CHAPTER 1

The in-betweeners: a short introduction

Adapted from:
Bispecific antibody platforms for cancer immunotherapy.
Lameris R, de Bruin RC, Schneiders FL, van Bergen en Henegouwen PM, Verheul HM, de Gruijl TD, van der Vliet HJ.
Traditionally, immune responses were categorized into two types: innate and adaptive. The traditional opinion that innate immunity is nonspecific and lacks immunologic memory whereas adaptive immunity is characterized by specific antigen recognition and immunologic memory is no longer accurate or sufficient. Innate-like lymphocytes, also called ‘in-betweeners’, including γδ T cells and NKT cells, combine conventional adaptive features with rapid, innate-like responses, and thereby play an important role in initiating and orchestrating immune responses [1–3].

**Invariant Natural killer T (iNKT) cells**

Invariant natural killer T (iNKT) cells represent a distinctive population of lymphocytes characterized by a (semi-)invariant TCR. Unlike conventional T-cells, iNKT cells recognize (glyco-)lipids presented by non-polymorphic CD1d molecules. Upon stimulation, iNKT cells rapidly secrete a wide range of cytokines and induce activation of effector cells (e.g. NK and CTL) in an IFN-γ dependent manner. [4–6] iNKT cells were shown to contribute to immune surveillance in early-stage tumors and chemically induced cancers and appear to play a pivotal role in controlling different forms of cancer, at least in mice. [7,8] Moreover, iNKT cells from patients with advanced cancer display quantitative and qualitative defects and circulating numbers correlate with patient survival. [7–10] Reciprocal interactions between dendritic cells (DC) and iNKT cells can reverse defects in the iNKT cell population. Indeed, *in vitro* results indicate rehabilitation of iNKT cell function after stimulation with monocyte derived DC (moDC) pulsed with the agonistic CD1d ligand α-galactosylceramide (α-GalCer) and exogenous IL-12. [11,12] It was shown that sustained activation of iNKT cells at the tumor site could be induced after systemic treatment with α-GalCer loaded on soluble CD1d fused to an anti-tumor scFv. Potent tumor inhibition of aggressive tumor grafts expressing the targeted antigen was observed in mice. [13,14] Although less clear than in mice, clinical studies evaluating injection of α-GalCer-pulsed moDC with or without adoptive transfer of *ex vivo* expanded iNKT cells have reported objective tumor regressions in several patients. [15–18] In patients with recurrent HNSCC, nasal submucosal administration of αGalCer-pulsed APCs combined with intra-arterial infusion of activated iNKT cells via tumor-feeding arteries produced an objective response in 5 out of 10 patients. [15] The number of infiltrating iNKT cells in extirpated tumor tissue correlated with clinical outcome. [18] The precise role of iNKT cells in human cancer treatment still has to be determined, however, clinical data underscore their role in tumor immune-surveillance and indicate beneficial effects with low toxicity in cancer
The In-betweeners: a short introduction
treatment.

**Figure 1.** iNKT cells are activated by lipid antigens presented in the context of a CD1d antigen presenting molecule. Activation of Vy9Vδ2-T cells is initiated by conformational changes of CD277 upon intracellular phosphoantigen accumulation (isopentenyl pyrophosphate (IPP)). Adapted from [19].

**Vy9Vδ2T-cells**
γδ T-cells, once regarded an evolutionary redundant T-cell subset *en route* to extinction, have recently been demonstrated to hold a unique position in the immune system. They can directly lyse stressed or infected cells, produce a diversified set of cytokines and chemokines to regulate both immune and non-immune cells, and can present antigens for αβ T-cell priming. [2] Vy9Vδ2-T cells constitute the predominant γδ-T cell subset in human peripheral blood and account for 1–5% of peripheral blood mononuclear cells (PBMC) of healthy adults. Vy9Vδ2-T cells can be activated and expanded by non-peptidic pyrophosphate antigens (pAg), of which there are both host and microbe-derived counterparts, typified by isopentenyl pyrophosphate (IPP) and hydroxymethyl-but-2-enylpyrophosphate (HMBPP) respectively. Furthermore, aminobisphosphonate compounds (e.g. zoledronic acid and pamidronate) sensitize target cells to Vy9Vδ2-T cell killing by promoting the intracellular accumulation of endogenous IPP by inhibiting mevalonate metabolism. [20] Recently, it was reported that CD277/ butyrophilin (BTN) 3A1 is required for the presentation of pAg to
Vy9Vδ2 T cells. [21,22] In stressed/malignant cells pAg production is frequently upregulated allowing discrimination from normal tissue. Indeed, Vy9Vδ2 T-cells have been shown to be able to recognize and eliminate malignant cells from multiple tumors types, including multiple myeloma (MM), NHL, prostate-, renal cell- and colon cancer. Of interest, quantitative and qualitative defects in the Vy9Vδ2 T-cell population have been observed in various malignancies [20] and negatively impact disease-free survival, e.g. in ovarian carcinoma. [23] Importantly however, these functional Vy9Vδ2 T-cell defects are reversible. [20] In patients with various metastatic cancers treatment with zoledronic acid and IL-2 promoted the differentiation of peripheral blood Vy9Vδ2 T-cells toward an effector/memory-like phenotype with augmented numbers correlating with arrested disease progression. Observed toxicities were minor and limited to transient flu-like symptoms. [24,25] Adoptive transfer of Vy9Vδ2 T-cells following ex vivo expansion by pAg, aminobisphosphonates or mAbs combined with IL-2, in patients with various types of metastatic cancer resulted in some clinical responses. [20] Of interest, Vy9Vδ2T-cell mediated lysis of hepatic tumor cell lines could be significantly enhanced by an anti-EpCAM-anti-CD3 BiTE in vitro. [26] Although clinical data are still scarce, preliminary findings clearly indicate that exploiting the natural abilities of Vy9Vδ2 T-cells in cancer immunotherapy is feasible and carries low toxicity.

Introduction to the chapters
Both iNKT cells and Vy9Vδ2-T cells have shown great promise in anti-tumor immune responses and interest in exploiting these lymphocyte subsets for the induction of potent and enduring antitumor immune responses has grown rapidly. Moreover, given the current spectacular results of immune checkpoint inhibitors and the rising interest in bispecific targeting constructs, specific activation of iNKT and Vy9Vδ2-T cells has more potential than ever before. Multiple studies have shown immunological, biochemical and even clinical responses in patients treated with specific activating ligands, underscoring their role in anti-tumor immunity. However, results lack consistency and many challenges remain to be overcome, since both iNKT and Vy9Vδ2-T cells can be influenced greatly by the environment in which they are in. In this dissertation we have studied iNKT and Vy9Vδ2-T cells in detail and investigated cross-talk between these subsets, evaluating whether their reciprocal interactions can interfere or enhance anti-tumor immune responses.
**Chapters 1 and 2** provide an introduction to iNKT and Vγ9Vδ2-T cells and review the clinical experience that has been obtained in the field of cancer immunotherapy.

In **Chapter 3** we provide a detailed description of how immature and mature moDC can be generated from peripheral blood and are optimally exposed to α-GalCer to allow activation and biased cytokine production in responding iNKT.

**Chapter 4** provides an updated analysis of a study in which circulating iNKT cell numbers were assessed in a group of HNSCC patients before the start of curative intent radiotherapy. It was shown that patients with HNSCC that have a severe deficiency of iNKT cells have a strikingly poor clinical outcome.

We studied the effects of iNKT cell activation on Vγ9Vδ2-T cells in **Chapter 5**, and found that co-activation of iNKT cells enhanced the IFN-γ production as well as the cytolytic potential of phosphoantigen-activated Vγ9Vδ2-T cells and that this effect was mediated via the production of TNF-α.

In **Chapter 6** we explored the capacity of Vγ9Vδ2-T cells to act as antigen presenting cells and present glycolipid antigen to iNKT cells. Vγ9Vδ2-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 Vγ9Vδ2-T cells, but depends on trogocytosis of CD1d-containing membrane fragments from pAg-expressing cells with which Vγ9Vδ2-T cells interact.

We further discuss the role of Vγ9Vδ2-T cells in cancer immunotherapy in **Chapter 7**, focusing on their putative use as a novel APC-platform for potentiation of iNKT cell based cancer immunotherapy.

In **Chapter 8** we investigated the effect of NBP on glycolipid Ag presentation by moDC and found that treatment of moDC with NBP during DC maturation resulted in a striking inhibition of iNKT cell activation. This inhibitory effect was found to be caused by a reduction in moDC apolipoprotein E (apoE, a known facilitator of trans-membrane transport of exogenously derived glycolipids) production and could be relieved by supplementing apoE in the culture medium.

**Chapter 9** describes the studied effects of intravenous administration of NBP and statins on circulating Vγ9Vδ2-T cells in advanced cancer patients. We found that administration of NBP resulted in the rapid activation of
circulating Vy9Vδ2-T cells, with circulating numbers dropping immediately after infusion and recovering within the next week. Pretreatment of patients with statins did not significantly reduce the observed activation nor did it prevent the occurrence of an acute phase response (APR).

References