Summarizing discussion and future perspectives
This thesis has focused on two unique lymphocyte subsets that play an important role in anti-tumor immune responses and interactions between these subsets. iNKT cells constitute a lymphocyte lineage sharing characteristics of both T cells and NK cells. They display a highly restricted TCR repertoire and recognize antigens in the context of a monomorphic CD1d antigen presenting molecule [1,2]. iNKT cells have been shown to play crucial roles in various immune responses, including antitumor responses, based on flexibility with regards to their predominant cytokine profile. As immune-regulatory cells, iNKT cells constitute a bridge between the innate and adaptive immune system by the rapid release of high levels of cytokines creating a micro-environment in which developing adaptive immune responses can be skewed [2,3]. As such, manipulating iNKT cells potentially constitutes a valuable therapeutic approach.

Vγ9Vδ2-T cells are the predominant γδ-T cells subset in human peripheral blood and have well-established anti-tumor effector functions [4]. They can be activated and expanded by phosphoantigens (pAg). Aminobisphosphonates (NBP) support the intracellular accumulation of endogenous pAg by inhibiting the mevalonate metabolism, inducing mobility/conformational changes of the ubiquitously expressed CD277/BTN3A1 that triggers Vγ9Vδ2-T cell activation and expansion [5]. Upon stimulation, Vγ9Vδ2-T cells acquire the capacity to kill solid tumors of diverse origins, produce a diverse set of cytokines and chemokines to regulate other cells, and are able to present antigens for αβ T cell priming. Their abundance and high in vitro anti-tumor potential warrant further studies to enhance their antitumor effects (in vivo and) in patients.

The fact that iNKT and Vγ9Vδ2-T cells are clinically relevant in multiple tumor types [6–9] has triggered clinical trials focusing on these immune subsets. Interestingly, therapeutic manipulation of both iNKT cells and Vγ9Vδ2-T cells has been shown to induce immunological, biochemical and even clinical responses in patients treated with specific activating ligands without causing substantial toxicity upon activation. However, clinical results lacked consistency. Several factors can/may play a role in the variable induction of antitumor immune responses in clinical trials that have been performed to date, including the low number of iNKT cells in patients with cancer, occurrence of anergy induction upon intravenous antigen stimulation and the role of micro-environmental conditions. In this thesis we have studied aspects of iNKT cells and Vγ9Vδ2-T cells as well as potential interactions between these subsets in an effort to gain more insight into cross-talk in the induction and effector phase between these unique immune subsets. In addition we explored new therapeutic approaches to exploit the antitumor effector functions of these cells in patients with cancer.
(summarized in figure 1).

Most striking clinical and preclinical effects with the iNKT cell activating ligand α-GalCer have thus far been obtained when α-GalCer was loaded onto dendritic cells (DC), either ex vivo or in situ, e.g. on skin-resident subsets [10–12]. As described in Chapter 3 we have developed and optimized protocols to generate immature and mature monocyte-derived DC (moDC) from peripheral blood and to optimally expose them to α-GalCer to allow activation and biased cytokine production in responding iNKT cells. Furthermore, as direct ligation of CD1d using monoclonal antibodies has also been shown to induce Th1-biased immune responses, this methodology was also described as a promising approach to the immunotherapy of cancer. The impact of NKT cells in terms of tumor control has been demonstrated by their prognostic value in patients with cancer.

In Chapter 4, we have provided an updated analysis with a median follow-up time of 8.7 years of a study in which circulating iNKT cell numbers were assessed in a group of 47 patients with HNSCC before the start of curative intent radiotherapy. This study demonstrated that patients with HNSCC and a severe deficiency of iNKT cells have a strikingly poor clinical outcome, with respect to overall survival, disease specific survival and loco-regional control compared to patients without this deficiency. Furthermore, it was found that the survival benefit of patients with intermediate/high iNKT cell numbers was not related to differences in HPV infection. Since the correlation between a low frequency of intra-tumoral iNKT cells and poor prognosis was also noted in neuroblastoma [8] and colorectal cancer [9], the predictive value of iNKT cells could be a more general phenomenon and, despite their small numbers, underscores their important role in the regulation of anti-tumor immune responses.

Numerous studies have been carried out to characterize the cross-talk between iNKT cells and other conventional effector cell subsets encompassing NK, T and myeloid subsets in the immune response against cancer (see Figure 1 for a schematic overview). In aggregate, these studies point to the versatile direct and indirect ways in which iNKT cells can contribute to the antitumor effector response and the elimination of tumor-mediated immune suppression, both systemically and in the tumor microenvironment.

In contrast, relatively little is known about cross-talk between iNKT cells and Vγ9Vδ2-T cells and how these subsets can mutually modulate each other’s antitumor effector functions.
Figure 1 Overview of direct and indirect antitumor effects of iNKT cells and mechanisms of cross-talk between iNKT and other myeloid and lymphoid immune effector subsets, resulting in reduced immune suppression and the boosting of antitumor immunity. Adopted from [13,14].

In Chapter 5 we therefore set out to explore whether the antitumor activity of Vy9Vδ2-T cells could be potentiated by iNKT cells.

We found that activation of iNKT cells using α-GalCer resulted in an enhancement of the activation of Vy9Vδ2-T cells induced by pAg. Co-activation of iNKT cells also enhanced the IFN-γ production as well as the cytolytic potential of Vy9Vδ2-T cells against tumor cells. This potentiating effect of iNKT cells was mediated by their production of TNF-α. These observations have thus expanded
the spectrum of immune cells affected by iNKT cells from conventional T cells, NK cells, B cells, DC and Tregs, to include Vy9Vδ2-T cells and have clearly shown how this interaction may contribute to antitumor immunity.

As mentioned before, clinical trials with α-GalCer–pulsed moDC have shown anecdotal antitumor activity in advanced cancer. However, there are several practical limitations that hamper further clinical exploration of treatment with α-GalCer–pulsed moDC. As Vy9Vδ2-T cells have been shown to be able to acquire professional antigen-presenting capacities upon their activation by pAg [15,16], we explored their capacity to present glycolipid antigen to iNKT cells in Chapter 6. Vy9Vδ2-T APC were indeed found to be able to present α-GalCer to iNKT cells resulting in their activation. However, glycolipid antigen presentation was shown not to result from the de novo synthesis of CD1d by Vy9Vδ2-T, but to depend on trogocytosis of CD1d-containing membrane fragments from pAg-expressing cells with which Vy9Vδ2-T cells interact. Although we found that α-GalCer loaded moDC clearly outperformed Vy9Vδ2-T cells functioning as APCs on a per cell basis, Vy9Vδ2-T cells had the advantage of a quicker maturation into APCs as well as that of quantitative superiority, being more numerous as compared to moDC precursors. Furthermore, culture supernatants of iNKT activated via Vy9Vδ2-T cells as APCs were more biased toward a Th1 type cytokine profile –with a higher IFNγ/IL-4 ratio– than those iNKT cells stimulated by moDC's. These features make Vy9Vδ2-T cells an interesting platform for consideration in future clinical trials as reviewed in Chapter 7.

We next determined the effects of NBP on glycolipid Ag-induced iNKT cell activation in vitro and found that NBP resulted in a striking reduction of moDC-mediated α-GalCer-induced iNKT activation (Chapter 8). This inhibitory effect was found to be caused by an NBP-induced reduction in the production of apolipoprotein E (apoE), which is a known facilitator of trans-membrane transport of exogenously derived glycolipids by DC. These data on the inhibitory effect of NBP on glycolipid-mediated iNKT cell activation should be taken into account in the design of future iNKT cell-based anti-cancer therapies, as NBP are regularly prescribed in cancer patients with bone metastases.

Though it is known that intravenous administration of NBP can induce powerful systemic activation of Vy9Vδ2-T cells in vivo and that these effects are strongly associated with the occurrence of an acute phase response (APR), the effect of NBP on the induction of APC markers on circulating Vy9Vδ2-T cells and the potential of statin pretreatment of patients to inhibit Vy9Vδ2-T cell activation and the related occurrence of an APR have not been extensively studied. In Chapter 9 we confirmed that in vivo treatment with NBP induced Vy9Vδ2-T cells
to develop towards an activated and Th1 cytokine producing effector phenotype accompanied by clinical signs of an APR. Circulating Vy9Vδ2-T cells did not acquire phenotypic characteristics of APC. Although treatment with simvastatin, started at least 1 week before the first NBP administration, was found to limit perforin, granzyme B and HLA-DR expression by Vy9Vδ2-T cells activated by NBP, simvastatin was not able to fully prevent either the development of an activated Th1 cytokine producing effector phenotype by Vy9Vδ2-T cells or the occurrence of APR upon NBP-infusion. This limited effect of statins could be related to an incomplete inhibition of the mevalonate pathway at the doses used in our study resulting in sufficient pAg accumulation in response to NBP administration for Vy9Vδ2-T cells to still become activated. Though higher therapeutic dosing of statins could be more effective, another approach to prevent the APR response in patients treated with NBP could consist of the direct blocking of pAg recognition by Vy9Vδ2-T cells, e.g. by using neutralizing Vy9Vδ2-TCR specific antibodies. For an overview of the findings from this thesis, see Figure 2.

Figure 2 Overview of the findings from this thesis outlining the interactions between iNKT and Vy9Vδ2-T cells with possible consequences for the treatment of cancer.

Future clinical perspectives
A general drawback of many of the clinical trials thus far performed with iNKT cells and Vy9Vδ2-T cells is that, though they are effective in inducing the specific (systemic) activation of these cell subsets, they provide no specific trigger for these cells to accumulate at the tumor site where they would be expected to exert their antitumor effect. Also, readouts of iNKT and Vy9Vδ2-T cell responses
are primarily from peripheral blood rather than (tumor) tissues. Analysis of iNKT cells and Vy9Vδ2-T cells in the tumor-environment (e.g. cytokine responses) is crucial, especially since down-regulation of the TCR upon activation complicates unambiguous identification of these cells. Indeed, promising clinical responses have been reported in patients with HNSCC after local activation of iNKT cells, suggesting that the activation of iNKT cells within the tumor micro-environment could be effective [17]. In light of this approach, therapies specifically targeting iNKT cells and Vy9Vδ2-T cells to the tumor microenvironment are of interest. Therapeutic antibody fragments in development, e.g. single-chain Fv fragments (scFvs), camelid VHH domains, human domain antibodies (dAbs), and an ever expanding array of bispecific/dual-targeting variants (e.g. Bi-specific T cell engagers (BiTEs)), are aiming at inducing Th1-biased responses in the generally suppressive tumor-microenvironment ([18,19] and reviewed in [20]). These targeting approaches could possibly be further supported by increasing the frequency of Vy9Vδ2-T or iNKT cells, for example by means of adoptive transfer or in vivo stimulation and expansion of Vy9Vδ2-T or iNKT cells, approaches that have been shown to be safe therapeutic modalities resulting in objective clinical responses in a variety of cancer types (reviewed in [21]).

One can envision that the stimulatory effect of iNKT cells on the effector function of Vy9Vδ2-T cells, as described in chapter 5, can be used to strengthen future Vy9Vδ2-T cell based immunotherapeutic approaches. Combined presence of activated iNKT and Vy9Vδ2-T cells at the effector site could conceivably potentiate anti-tumor immune responses. This strategy is broadly applicable, since both iNKT and Vy9Vδ2-T cells can kill a wide variety of tumor targets and have well conserved Ag recognition receptors. Another advantage of this strategy, beside the enhancement of the anti-tumor effector functions of Vy9Vδ2-T cells, is the ability of Vy9Vδ2-T cells to act as professional APC at the tumor site. The APC function in human Vy9Vδ2-T cells has been described by Brandes et al. [15] and might be highly relevant to immunotherapy. As described above, we have shown in chapter 6 [22] that Vy9Vδ2-T APC can present antigens to iNKT, as a result of trogocytosis of CD1d-containing membrane fragments from pAg-expressing cells. CD1d-expressing Vy9Vδ2-T cells were able to activate iNKT in a CD1d-restricted and a-GalCer-dependent fashion. In order to achieve a more effective APC platform for iNKT cell-based immunotherapy, one could imagine using retroviral CD1d transduction of Vy9Vδ2-T cells, hypothesizing that the limited CD1d-restricted APC function of Vy9Vδ2-T cells could be related to the relatively limited surface density of CD1d, when compared with transfectants or moDC.
Other aspects of cross-talk between Vγ9Vδ2-T and iNKT cells in the tumor micro-environment should be carefully studied when aiming for a (Th1-biased) anti-tumor immune response. Vγ9Vδ2-T cells show remarkable plasticity that is regulated by different stimuli. For instance, it has been found that activation of Vγ9Vδ2-T cells in the presence of differential stimuli could either promote Th17- or Treg-like-Vγ9Vδ2-T cells, resulting in potentially tumor-promoting responses, favoring tumor cell proliferation and metastatic spread (reviewed in [23]). These observations clearly suggest that identification and elimination of these stimuli in the micro-environment could be important in establishing an anti-tumor immune response without unwanted collateral effects. This concept of environmental programming is also valid for iNKT cells, as for instance Tahir et al. found that the functional defect in iNKT cells in patients with advanced prostate cancer, appeared to be reversible by administration of the pro-inflammatory cytokine IL-12 [24].

Furthermore, repeated i.v. administration of α-GalCer induced iNKT cell anergy, while repeated administration of CD1d targeted antibody constructs resulted in sustained iNKT cell activation [25,26], suggesting that tumor-targeting of iNKT cells or of α-GalCer–pulsed APC might constitute a powerful but as yet unexplored clinical approach. Other approaches that warrant further exploration with respect to their capacity to enhance the efficacy of iNKT cell based immunotherapy include alternative routes of administration of α-GalCer [12], and the use of alternative glycolipid ligands such as the non-glycosidic compound threitolceramide, which was shown to efficiently activate iNKT cells, resulting in maturation of dendritic cells and the priming of Ag-specific T and B cells, while preventing ligand-induced iNKT cell lysis of pulsed dendritic cells as well as activation-induced anergy of iNKT [27].

New and highly promising in the field of immunotherapy are immune checkpoint inhibitors (e.g. anti-CTLA4 and anti-PD1/PD-L1 mAbs), leading to durable immune responses and long-term disease control in different types of cancer (e.g. in melanoma [28,29]). However, impressive responses occur in a limited group of patients, emphasizing the need for further improvement of this approach. We hypothesize that checkpoint inhibitors could have a beneficial effect on antitumor responses of iNKT and Vγ9Vδ2-T cells, and vice versa that specific activation of iNKT and Vγ9Vδ2-T cells could possibly enhance the immune responses of checkpoint inhibitors. As mentioned previously, therapies specifically targeting iNKT and Vγ9Vδ2-T cells to the tumor microenvironment are of interest and could be of benefit in combination with immune checkpoint blockade. The development of therapeutic bispecific/dual-targeting antibody
Summarizing discussion and future perspectives

fragments (BiTEs) seems very promising, as for example blinatumomab is already registered for treatment of relapsed/refractory B-precursor acute lymphoblastic leukemia (ALL) leading to durable remission by engaging T cells through CD3 of the TCR complex to induce lysis of CD19 expressing cells [30]. Many of the current bispecific approaches target CD3 and do not discriminate between pro-inflammatory T cells and regulatory T cells and can thus also target Tregs to the tumor microenvironment. Specific targeting of conserved iNKT or Vy9Vδ2-T cells could avoid this detrimental targeting of Tregs and perhaps further potentiate the generation of a durable pro-inflammatory anti-tumor response.

Concluding Remarks

In summary, in this dissertation we have studied the functional interactions between iNKT and Vy9Vδ2-T cells, and how these can interfere with or enhance anti-tumor immune effector responses mediated by these conserved T cell subsets. Although we have shown that concurrent activation of iNKT cells and Vy9Vδ2-T cells is very potent, many challenges remain to overcome the susceptibility of these cells to the immunosuppressive environment that cancer cells induce. Crosstalk between these cells raises possibilities for novel iNKT and Vy9Vδ2-T cell based therapeutic approaches. In order for these approaches to be successful, it is essential to also address the immune suppressive microenvironment by novel or additional strategies. Indeed, targeting these invariant T cell subsets to the tumor may conceivably contribute to this, by tipping the balance in the tumor-microenvironment toward a Th1 biased response, thereby enhancing anti-tumor immunity. Between these in-betweeners novel solutions may thus arise for the effective immunotherapy of cancer.

References

5. C. Harly, Y. Guillaume, S. Nedellec, C.-M. Peigné, H. Mönkkönen, J. Mönkkönen, et al., Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major
CHAPTER 10


21. C.L. Deniger DC, Moyes JS, Clinical applications of gamma delta T cells with multivalent


