conventional antibodies requires a variable heavy-light chain domain interaction for optimal performance and is susceptible to instability. Moreover, this required variable heavy-light chain domain interaction necessitates the use of more complex manufacturing systems resulting in increased production costs when compared to the manufacturing costs for VHHs. High manufacturing costs is also a well-known obstacle for the use of CAR-T cell therapy as this approach requires the labor-intensive adoptive transfer of most often autologous immune cells. In contrast, our developed bispecific VHH is expected to be applicable not only ”off-the-shelf” but also to a large patient group as all individuals have Vγ9Vδ2-T cells which despite inter- and intra-individual sequence variation in the Vγ9Vδ2-TCR δ2-CDR3 region[39] can be targeted by our bispecific VHH.

Concluding remarks

In this thesis we have reported on the role that iNKT and Vγ9Vδ2-T cells play in cancer and how the function of these cells can be manipulated for immunotherapeutic purposes using specific VHHs. The Vγ9Vδ2-T cell and CD1d-specific VHHs described in this thesis constitute promising tools not only for the further characterization of these immune cell subsets, but also for the future clinical exploration in various conditions in which these conserved cell subsets have been implicated to play a role.

References

1. Lanier LL, Sun JC. Do the terms innate and adaptive immunity create conceptual barriers? Nat Rev Immunol 2009; 9:302–3.
2. Wu Y-L, Ding Y-P, Tanaka Y, Shen L-W, Wei C-H, Minato N, Zhang W. γδ T cells and their potential for immunotherapy. Int J Biol Sci 2014; 10:119–35.
3. Taniguchi M, Harada M, Dashtsoodol N, Kojo S. Discovery of NKT cells and development of NKT cell-targeted anti-tumor immunotherapy. Proc Jpn Acad Ser B Phys Biol Sci 2015; 91:292–304.
4. Terabe M, Berzofsky JA. The role of NKT cells in tumor immunity. Adv Cancer Res 2008; 101:277–348.
5. Wilson SB, Delovitch TL. Janus-like role of regulatory iNKT cells in autoimmune disease and tumour immunity. Nat Rev Immunol 2003; 3:211–22.
6. McEwen-Smith RM, Salio M, Cerundolo V. The Regulatory Role of Invariant NKT Cells in Tumor Immunity. Cancer Immunol Res 2015; 3:425–35.
7. Bonneville M, Scotet E. Human Vgamma9Vdelta2 T cells: promising new leads for immunotherapy of infections and tumors. Curr Opin Immunol 2006; 18:539–46.
8. Braza MS, Klein B. Anti-tumour immunotherapy with Vγ9Vδ2 T lymphocytes: from the bench to the bedside. Br J Haematol 2013; 160:123–32.
9. Legut M, Cole DK, Sewell AK. The promise of γδ T cells and the γδ T cell receptor for cancer immunotherapy. Cell Mol Immunol 2015; 12:656–68.
10. Harmsen MM, De Haard HJ. Properties, production, and applications of camelid single-domain antibody fragments. Appl Microbiol Biotechnol 2007; 77:13–22.
11. Transue TR, De Genst E, Ghahroudi MA, Wyns L, Muyldermans S. Camel single-domain antibody inhibits enzyme by mimicking carbohydrate substrate. Proteins 1998; 32:515–22.
12. van der Linden RH, Frenken LG, de Geus B, Harmsen MM, Ruuls RC, Stok W, de Ron L, Wilson S, Davis P, Verrips CT. Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. Biochim Biophys Acta 1999; 1431:37–46.
13. Weinkove R, Brooks CR, Carter JM, Hermans IF, Ronchese F. Functional invariant natural killer T-cell and CD1d axis in chronic lymphocytic leukemia: implications for immunotherapy. Haematologica 2013; 98:376–84.
14. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF, Westra WH, Chung CH, Jordan RC, Lu C, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 2010; 363:24-35.
15. Metelitsa LS, Wu H-W, Wang H, Yang Y, Warsi Z, Asgharzadeh S, Groshen S, Wilson SB, Seeger RC. Natural killer T cells infiltrate neuroblastomas expressing the chemokine CCL2. J Exp Med 2004; 199:1213–21.
16. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, Imamura M. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. Clin Cancer Res 2005; 11:7322–7.
17. Yue SC, Shaulov A, Wang R, Balk SP, Exley MA. CD1d ligation on human monocytes directly signals rapid NF-kappaB activation

and production of bioactive IL-12. Proc Natl Acad Sci U S A 2005; 102:11811–6.

18. Spanoudakis E, Hu M, Naresh K, Terpos E, Melo V, Reid A, Kotsianidis I, Abdalla S, Rahemtulla A, Karadimitris A. Regulation of multiple myeloma survival and progression by CD1d. Blood 2009; 113:2498–507.
19. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat Rev Immunol 2013; 13:101–17.
20. Roozendaal R, Mebius RE, Kraal G. The conduit system of the lymph node. Int Immunol 2008; 20:1483–7.
21. Cerundolo V, Silk JD, Masri SH, Salio M. Harnessing invariant NKT cells in vaccination strategies. Nat Rev Immunol 2009; 9:28–38.
22. van der Vliet HJJ, Balk SP, Exley MA. Natural killer T cell-based cancer immunotherapy. Clin Cancer Res 2006; 12:5921–3.
23. van der Vliet HJJ, Molling JW, von Blomberg BME, Nishi N, Kölgen W, van den Eertwegh AJM, Pinedo HM, Giaccone G, Scheper RJ. The immunoregulatory role of CD1d-restricted natural killer T cells in disease. Clin Immunol 2004; 112:8-23.
24. Gogoi D, Chiplunkar S V. Targeting gamma delta T cells for cancer immunotherapy: bench to bedside. Indian J Med Res 2013; 138:755–61.
25. Nussbaum SR, Younger J, Vandepol CJ, Gagel RF, Zubler MA, Chapman R, Henderson IC, Mallette LE. Single-dose intravenous therapy with pamidronate for the treatment of hypercalcemia of malignancy: comparison of 30-, 60-, and 90-mg dosages. Am J Med 1993; 95:297–304.
26. Adami S, Bhalla AK, Dorizzi R, Montesanti F, Rosini S, Salvagno G, Lo Cascio V. The acute-phase response after bisphosphonate administration. Calcif Tissue Int 1987; 41:326–31.
27. Hewitt RE, Lissina A, Green AE, Slay ES, Price DA, Sewell AK. The bisphosphonate acute phase response: rapid and copious production of proinflammatory cytokines by peripheral blood gd T cells in response to aminobisphosphonates is inhibited by statins. Clin Exp Immunol 2005; 139:101–11.
28. Sauty A, Pecherstorfer M, Zimmer-Roth I, Fioroni P, Juillerat L, Markert M, Ludwig H, Leuenberger P, Burckhardt P, Thiebaud D. Interleukin-6 and tumor necrosis factor alpha levels after bisphosphonates treatment in vitro and in patients with malignancy. Bone 1996; 18:133–9.
29. Gober H-J, Kistowska M, Angman L, Jenö P, Mori L, De Libero G. Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 2003; 197:163-8.
30. Thompson K, Rogers MJ. Statins prevent bisphosphonate-induced gamma,delta-T-cell proliferation and activation in vitro. J Bone Miner Res 2004; 19:278–88.
31. Coscia M, Vitale C, Peola S, Foglietta M, Rigoni M, Griggio V, Castella B, Angelini D, Chiaretti S, Riganti C, et al. Dysfunctional Vγ9Vδ2 T cells are negative prognosticators and markers of dysregulated mevalonate pathway activity in chronic lymphocytic leukemia cells. Blood 2012; 120:3271–9.
32. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, Tony H-P. Gammadelta T cells for immune therapy of patients with lymphoid malignancies. Blood 2003; 102:200–6.
33. Wang H, Morita CT. Sensor Function for Butyrophilin 3A1 in Prenyl Pyrophosphate

Stimulation of Human Vγ2Vδ2 T Cells. J Immunol 2015; 195:4583–94.
34. Fournié J-J, Sicard H, Poupot M, Bezombes C, Blanc A, Romagné F, Ysebaert L, Laurent G. What lessons can be learned from γδ T cell-based cancer immunotherapy trials? Cell Mol Immunol 2013; 10:35–41.
35. Latha TS, Reddy MC, Durbaka PVR, Rachamallu A, Pallu R, Lomada D. γδ T Cell-Mediated Immune Responses in Disease and Therapy. Front Immunol 2014; 5:571.
36. Roovers RC, Vosjan MJWD, Laeremans T, el Khoulati R, de Bruin RCG, Ferguson KM, Verkleij AJ, van Dongen GAMS, van Bergen en Henegouwen PMP. A biparatopic anti-EGFR nanobody efficiently inhibits solid tumour growth. Int J Cancer 2011; 129:2013–24.
37. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. N Engl J Med 2008; 358:1160–74.
38. Van den Eynde M, Baurain JF, Mazzeo F, Machiels JP. Epidermal growth factor receptor targeted therapies for solid tumours. Acta Clin Belg 66:10–7.
39. Gründer C, van Dorp S, Hol S, Drent E, Straetemans T, Heijhuurs S, Scholten K, Scheper W, Sebestyen Z, Martens A, et al. γ9 and δ2CDR3 domains regulate functional avidity of T cells harboring γ9δ2TCRs. Blood 2012; 120:5153–62.
40. Kijanka M, Dorresteijn B, Oliveira S, van Bergen en Henegouwen PM. Nanobody-based cancer therapy of solid tumors. Nanomedicine 2015; 10:161–74.
41. Motohashi S, Kobayashi S, Ito T, Magara KK, Mikuni O, Kamada N, Iizasa T, Nakayama T, Fujisawa T, Taniguchi M. Preserved IFN-alpha production of circulating Valpha24 NKT cells in primary lung cancer patients. Int J cancer 2002; 102:159–65.
42. Molling JW, Kölgen W, van der Vliet HJJ, Boomsma MF, Kruizenga H, Smorenburg CH, Molenkamp BG, Langendijk JA, Leemans CR, von Blomberg BME, et al. Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. Int J cancer 2005; 116:87–93.
43. Cordova A, Toia F, La Mendola C, Orlando V, Meraviglia S, Rinaldi G, Todaro M, Cicero G, Zichichi L, Donni PL, et al. Characterization of human γδ T lymphocytes infiltrating primary malignant melanomas. PLoS One 2012; 7:e49878.
44. Raspollini MR, Castiglione F, Rossi Degl'innocenti D, Amunni G, Villanucci A, Garbini F, Baroni G, Taddei GL. Tumour-infiltrating gamma/delta T-lymphocytes are correlated with a brief disease-free interval in advanced ovarian serous carcinoma. Ann Oncol 2005; 16:590–6.
45. Oberg H-H, Peipp M, Kellner C, Sebens S, Krause S, Petrick D, Adam-Klages S, Röcken C, Becker T, Vogel I, et al. Novel bispecific antibodies increase γδ T-cell cytotoxicity against pancreatic cancer cells. Cancer Res 2014; 74:1349–60.
46. Lameris R, de Bruin RCG, Schneiders FL, van Bergen en Henegouwen PMP, Verheul HMW, de Gruijl TD, van der Vliet HJ. Bispecific antibody platforms for cancer immunotherapy. Crit Rev Oncol Hematol 2014; 92:153–65.
47. Tijink BM, Laeremans T, Budde M, Stigter-van Walsum M, Dreier T, de Haard HJ, Leemans CR, van Dongen GAMS. Improved tumor targeting of anti-epidermal growth factor receptor Nanobodies through albumin

binding: taking advantage of modular Nanobody technology. Mol Cancer Ther 2008; 7:2288–97.

48. Chaudhry MS, Karadimitris A. Role and regulation of CD1d in normal and pathological B cells. J Immunol 2014; 193:4761–8.
49. Ni C, Wu P, Wu X, Zhang T, Zhang T, Wang Z, Zhang S, Qiu F, Huang J. Thymosin alpha1 enhanced cytotoxicity of iNKT cells against colon cancer via upregulating CD1d expression. Cancer Lett 2015; 356:579–88.
50. Stirnemann K, Romero JF, Baldi L, Robert B, Cesson V, Besra GS, Zauderer M, Wurm F, Corradin G, Mach J-P, et al. Sustained activation and tumor targeting of NKT cells using a CD1d-anti-HER2-scFv fusion protein induce antitumor effects in mice. J Clin Invest 2008; 118:994–1005.
51. Yoneda K, Morii T, Nieda M, Tsukaguchi N, Amano I, Tanaka H, Yagi H, Narita N, Kimura H, Kawano T, et al. The peripheral blood Valpha24+ NKT cell numbers decrease in patients with haematopoietic malignancy. Leuk Res 2005; 29:147–52.
52. Azakami K, Sato T, Araya N, Utsunomiya A, Kubota R, Suzuki K, Hasegawa D, Izumi T, Fujita H, Aratani S, et al. Severe loss of invariant NKT cells exhibiting anti-HTLV-1 activity in patients with HTLV-1-associated disorders. Blood 2009; 114:3208–15.
53. Kawano T, Nakayama T, Kamada N, Kaneko Y, Harada M, Ogura N, Akutsu Y, Motohashi S, Iizasa T, Endo H, et al. Antitumor cytotoxicity mediated by ligand-activated human V alpha24 NKT cells. Cancer Res 1999; 59:5102–5.
54. Molling JW, Langius JAE, Langendijk JA, Leemans CR, Bontkes HJ, van der Vliet HJJ, von Blomberg BME, Scheper RJ, van den Eertwegh AJM. Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. J Clin Oncol 2007; 25:862–8.
55. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, Balk SP, Exley MA. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. J Immunol 2001; 167:4046–50.
56. Giaccone G, Punt CJA, Ando Y, Ruijter R, Nishi N, Peters M, von Blomberg BME, Scheper RJ, van der Vliet HJJ, van den Eertwegh AJM, et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. Clin Cancer Res 2002; 8:3702–9.
57. Dhodapkar M V, Geller MD, Chang DH, Shimizu K, Fujii S-I, Dhodapkar KM, Krasovsky J. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. J Exp Med 2003; 197:1667–76.
58. Crough T, Purdie DM, Okai M, Maksoud A, Nieda M, Nicol AJ. Modulation of human Valpha24(+)Vbeta11(+) NKT cells by age, malignancy and conventional anticancer therapies. Br J Cancer 2004; 91:1880–6.
59. Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. J Immunol 2002; 168:6494–9.
60. Argentati K, Re F, Serresi S, Tucci MG, Bartozzi B, Bernardini G, Provinciali M. Reduced

number and impaired function of circulating gamma delta T cells in patients with cutaneous primary melanoma. J Invest Dermatol 2003; 120:829–34.

61. Nicol AJ, Tokuyama H, Mattarollo SR, Hagi T, Suzuki K, Yokokawa K, Nieda M. Clinical evaluation of autologous gamma delta T cell-based immunotherapy for metastatic solid tumours. Br J Cancer 2011; 105:778–86.
62. Thedrez A, Lavoué V, Dessarthe B, Daniel P, Henno S, Jaffre I, Levêque J, Catros V, Cabillic F. A quantitative deficiency in peripheral blood Vγ9Vδ2 cells is a negative prognostic biomarker in ovarian cancer patients. PLoS One 2013; 8:e63322.
63. Chang DH, Osman K, Connolly J, Kukreja A, Krasovsky J, Pack M, Hutchinson A, Geller M, Liu N, Annable R, et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. J Exp Med 2005; 201:1503–17.
64. Onea AS, Jazirehi AR. CD19 chimeric antigen receptor (CD19 CAR)-redirected adoptive T-cell immunotherapy for the treatment of relapsed or refractory B-cell Non-Hodgkin's Lymphomas. Am J Cancer Res 2016; 6:403–24.
65. Wilke CM, Wu K, Zhao E, Wang G, Zou W. Prognostic significance of regulatory T cells in tumor. Int J cancer 2010; 127:748–58.
66. Koristka S, Cartellieri M, Theil A, Feldmann A, Arndt C, Stamova S, Michalk I, Töpfer K, Temme A, Kretschmer K, et al. Retargeting of human regulatory T cells by single-chain bispecific antibodies. J Immunol 2012; 188:1551-8.