PART I

Relevance of invariant Natural Killer T cells in human cancer
Chapter 1

Introduction
The relation between cancer and the immune system

Cancer is a progressive disease with increasing incidence and is gradually outnumbering cardiovascular disease as the leading cause of death in the Western community. Population ageing, lifestyle and environmental factors all play a part in cancer development. In addition, recent advances in cancer detection, using novel diagnostic and screening tests, contribute to the growing incidence of cases. This leads to an increasing demand for therapeutic intervention. Treatment of patients at an early stage of the disease with surgery, radiotherapy, and/ or chemotherapy usually results in a good clinical outcome. Unfortunately, when patients are presented at a well advanced stage of disease (which is often the case) these conventional therapies show limited efficacy, resulting in high mortality rates. This has stimulated interest in developing complementary therapeutic strategies.

Within the concept of anti-tumor immune therapy it is attempted to exploit the patient’s immune system to eradicate malignancies from the body. This is often performed in an adjuvant setting simultaneous with or following conventional treatment. In humans an effective immune response is presumably essential to naturally eradicate malignant cells. This is illustrated by observations in immunocompromised individuals, who have a higher incidence of tumors (reviewed in [1]). Direct evidence for a relation between immune suppression and cancer genesis is lacking to date. Nonetheless, data suggestive of this relation keep emerging. In a recent meta-analysis comparing 7 HIV/ AIDS patient cohorts (n=444172) with 5 transplant recipient cohorts (n=31977) a striking similarity was observed of the patterns of increased risk for a broad variety of malignancies, especially those of viral etiology [2]. Both populations differed in lifestyle and other host risk factors for the development of cancer. Hence, the authors concluded that in immune-compromised individuals, immune deficiency is the predominant risk factor for development of advanced stage cancer. Also in cancer progression an important role for the immune system is most likely, since individuals with a large amount of tumor infiltrating activated T and/ or NK cells have a more favorable prognosis of e.g. colorectal cancer [3-6], head and neck squamous cell carcinoma (HNSCC) [7;8] or melanoma [9;10]. Finally, paraneoplastic phenomena show that malignant cells may induce vigorous immune responses. For example, CD8⁺ cytotoxic T lymphocytes (CTL) and/ or antibodies known to be reactive against tumor antigens that are also expressed by neuronal cells have been detected in patients with neurologic dysfunction in the setting of a remote cancer (reviewed in [11]). A similar link is known for the T cell response against melanoma cells and the autoimmune T cell response against normal melanocytes, known as the de-pigmentation disorder vitiligo (reviewed in [12]).

The concept of “Cancer immunoediting”

A comprehensive view on the complex relation between cancers and the immune system has arisen from a large body of mouse studies through the concept of “cancer immunoediting”. In this model three phases can be distinguished: elimination, equilibrium and escape [13]. First, in the elimination phase (more commonly known as tumor immune surveillance) emerging malignant cells are eradicated by the immune system. For example, Rag2-deficient (Rag2⁻/⁻) mice which lack both B and T cells, are predisposed to spontaneously develop adenomas of the intestine and lung. When Rag2⁻/⁻ mice are also deficient for the immune effector cytokines IFN-α and IFN-γ (i.e. STAT1⁻/⁻), susceptibility increases and mice also develop adenomas of the breast and/ or colon [14]. Mice that lack the T and NK cell effector molecule perforin (pfp⁻/⁻) are prone to develop B cell lymphomas [15]. Interestingly, when the lymphoma cells are transplanted back into immunocompetent Wildtype recipients, they are easily eradicated by CTL [16]. A predisposition to cancer genesis has also been observed in mice lacking TNF-related apoptosis-inducing ligand (TRAIL⁻/⁻) [17], in mice with defective FAS/ FAS Ligand interactions [18], in severe combined immunodeficiency (SCID) mice (lacking B and T cells) [19], in mice deficient in MHC class I expression and/ or antigen presentation [20;21], in mice that lack both GM-CSF and IFN-γ
and in mice lacking IL-12 receptorβ2 [23]. Susceptibility to carcinogen induced tumors is also increased in immunodeficient mice. For example, mice deficient in T and B cells, or more specifically deficient in T cell subsets that are closely related to the innate immune system like γδ-T cells or (invariant) CD1d restricted T cells, are more susceptible to methylcholanthrene-A (MCA) induced sarcomas. This has also been observed for mice deficient in cytotoxic effector molecules like IFN-γ, TRAIL or perforin (reviewed in [24]). Attempts of the immune system to eliminate a tumor can also be unmasked by the depletion of immunosuppressive CD4+ CD25+ regulatory T cells (Treg). For example, when vigorously growing intradermal fibrosarcomas were depleted of Treg, they were rapidly eradicated by CTL [25].

Second, even though immunocompetent wildtype mice are more capable of eradicating malignant cells than immunodeficient mice, the elimination phase can still lead to a suboptimal end result. In this case a temporary state of equilibrium arises between the immune system and the tumor mass. In other words, the tumor does not proliferate but becomes dormant and is kept under check of the immune system. Evidence for this phase is not yet well established but still noteworthy. For example, when mice that appear to be tumor free for a prolonged period after low dose MCA treatment are depleted of CD4+ and CD8+ T cells they succumb to rapidly developing sarcomas [26]. In humans, it has been suggested that pre-malignant myeloid progenitor cells, known as monoclonal gammopathy of undetermined significance (MGUS), can be kept under check of cytotoxic T cells for years before they develop into malignant myeloma cells [27-29].

Finally, when tumors are able to effectively disable the immune response directed against them, they can escape the immune system and thrive within the body. For example, tumors can induce effector cell apoptosis or down-modulate target molecules for these effector cells (reviewed in [30-32]). Once established, tumors that have escaped immune elimination can exploit the immune system to generate an environment in which they can thrive. Human head and neck, non-small cell lung and breast cancers can release chemotactic factors to recruit CD34+ myeloid suppressor cells. These cells support tumor development by providing molecules and factors essential for tumor growth and angiogenesis but also by a profound inhibitory activity on tumor infiltrating (effector) T cells (reviewed in [33]). The differentiation of CD34+ myeloid suppressor cells can be promoted by microvesicles, which are abundant in body fluids of patients with e.g. melanoma and colorectal cancer [34]. A frequently studied escape strategy of tumors is to facilitate the accumulation of Treg within their microenvironment [35-38].

**CD4+CD25+FoxP3+ regulatory T cells vs. invariant CD1d restricted natural killer T cells**

Treg represent a functionally distinct lineage of immunoregulatory T cells and are crucial for the maintenance of immunological tolerance. They are characterized by the expression of the transcription factor Forkhead box P3 (FoxP3). Mutations in the gene encoding FoxP3 cause immune deregulation, polyendocrinopathy, enteropathy and X-linked syndrome. Furthermore, Treg constitutively express CD25 (IL-2Rα), glucocorticoid-induced tumor necrosis factor receptor (GITR) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). Treg can actively suppress the function of other immune cells via the secretion of IL-10 and TGF-β, CTLA-4 induced reverse signaling through B7 (CD80/86) on DC, perforin and granzyme dependent lysis of (effector) T cells and inhibition of IL-2 mRNA in responding T cells [39;40]. Currently they are regarded to be the predominant mediators of tumor-driven immune suppression. Accordingly, an increased frequency of Treg cells has been related to poor clinical outcome of cancer [41-46]. Therefore, investigators are increasingly focusing on the elimination of Treg as a stand-alone anti-cancer immune therapy, or preceding therapies aimed at the increase in number/ function of effector cell populations [47;48].

The concept of cancer immunoediting and the pivotal role herein likely played by Treg strengthen the view of tumor immunologists that it is of vital importance for the cancer patient
to maintain or drive the immune balance in disfavor of the (developing) disease. A relatively recently discovered additional immunoregulatory T-cell lineage, CD1d restricted invariant natural killer T (iNKT) cells, is particularly attractive in this respect. As their name implies, iNKT cells share phenotypic properties with both T-cells and natural killer (NK) cells. They express a T-cell receptor (TCR) together with NK receptors (reviewed in [49]). In contrast to conventional T cells (including Treg), that can recognize antigens presented by polymorphic MHC molecules, iNKT cells express a canonical TCR-Vα-chain (Vα24.Jα18 in humans, preferably paired with Vβ11; Vα14.Jα18 in mice, paired with Vβ2, Vβ7 or Vβ8.2) and as such recognize glycolipid antigens presented by the evolutionary conserved monomorphic CD1d molecule. The glycolipid α-galactosylceramide (αGalCer) originally isolated from the marine sponge Agelas mauritianus is a strong synthetic ligand for iNKT cells, which induces iNKT cell proliferation and activation and secretion of a broad spectrum of cytokines [50;51]. Recently, a (currently challenged [52;53]) endogenous ligand, the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) was identified as an important antigen in the development of iNKT cells [54].

The main function of iNKT cells lies in regulation of immune responses through the production of a wide variety of both pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines very swiftly upon their activation. Owing to this broad spectrum of cytokines, iNKT cells have the capacity to enhance host immunity to microorganisms and cancers as well as to prevent autoimmunity (Reviewed in [55]). Human and mouse iNKT cells can either be CD4+ or CD4−CD8− (double negative (DN)) and in humans a small proportion can express CD8. Direct ex vivo analyses suggested that CD4+ iNKT cells produce both pro-inflammatory cytokines (TNF-α, IFN-γ) as well as anti-inflammatory cytokines (IL-4, IL-13) whereas the DN and CD8+ iNKT cell subsets, primarily produce pro-inflammatory cytokines [56-59].
iNKT cells and the response against microbes

For nearly a decade, iNKT cell biology has been studied solely using αGalCer as an antigen, due to the lack of known natural ligands. Still, indirect evidence suggested the existence of microbial antigens for iNKT cells. For example, the initial influx of iNKT cells was required for the formation of granulomatous lesions caused by *Mycobacterium tuberculosis* [60] or *Cryptococcus neoformans* [61]. Clearance of *C. neoformans* was significantly delayed in TCR-Vα14−/− mice (lacking iNKT). Furthermore, CD1d−/− mice (lacking iNKT and other CD1d restricted T cells) were more susceptible to infection with *Borrelia burgdorferi*, the causative agent of Lyme disease [62] or with *Pseudomonas aeruginosa* in the lungs [63]. A critical role for iNKT cells in the defense against *Streptococcus pneumoniae* has also been described [64]. Immunoboosting of Wildtype mice with αGalCer resulted in IFN-γ release and the subsequent systemic clearance of *C. neoformans* [65] and also the pulmonary clearance of *P. aeruginosa* via enhanced phagocytosis by alveolar macrophages and the increased production of TNF-α [66]. Enhancing effects of αGalCer treatment have been described for the response against parasites like *Plasmodium spp*, the causative agent of Malaria [67;68], and *Trypanosoma cruzi*, the causative agent of Chagaz disease [69]. Similarly, the administration of αGalCer induced inhibition of viral replication in Hepatitis B virus (HBV) transgenic mice in a non-cytopathic manner through the release of IFN-α/β and IFN-γ [70;71], boosted the response against respiratory syncytical virus (RSV) [72] and ameliorated disease symptoms of diabetogenic encephalomyocarditis virus [73]. In agreement with the observations on bacterial and fungal infections, quite recently glycolipids were isolated from e.g. *Ehrlichia muris*, *Sphingomonas capsulata* or *Borrelia burgdorferi*, that have been demonstrated to trigger iNKT cell activation in vitro [74-76]. This suggests that iNKT cells are indeed involved in the natural response against these microbial pathogens (reviewed in [77]).

iNKT cells and auto-immune disorders

In patients with autoimmune disorders that are characterized by auto reactive tissue damage the level of circulating iNKT cells, or of those residing in inflamed tissues, is significantly diminished [78-80]. This implies that they are likely involved somewhere in the auto-immune disease process, but to date their role in auto-immune disorders is ambiguous. For example, in non-obese diabetic (NOD) mice, that spontaneously develop type 1 diabetes, a numerical and functional deficit in iNKT cells preceded diabetes development [81]. Furthermore, germline deletion of CD1d locus in NOD mice accelerated the onset of diabetes and resulted in exacerbated disease [82]. Both in NOD mice [83] and in humans with type 1 diabetes, the residual iNKT cells were found to be selectively deficient in IL-4 secretion [84]. Treatment of NOD mice with αGalCer, even when initiated after the onset of disease, protected them from type 1 diabetes and prolonged the survival of pancreatic islets transplanted into newly diabetic NOD mice. Protection from type 1 diabetes by αGalCer was associated with the suppression of both T- and B-cell autoimmunity to islet beta cells and with a polarized Th2-like response in spleen and pancreas of these mice [85]. In experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS), treatment of mice with αGalCer could either prevent or aggravate disease, depending on the timing of treatment and on the mouse strain [86;87]. These contrasting outcomes were likely related to differences in the Th1 (IFN-γ secretion)/Th2 (IL-4, IL-10 secretion) balance. In line with this, treatment of iNKT cells with an αGalCer analogue that induced elevated levels of IL-4 and reduced levels of IFN-γ resulted in superior protection against EAE [88]. Inflammatory bowel disease has been proposed to be maintained by autoreactive iNKT cells in response to increased CD1d expression on intestinal epithelial cells resulting from tissue damage [89;90]. In this model epithelial tissue damage and Hsp110 release are enhanced, resulting in the constitutive up-regulation of CD1d expression and subsequent activation of resident auto-reactive iNKT cells to secrete Th1 cytokines, leading to chronic inflammation. However,
αGalCer treatment reduced clinical manifestations of dextran sodium sulfate induced colitis in mice [91]. The discrepancies in these and other, not mentioned, studies reflect the difficulty in understanding the role of iNKT cells in auto-immune disorders. Further insight in the effects of αGalCer dosage and administration route on iNKT cells is needed, since e.g. a single injection of αGalCer can induce iNKT cell anergy, a risk that has not been addressed in the studies above [92]. Also, the involvement of different iNKT cell subsets needs to be clarified. Finally, the phenomena observed in the studies above have been addressed by focusing on the iNKT cells’ Th1/Th2 balance. The role of this Th1/Th2 balance in autoimmunity has been poorly understood, especially the role of IFN-γ, since it sometimes appears to enhance and sometimes to hamper autoimmunity. Currently IL-17 secreting “Th17” cells, a novel Th lineage distinct from Th1 and Th2 cells, are increasingly being regarded as the driving force behind many autoimmune responses previously attributed to IFN-γ [93]. How (IFN-γ secreting) iNKT cells relate to Th17 cells thus needs to be addressed. Recently, an IL-17 secreting (IFN-γ/IL-4 low) subpopulation of iNKT cells was identified that preferentially resided in mouse lungs and that was critical for the development of IL-17 mediated airway neutrophilia upon αGalCer or LPS instillation [94].

iNKT cells and anti-tumor immune responses

Systemic αGalCer injection to activate iNKT cells has been reported to lead to the inhibition of hepatic metastasis formation of B16.F10 melanoma [95;96]. The effect could be enhanced by combining αGalCer treatment with IL-12 [97]. In addition, subsequent studies have shown anti-tumor effects of adoptively transferred iNKT cells into tumor inoculated iNKT cell deficient recipients. The anti-tumor effects depended on the iNKT subtype in combination with systemic αGalCer treatment [98] or on activation with IL-12 while treatment with recombinant cytokines produced by IL-12 activated iNKT cells had no effect [99]. Although the anti-tumor effects were mostly NK-cell dependent, T-cell dependent effects on intravenously injected plasmacytoma cells have also been described [100]. In vivo studies have shown that iNKT cells can indeed enhance NK cell activation as well as alloresponses dependent on αGalCer presented by dendritic cells (DC) [101;102]. In line with these findings, adoptive transfer of αGalCer pre-pulsed DC induced strong anti-tumor responses against B16.F10 lung metastases and MCA induced sarcomas in various tumor models [103;104]. Interestingly, iNKT cells also play an important role in immuno-surveillance (referred to above as the tumor elimination phase) in the absence of αGalCer, as shown by studies in iNKT-deficient mice. These mice were found to be more susceptible to MCA induced sarcomas, while protection could be restored by adoptive transfer of iNKT cells derived from wild-type animals. Protection depended on CD1d, IFN-γ production by iNKT cells, and NK and CD8 T-cell function [105].

These intriguing findings from pre-clinical studies prompted several groups to study iNKT cells in human cancer patients. Inconsistent data were obtained from these studies from mostly relatively small and sometimes poorly defined cohorts, with some investigators stating that iNKT cells in peripheral blood of cancer patients were numerically and/or functionally compromised whereas others observed no differences compared to healthy controls. In this thesis, the relation of iNKT cells to disease status and clinical outcome of cancer was further established, as was the promise of treating patients with autologous adoptive transfer of high purity pro-inflammatory iNKT cells.
To address the concluding issues, this thesis consists of the following objectives

1. *Are the numbers of peripheral blood iNKT cells indeed decreased in cancer patients compared to healthy adults?*
   a) After correction for confounding factors like age, gender, tumor type, disease stage or tumor load?
   b) Are the residual iNKT cells in cancer patients still functional *in vitro*?

2. *Is there a putative protective role of peripheral blood iNKT cells against cancer?*
   a) Regarding progression of pre-malignant cervical lesions?
   b) Regarding recurrence of disease after curative radiation therapy HNSCC?

3. *Is anti-cancer immunotherapy via autologous adoptive transfer of ex vivo expanded iNKT cells feasible?*
   a) Can high purity pro-inflammatory iNKT cells be generated *ex vivo* from cancer patients’ blood samples?
   b) Set up a system for the *in vitro* expansion of functional oligoclonal mouse iNKT cells, representative of *in vivo* iNKT cells.
   c) Does adoptive transfer of these expanded iNKT cells lead to protection in pre-clinical tumor models?
Reference List


Chapter 1


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