Cancer Drug Discovery and Development

Masaki Terabe
Jay A. Berzofsky *Editors*

Natural Killer T cells

Balancing the Regulation of Tumor Immunity

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Editors

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Preface

Natural killer T (NKT) cells discussed in this book are CD1d-restricted T cells, not to be confused with NK cells. Unlike conventional T cells restricted by conventional class I or II major histocompatibility complex (MHC) antigens, they respond to antigens presented by CD1d, which is a non-classical MHC class I-like molecule. It has been two decades since NKT cells were first identified as NK1.1 expressing T cells. One of the highlights of the early discoveries on NKT cells is the identification of their unique TCR gene segment usage, namely Vα14Jα281 (now called Vα14Jα18), forming an invariant TCR alpha chain (now these NKT cells are called type I NKT cells or iNKT cells) and its prototypic agonistic antigen alpha-galactosylceramide (KRN7000). This stunning finding shed light on some important characteristics of this relatively small but pivotal T cell population. First, NKT cells recognize lipid, not protein, antigens, indicating that this T cell subset surveys a range of antigens distinct from that of conventional T cells. Given that lipids are major components of any cells or viruses, it makes sense the immune system has a T cell repertoire that surveys lipids. Second, despite the fact that NKT cells are only approximately 1% of mouse spleen cells, they can strongly regulate tumor immunity. Third, because of the expression of the invariant TCR alpha chain, one can stimulate most type I NKT cells with a single antigen such as KRN7000.

Overall, NKT cells bridge the gap between the innate and adaptive immune systems. Like innate immune cells, they can respond quickly to be among the first responders on the scene, and rapidly produce cytokine. As adaptive immune cells, they provide the T cell arm of the immune system the capacity to recognize lipid antigens, as mentioned. They also play a regulatory role as well as an effector one, with the ability to activate T and B cells, NK cells, and dendritic cells, but also the ability to downregulate these under certain conditions.

An important technical break though made for NKT cell research was the invention of CD1d-tetramer and CD1d-dimer by which we can stain and identify NKT cells with flow cytometry. With this now widely used tool, we learned that NK1.1 can be no longer be used as a marker for this T cell population, but the only way to define NKT cells is as “CD1d restricted T cells.” Now we understand that there are many subsets of NKT cells that can be defined by their TCR gene segment usage

(e.g., Vβ2, 7, or 8) or surface molecule expression pattern (such as $CD4^+$ or $CD4^-8^-$, or $NK1.1^+$ or $NK1.1^-$) or cytokine profile. Among the subsets of CD1d-restricted T cells, ones that lack the semi-invariant TCR and use more diverse TCRs, but still recognize lipids presented by CD1d, were discovered and are now called type II NKT cells. These seem to play more of a regulatory role, especially in tumor immunity, but can have effector functions as well.

With the fact that KRN7000 is a strong inducer of tumor immunity through activating NKT cells as well as for a long time the only tool available to specifically activate NKT cells, knowledge of NKT cells has been accumulated through functional studies in the context of tumor immunology including clinical trials of KRN7000 in cancer patients. Although the main focus of this book is to review what we have learned about NKT cells with studies in cancer immunology and to examine their clinical utility, the knowledge should be informative to understand this underappreciated T cell population in many other fields outside of tumor immunology.

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Contents

1 Introduction: Mechanisms of NKT-Cell- Mediated Adjuvant Activity and Function of iPS-Derived NKT Cells 1
Masaru Taniguchi, Shin-ichiro Fujii, Toshinori Nakayama, Shinichiro Motohashi, Nyambayar Dashtsoodol, Hiroshi Watarai, and Michishige Harada

2 Structure and Recognition of Antigens for Invariant NKT Cells 15
Bo Pei and Mitchell Kronenberg

3 Invariant NKT Cell-Based Vaccine Strategies 39
John-Paul Jukes, Jonathan D. Silk, Mariolina Salio, and Vincenzo Cerundolo

4 Immune Regulation of Tumor Immunity by NKT Cells 55
Jessica J. O'Konek, Jay A. Berzofsky, and Masaki Terabe

5 The Regulation of CD1d$^+$ and CD1d$^-$ Tumors by NKT Cells: *The Roles of NKT Cells in Regulating CD1d$^+$ and CD1d$^-$ Tumor Immunity* 71
Jianyun Liu, Gourapura J. Renukaradhya, and Randy R. Brutkiewicz

6 DC-Based Immunotherapy Targeting NKT Cells 95
Shin-ichiro Fujii and Kanako Shimizu

7 Therapeutic Approaches Utilising NKT Cells 111
Stephen R. Mattarollo and Mark J. Smyth

8 NKT Cells of Cancer Patients and How Models Can Inform Therapeutic Plans 129
Mark A. Exley, Lydia Lynch, and Michael Nowak

9 Understanding the Role of Natural Killer T Cells in Hematologic Malignancies: Progress and Challenges 153
Natalia Neparidze and Madhav V. Dhodapkar

10 Clinical Trials with α-Galactosylceramide (KRN7000) in Advanced Cancer 169
Famke L. Schneiders, Rik J. Scheper, Hetty J. Bontkes, B. Mary E. von Blomberg, Alfons J. M. van den Eertwegh, Tanja D. de Gruijl, and Hans J. van der Vliet

11 Clinical Trials of Invariant Natural Killer T Cell-Based Immunotherapy for Cancer 185
Shinichiro Motohashi, Yoshitaka Okamoto, and Toshinori Nakayama

Index 199

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Chapter 1
Introduction: Mechanisms of NKT-Cell-Mediated Adjuvant Activity and Function of iPS-Derived NKT Cells

Masaru Taniguchi, Shin-ichiro Fujii, Toshinori Nakayama, Shinichiro Motohashi, Nyambayar Dashtsoodol, Hiroshi Watarai, and Michishige Harada

Abstract Natural killer T (NKT) cells are characterized by the expression of an invariant Vα14Jα18 receptor that recognizes glycolipids such as α-galactosylceramide (α-GalCer) in conjunction with CD1d molecule. Functionally, NKT cells can act as innate immune cells but can also bridge the innate and acquired immune systems. A prime example of one such function is adjuvant activity: NKT cells augment anti-tumor responses by their production of IFN-γ, which acts on NK cells to eliminate MHC^- target tumor cells and also on CD8 cytotoxic T cells to kill MHC^+ tumor cells. Thus, when NKT cells are activated with α-GalCer-pulsed dendritic cells, both MHC^- and MHC^+ tumor cells can be effectively eliminated. Since both types of tumor cells are simultaneously present in cancer patients, NKT cells are only the cell type that can circumvent the difficult problem of eliminating them. Based on these findings, we have developed NKT-cell-targeted adjuvant cell therapies with strong anti-tumor activity in humans. However, two-thirds of patients were not eligible for this therapy because they no longer had sufficient numbers of NKT cells. To overcome the problem of the limited number of NKT cells in cancer patients, we successfully established a method to generate NKT cells with adjuvant activity from induced pluripotent stem (iPS) cells. Thus, the iPS technology has shown great promise for future cancer therapy.

1 Introduction

NKT cells are characterized by their expression of a unique single invariant antigen receptor encoded by Vα14Jα18 (Imai et al. 1986; Taniguchi et al. 2003a, b) mainly associated with Vβ8.2 in mice and Vα24Jα18 with Vβ11 in humans

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(Lantz and Bendelac 1994). This receptor recognizes glycolipid ligands in association with the monomorphic MHC-like molecule, CD1d (Bendelac 1995; Bendelac et al. 1995; Exley et al. 1997). These characteristics are quite distinct from conventional T cells, which have a highly diverse repertoire and mainly recognize peptide antigens in conjunction with polymorphic MHC molecules. An important finding is that the invariant Vα14 TCRα is used only by NKT cells and not by conventional T cells. Thus, when a pre-rearranged Vα14Vβ8.2 gene is introduced into recombination activating gene (RAG)-deficient mice only NKT cells but not conventional T cells or NK cells develop, defining NKT as a unique lymphocyte subset (Kawano et al. 1997). Conversely, Jα18 knockout mice specifically lack NKT cells but have normal numbers of conventional T cells (Cui et al. 1997).

Different from innate and acquired immune systems which normally recognize pathogens or external antigens, NKT cells are inherently autoreactive, recognizing unknown endogenous ligands in conjunction with CD1d, and are thus always activated and express cell surface activation markers such as CD69, CD44, NK1.1 (Matsuda et al. 2000) and the high-affinity IL-12 receptor (Kawamura et al. 1998). They also accumulate cytokine mRNAs for IL-4 and IFN-γ (Matsuda et al. 2003), but do not produce any cytokines and do not mediate any known functions under physiological conditions. However, once NKT cells receive second signals through Toll-like receptors (TLR) upon pathogen infection, they quickly release Th1 cytokines, which act on other cell types in both innate and acquired immune systems to mediate adjuvant activity, enhancing pro-inflammatory Th1-type immune responses on anti-pathogen or anti-tumor responses. Based on their selective expression of an invariant antigen receptor, conserved function among species, and glycolipid recognition with monomorphic CD1d, NKT cells are identified as a novel lymphocyte subset (Taniguchi et al. 2003a, b).

Here, we describe the discovery and properties of NKT cells, their ligand recognition and their adjuvant activity, features that are now being manipulated therapeutically to treat human cancer. We also describe the iPS technology to develop large numbers of NKT cells in vitro, which is an important advance in overcoming the problem on the limited number of NKT cells in advanced cancer patients.

2 Discovery of NKT Cells

NKT cells were discovered based on four independent lines of investigation, all of which ultimately contributed to the identification of NKT cells as a novel cell type. In 1986, we identified an invariant Vα14 antigen receptor gene by the isolation of complementary DNAs (cDNAs) from suppressor T cell hybridomas (Imai et al. 1986; Koseki et al. 1989). Vα14 cDNAs isolated from the 12 out of 13 independently established hybridomas contained the same Vα14 and Jα18 gene segments with a one-nucleotide N region (Imai et al. 1986; Koseki et al. 1990). As the N region in all of these Vα14Jα18 cDNAs provided the third base of a glycine codon, the use of any nucleotide in the N region encoded an invariant Vα14 α-chain at the

amino acid level. The findings were quite unusual, because T-cell receptors typically have an enormous diversity since they are composed of various V, D and J gene segments with diverse N regions. Therefore, it seemed at the time that the most likely explanation for the dominant expression of one particular invariant Vα14 receptor was that it was an artifact due to the use of hybridomas.

To exclude this possibility, we investigated the usage of the invariant Vα14 receptor in unprimed normal mice by using an RNase protection assay with an invariant Vα14 antisense probe, which was synthesized in vitro using a T7 phage promoter linked to a hybridoma-derived Vα14 cDNA of C57BL/6 (B6) origin and labeled with ^{32}P. With this radioactive antisense probe, we expected to detect a 630 bp band in B6 mice, but a 400 bp band in BALB/c mice due to the presence of allelic polymorphisms in the BALB/c Vα14 region, such that RNase would cleave the single-strand mismatched portion of the B6-derived probe. As expected, we detected a 630 bp band in B6 and a 400 bp band in BALB/c mice. What was unexpected is that the intensity of the bands was calculated to be about 1–4% of total T-cell receptor α-chains (TCRα) in both B6 and BALB/c mice. Remarkably, based on this type of analysis, Vα14$^+$ cells were estimated to constitute 1–3% of T cells in spleen, 10–20% in liver, 40% in bone marrow (BM), and 0.4% in the thymus of unprimed mice (Koseki et al. 1989, 1990; Makino et al. 1995).

There are approximately 100 Vα gene segments and the diversity of the TCRα repertoire is calculated to be roughly 10^8. Thus, the frequency of cells expressing any one particular TCRα is expected to be about 1 in 10^6. However, based on our experimental data, the frequency of the expression of the invariant Vα14 TCRα in unprimed mice is more than 10^4 times higher than expected (Koseki et al. 1989, 1990). In general, clonal expansion of a lymphocyte with a particular TCR is considered to be the result of antigen stimulation. However, we observed clonal expansion of Vα14$^+$ cells in unprimed mice, suggesting that the Vα14$^+$ cells are likely to belong to an autoreactive repertoire.

As it turned out, all inbred laboratory mouse strains, no matter what their MHC haplotypes, possessed considerable numbers (1–4% of total TCRα) of Vα14$^+$ cells, although the numbers of Vα14$^+$ cells were greatly reduced in β2-microglobulin-deficient mice (Adachi et al. 1995). Since the invariant Vα14 TCRα expression was independent of MHC haplotype or of antigen priming, this led to the idea that Vα14$^+$ cells were likely to be selected by an endogenous ligand(s) in conjunction with monomorphic rather than the polymorphic MHC, which is important for the selection of conventional T cells. Moreover, using BM chimera experiments it was demonstrated that Vα14$^+$ cells were selected by BM-derived cells, including thymocytes, but not by thymic epithelial cells (Adachi et al. 1995), suggesting that the selection processes for Vα14$^+$ cells were unique and distinct from those for conventional T cells, which are selected by thymic epithelial cells.

In 1987 and thereafter, several reports (Fowlkes et al. 1987; Budd et al. 1987; Sykes 1990; Ballas and Rasmussen 1990; Levitsky et al. 1991) indicated that a small population of CD4$^-$CD8$^-$ double-negative (DN) thymocytes exclusively expressed TCR Vβ8.2, CD44 and NK1.1 and produced both Th1 and Th2 cytokines (Arase et al. 1993). Lantz and Bendelac (1994) provided evidence that hybridomas

established from these Vβ8$^+$CD44$^+$ thymocytes expressed the invariant Vα14 TCRα mRNA, confirming that Vα14$^+$ cells, Vβ8$^+$ DN thymocytes, and Vβ8$^+$ CD44$^+$ hybridomas represent the same cell type. Moreover, in 1994 it was found that the hybridomas established by Lantz and Bendelac all recognized CD1d, indicating that the mode of antigen recognition by NKT cells is completely distinct from that of conventional T cells (Lantz and Bendelac 1994; Bendelac 1995; Bendelac et al. 1995). Thus, this population was designated Vα14 NKT cells.

3 Ligand for NKT Cells

In 1997, we identified α-galactosylceramide (α-GalCer) as an exogenous glycolipid ligand for NKT cells that is presented by CD1d (Kawano et al. 1997). Concerning the nature of the endogenous ligand for NKT cells, we speculated that the ligand is not a peptidic antigen, because NKT cells develop in TAP (transporter associated with antigen processing)-deficient mice (Adachi et al. 1995). TAP is essential for the translocation of peptides from the cytosol, where they are generated by proteasome cleavage of ubiquitinated substrates, into the endoplasmic reticulum. There the peptides form a stable complex with the MHC class I heavy chain and β2 microglobulin, which can leave the ER and transit to the cell surface where the MHC–peptide complex is used in the selection of CD8 T cells. Since NKT cell development is TAP-independent, it is likely that NKT cells recognize nonpeptide antigens, such as glycolipids, lipids, or carbohydrates. Also, for the interaction between TCR and the antigen/MHC complex, it is essential to have a hydrophilic moiety. However, CD1d has two large hydrophobic pockets that do not allow for peptide binding. Based on the findings, we synthesized six glycolipids composed of three different sugar moieties (galactose, glucose, or mannose) linked to the ceramide with either an α- or β-configuration. These synthetic glycolipids were tested for their ability to stimulate NKT cells from NKT mice, which have only NKT cells but no conventional T cells, NK cells, or B cells in their immune system.

We found several important rules for the ligand to stimulate NKT cells. One is that the α- but not β-anomeric conformation between the sugar and the ceramide is important, because only α-GalCer but not β-GalCer stimulates NKT cells. The second important point is that the configuration of the 2′OH on the sugar moiety is essential, because α- or β-mannosylceramide, which has a different 2′OH configuration from galactose or glucose failed to stimulate NKT cells, suggesting that the 2′OH on galactose or glucose is essential. The third point is that the 3′OH on the sphingosine is critical, because α-GalCer lacking this group failed to stimulate NKT cells.

We also used alanine mutagenesis of CD1d to investigate the amino acids in the α-helixes of CD1d that are important for the interaction with α-GalCer and the Vα14 receptor. We tested NKT cell activation by using α-GalCer-loaded mutant CD1d and found that Arg 79, Asp 80, Glu 83 and Asp 153 failed to stimulate mouse NKT cells, suggesting that these amino acids are important either for the

binding of α-GalCer with mouse CD1d or for the binding with the Vα14 receptor (Kamada et al. 2001).

In 2007, the crystal structure of a trimolecular complex consisting of α-GalCer with human CD1d and human invariant Vα24Vβ11 was reported (Borg et al. 2007) Interestingly, four amino acids (Asp94, Arg95, Gly96, and Ser97) at the beginning of the Jα18 region of the invariant Vα24Jα18 TCRα were found to be important for binding with α-GalCer and CD1d: Asp94 in Jα18 (Jα18-Asp94) interacts with Arg79 on CD1d (CD1d-Arg79); Jα18-Arg95 interacts with three amino acids on CD1d (CD1d-Ser76, CD1d-Arg79, and CD1d-Asp80) and also with the 3′OH on sphingosine of α-GalCer; Jα18-Gly96 interacts with the 2′OH on galactose of α-GalCer; and Jα18-Ser97 interacts with CD1d-Glu150. Thus, the NKT TCRα chain is responsible for the binding with both CD1d and its ligand. On the other hand, the TCRβ chain does not contribute to α-GalCer binding, although Tyr48, Tyr50 and Glu57 on TCRβ are responsible for binding with CD1d at Glu83 and Lys86.

The human CD1d amino acids Arg79, Asp80, and Glu83, which are important for binding with either α-GalCer or the human NKT TCR, are well conserved in mice. Similarly, the first four amino acids (Asp94, Arg95, Gly96, and Ser97) in the mouse and human Jα18 are conserved. Thus it is likely that the important amino acids, such as Arg79, Asp80, and Glu83 in mouse CD1d we detected in the functional studies of mutant CD1d (Kamada et al. 2001) also interact with the first four amino acids in mouse Jα18, although the crystal structure of the mouse Vα14/Vβ8 and α-GalCer–mouse CD1d complex has not yet been solved.

In support of the notion that the NKT cell system is highly conserved between mouse and human, NKT cells can act across species barriers, such that human NKT cells are fully activated by mouse dendritic cells (DCs), and human DCs can activate mouse NKT cells (Brossay et al. 1998; Kawano et al. 1999). Therefore, the monomorphic nature of CD1d among different species, the conserved sequences of CD1d and the invariant antigen receptor on NKT cells, and the conserved functions of NKT cells in mouse and human, allow the results of studies of mouse models to be readily extrapolated to humans. Thus α-GalCer is being used as a drug for clinical applications (Nieda et al. 2004; Chang et al. 2005; Ishikawa et al. 2005; Motohashi et al. 2006, 2009; Motohashi and Nakayama 2008).

4 Mechanisms of NKT Cell-Mediated Adjuvant Activity Augmenting Anti-Tumor Responses

Although α-GalCer is an exogenous NKT cell ligand, there are good reasons to speculate that there are endogenous self-ligands. NKT cells appear to be activated in vivo; freshly isolated NKT cells show up-regulated expression of the IL-12R and activation markers, and accumulation of cytokine mRNAs (Matsuda et al. 2000, 2003; Kawamura et al. 1998). NKT cell recognition of self-ligands does not elicit

any effector functions; however, it appears to prime NKT cells to ensure a rapid response against pathogens. This in vivo phenotype of NKT cells indicates that in the steady state the NKT cells are equipped and ready to mediate their functions, but require additional second signals derived from their environment.

These second signals are the key to determine NKT cell functions. DCs in the steady state are immature; they are able to capture antigens but unable to activate conventional T cells. By contrast, NKT cells can be activated by immature DCs loaded with α-GalCer and reciprocally induce maturation of the immature DCs. A single injection of free α-GalCer in vivo induces a burst of early (at 6 h) IL-12 production by DCs followed by IFN-γ production by NKT cells (at 16–24 h) (Tomura et al. 1999; Kitamura et al. 1999; Gonzalez-Aseguinolaza et al. 2000; Trobonjaca et al. 2001; Strober et al. 2003; Hermans et al. 2003; Fujii et al. 2003, 2004). This response is due to up-regulation of co-stimulatory molecules (CD40) on DCs and CD40 ligand (CD40L) expression on NKT cells, both of which are detectable within 2–6 h after α-GalCer injection (Fujii et al. 2004). The DC maturation is NKT cell-dependent because it does not occur in NKT cell-deficient $J\alpha 18^{-/-}$ mice. The maturation of DCs is blocked in the tumor patients, because of their production of immunosuppressive cytokines such as TGF-β and IL-10 (Khong and Restifo 2002; Yang and Carbone 2004). Thus, the in vivo maturation of DCs by activated NKT cells is one of the important strategies to augment protective immunity in cancer patients.

Because of their self-reactivity and ability to quickly release large amounts of cytokines, NKT cells link the two immune systems, serving as a bridge between the innate and acquired systems, and thus augment protective immune responses, including anti-tumor immune responses, through activation of NK and CD8 killer T cells (Taniguchi et al. 2003a, b). This augmentation of protective immunity is known to be an NKT cell-mediated adjuvant effect and is critical for tumor eradication. Since tumor cells do not provide any adjuvant effects or "danger signals" to activate the immune system, they fail to induce tumor-specific immune responses that are potent enough to eradicate tumor cells even if tumor-specific T cells are present. Moreover, there are in general two types of tumor cells in a tumor mass: one is MHC^+ and the other is MHC^- (Khong and Restifo 2002; Yang and Carbone 2004), and effective tumor immunity requires that both types of tumor cells are eliminated at once. NKT cells are the only cell type that is able to both interact with immature DCs, inducing their maturation, and also augment the function of both NK cells and CD8 T cells (Fig. 1.1).

IFN-γ produced by activated NKT cells is a key cytokine for mediating adjuvant activity. By this mechanism, NKT cells can induce maturation of DCs, which thereby acquire the ability to present tumor antigens to CD8 T cells. The activated CD8 T cells can then eliminate MHC^+ tumor cells. IFN-γ can also activate NK cells, which kill MHC^- tumor targets. The efficacy of this approach has been demonstrated in several studies where treatment of tumor-bearing mice with α-GalCer-pulsed DCs to activate endogenous NKT cells leads to the eradication of established metastatic tumors (Toura et al. 1999). Thus, the activation of endogenous NKT cells is a promising strategy for treatment of cancer by selectively triggering protective anti-tumor immunity.

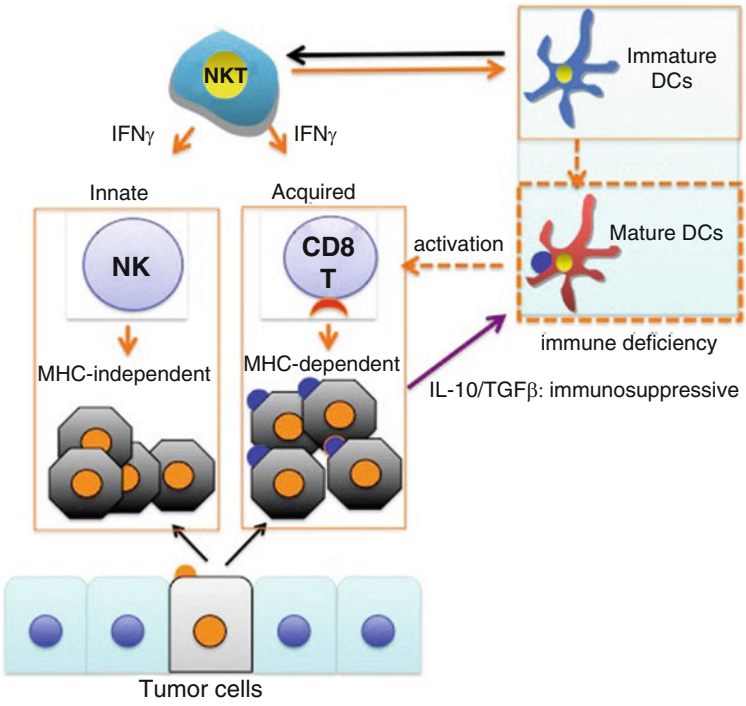

Fig. 1.1 The NKT-cell-mediated adjuvant activity. The NKT cell can bridge innate and acquired immunity and play a role in controlling protective responses. In general, two types of tumor cells are simultaneously present in a tumor mass: one is MHC^+ and the other is MHC^-, and effective tumor immunity requires that both types of tumor cells are eliminated at once. NKT cells are the only cell type that is able to both interact with immature DCs, inducing their maturation, and also augment the function of both NK cells and CD8 T cells. IFN-γ produced by activated NKT cells is a key cytokine for mediating adjuvant activity. By this mechanism, NKT cells can induce maturation of DCs, which thereby acquire the ability to present tumor antigens to CD8 T cells. The activated CD8 T cells can then eliminate MHC^+ tumor cells. IFN-γ can also activate NK cells, which kill MHC^- tumor targets. Thus, the manipulation of NKT cells is an important strategy to augment protective immunity in cancer patients

4.1 NKT Cell-Targeted Adjuvant Cell Therapy for Patients with Advanced Lung Cancer

Based on the above translational studies in tumor-bearing mice and the highly conserved nature of the NKT/CD1d system, α-GalCer was approved for use as a drug for clinical applications. Several clinical trials involving injection of α-GalCer-pulsed DCs have been carried out in patients with cancer, including colon cancer, multiple myeloma, anal cancer, and renal cell cancer (Nieda et al. 2004; Chang et al. 2005). Although no clear tumor reduction was detected, tumor markers were significantly decreased.

In collaboration with Toshinori Nakayama and Shinichiro Motohashi at Chiba University, we have completed a phase I/IIa clinical trial of NKT-cell-targeted adjuvant therapy using α-GalCer-pulsed DCs (total 4×10^9 cells per patient in four consecutive injections at 1 week intervals) on 17 patients with advanced lung cancer, including stage IV, IIIB primary cancer, and recurrent tumor after surgery (Ishikawa et al. 2005; Motohashi et al. 2006; Motohashi and Nakayama 2008; Motohashi et al. 2009). The patients' peripheral blood mononuclear cells were cultured with GMP grade GM-CSF and IL-2 for 2 weeks to increase the number of DCs, pulsed with α-GalCer for 24 h, and then administered intravenously into the donor. Significant increases in the number of IFN-γ-producing cells were detected in 60% of enrolled patients (10 out of 17) and this correlated with a prolonged median survival time (MST) of 31.9 months without tumor progression and metastasis, with only a primary treatment and no further additional treatment. Interestingly, none of these ten patients with longer survival time showed tumor regression (Motohashi et al. 2009). By contrast, the patient group with low IFN-γ production had an MST of 9.7 months, which is equivalent to the MST of 10 months after treatment with commercially available molecular target drugs such as anti-VGEFR antibody (13.1 months), anti-EGFR (10.1 months), Folic acid inhibitor (8.3 months), EGFR inhibitor (6.7 months). Thus, IFN-γ appears to be a good biological marker to predict a favorable clinical course.

However, despite the clear anti-tumor activity of the NKT cell-targeted therapy, two-thirds of patients were not eligible because they no longer had sufficient NKT cells (<10 cells/ml of blood).

5 Generation of Induced Pluripotent Stem Cells from Mature NKT Cells

To overcome the problem of the limited number of NKT cells in cancer patients, we attempted to establish methods to supplement NKT cells. Our previous studies on the generation of NKT cells from mouse embryonic stem (ES) cells containing a nucleus derived from a mature NKT cell (NKT cloned ES), which were established by nuclear transfer from B6 liver NKT cells, had clearly demonstrated that the NKT cloned ES cells preferentially gave rise to NKT cells both in vivo and in vitro, mediated adjuvant activity in vivo, and regressed tumors (Watarai et al. 2010a). Since induced pluripotent stem (iPS) cells are functionally equivalent to ES cells in many respects (Takahashi and Yamanaka 2006; Okita et al. 2007; Wernig et al. 2007), these results suggested the feasibility of using iPS cells for NKT cell-targeted adjuvant therapy.

Although several attempts have been made to generate iPS cells from mature B and T cells by the introduction of *Oct3/4, Sox2, Klf4* and *c-Myc*, the efficiency is very low (Hong et al. 2009). Moreover, there are no reports of the successful regeneration of functional B or T cells from lymphocyte-derived iPS cells (Hanna et al. 2008; Brown et al. 2010; Seki et al. 2010). Since NKT cells have a higher

reprogramming activity (71%) compared to conventional T cells (12%), we thought that NKT cells would be suitable for generating iPS cells. In fact, we successfully generated iPS cells from mature NKT cells and then were able to generate functional NKT cells from the iPS cells in vitro. NKT cells (10^6) isolated from spleen cells of normal B6 or B6 NKT clone mice were first activated by anti-CD3/CD28 together with IL-12 and IL-2 for a week, followed by reprogramming according to the conventional protocol (Takahashi and Yamanaka 2006). We established three independent iPS cell lines, which were NKT cell-derived, based on the presence of rearranged *Vα14Jα18* sequences. The expression of SSEA1, Oct3/4 and Nanog, and also that of endogenous *Oct3/4*, *Sox2*, *Klf4*, *Nanog*, *Ecat1*, *Gdf3*, *Rex1* and *Zfp296* mRNA in the iPS cells were detected at similar levels to those observed in ES cells. Genome-wide gene expression profiles of the iPS cell lines showed a higher correlation coefficient to ES cells than to splenic NKT cells.

5.1 Generation of Functional NKT Cells from iPS Cells

The potential of the iPS cell lines to re-differentiate into mature functional NKT cells in vitro was investigated using a modified standard protocol (Watarai et al. 2008, 2010a) of a 25-day culture system containing IL-7 and Flt3L as supportive cytokines and OP9/Dll-1 as stromal cells (Watarai et al. 2010b). With this culture system, the iPS cells (10^5) preferentially developed into NKT cells but not other cell types and gave rise to 3×10^7 NKT cells, a 300-fold expansion. The in vitro generated NKT cells were mostly of the immature $CD4^+CD8^+$ phenotype ($CD24^{hi}$, $CD44^{lo}$, $CD62L^{hi}$, $CD122^{lo}$, $CD69^{lo}$, and $NKG2D^-$), but ~30% of them expressed NK1.1. Moreover, despite their immature cell surface phenotype, the iPS-derived NKT cells produced significant amounts of IFN-γ and IL-4 upon α-GalCer stimulation. Therefore, the iPS-derived NKT cells represent functional NKT cells.

One of the most important findings of these studies is that the iPS-derived NKT cells with immature phenotype acquired the potential to produce large amounts of IFN-γ, which is essential for the adjuvant effect on anti-tumor responses. Interestingly, the iPS-derived NKT cells obtained from the 25-day cultures could produce IFN-γ levels similar to splenic NKT cells, whereas iPS-derived NKT cells in the 20-day culture system mainly produced IL-4 but not IFN-γ. Therefore, acquisition of the ability to produce IFN-γ is apparently due to the duration of the culture. Additionally, the immature cell surface phenotype of the iPS-derived NKT cells does not correlate with their functional activity.

Examined collectively, the 25-day culture system shows several advantages; the yield of NKT cells was ten times higher and the cells produced five times more IFN-γ per cell than those obtained in the 20-day culture system. Although our system for generation of NKT cells in vitro is a mouse model, it should be helpful in establishing methods for generation of iPS cells from human NKT cells and protocols for developing NKT cells from iPS cells suitable for a clinical setting.

5.2 *In Vivo Function of iPS-Derived NKT Cells*

The in vivo survival and function of iPS-derived NKT cells were investigated after transfer into NKT cell-deficient ($J\alpha18^{-/-}$) mice (Watarai et al. 2010b). Similar to the ability of normal wild type mature NKT cells to repopulate the $J\alpha18^{-/-}$ mice, a considerable number of iPS-derived NKT cells could repopulate in the recipient liver 2 weeks after adoptive transfer. Although the in vitro-generated iPS-derived NKT cells that we injected had an immature phenotype ($CD24^{hi}$, $CD44^{lo}$, $CD62L^{hi}$, $CD69^{lo}$, $CD122^{lo}$, and $NKG2D^{-}$), the NKT cells recovered from the liver were $CD24^{lo}$, $CD44^{hi}$, $CD62L^{lo}$, $CD69^{+}$, $CD122^{+}$, and $NKG2D^{+}$, phenotypically similar to mature liver NKT cells in wild type mice, indicating that their maturation occurs in vivo. Similar to the in vitro activity of iPS-derived NKT cells obtained in the 25-day cultures, the iPS-derived NKT cells transferred in vivo produced large amounts of IFN-γ upon antigen stimulation with α-GalCer intravenously, expanded significantly in number (~tenfold increase: 0.3–0.4% to 4.9–5.7%). These iPS-derived NKT cells had also down-modulated TCRβ expression, consistent with their in vivo activation.

5.3 *Adjuvant Activity of iPS-Derived NKT Cells*

Since the iPS-derived NKT cells produced IFN-γ upon stimulation with α-GalCer, we investigated their adjuvant effects on anti-tumor responses in mice. The mice that had received iPS-derived NKT cells (4×10^6) were immunized with cell-associated OVA (spleen cells from $TAP^{-/-}$ mice that were treated with hypertonic medium in the presence of OVA) together with α-GalCer (TOG). A week later, the mice were challenged with $OVA_{257–264}$ peptide and analyzed for IFN-γ production by OVA-specific CD8 T cells and by NK cells (Watarai et al. 2010b). We indeed observed significant production of IFN-γ by the NK cells and OVA-specific CD8T cells, as well as an increase in the number of these CD8 T cells (>70 times higher than the control group). Thus, iPS-derived NKT cells were shown to function as a cellular adjuvant for both innate and adaptive immune responses.

Under these conditions, iPS-derived NKT cells significantly augmented antigen-specific anti-tumor CD8 T-cell responses against EL-4-derived OVA-bearing EG7 tumor cells (OVA serves as tumor antigen in this system) but not against EL4 in TOG-immunized B6 mice. The growth of OVA-bearing EG7 tumor cells in $J\alpha18^{-/-}$ mice was significantly suppressed by adoptive transfer of iPS-derived NKT cells, whereas the growth of EL4 was not. Therefore, the iPS-derived NKT cells are functionally competent to mediate adjuvant activity in vivo.

These studies have demonstrated that it is possible to expand functional murine NKT cells in vitro through an iPS phase. Since the functions and α-GalCer reactivity of NKT cells are highly conserved between mice and humans, this finding has opened a pathway that may finally allow us to realize the full potential of NKT cell-targeted adjuvant cell therapy in human cancer patients.

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Chapter 2
Structure and Recognition of Antigens for Invariant NKT Cells

Bo Pei and Mitchell Kronenberg

Abstract It has been two decades since natural killer T (NKT) cells were identified and distinguished from conventional T cell populations by the invariant Vα14 rearrangement in their T cell antigen receptor (TCR). NKT cells recognize lipid antigens presented by CD1d, a member of a third family of antigen-presenting molecules. The first antigen known to activate NKT cells is a α-galactosyl ceramide (αGalCer), a highly potent synthetic glycosphingolipid (GSL) antigen closely related to a natural product, probably derived from a bacteria. Synthetic antigens related to αGalCer are being developed for clinical applications, and there is great interesting understanding why different variants cause different cytokine responses. Microbial glycosphingolipid antigens for NKT cells have been found in environmental microbes and also in pathogens such as *Borrelia burgdorferi*. For the microbial and synthetic antigens, when the TCR binds, it forces the sugar and CD1d into a fixed orientation. Self-antigens for NKT cells also have been defined, but these have diverse structures and it remains controversial if there is a single type of self-agonist responsible for the selection and peripheral activation of these cells.

1 Introduction

In vertebrates, antigen-presenting molecules play a critical role in host immune defense against microbial pathogens by presenting antigens (Ags) to T lymphocytes. Major histocompatibility complex (MHC)-encoded class I and class II molecules present peptides to T lymphocytes, while members of the CD1 family of molecules present lipid Ags (Kronenberg 2005; Rudolph et al. 2006; Bendelac et al. 2007).

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Considered as a third family of antigen-presenting molecules, in addition to MHC-encoded class I and class II proteins, CD1 polypeptides are expressed by a variety of cell types, including cortical thymocytes, myeloid cells, hepatocytes, intestinal epithelial cells, and keratinocytes. The highest amounts of CD1 expression, however, are found on marginal zone B (MZB) cells and various types of professional antigen-presenting cells (APC), including dendritic cells (DCs) and Langerhans cells (Silk et al. 2008a; De Libero et al. 2009).

Five human CD1 genes have been identified, with their protein products corresponding to CD1a, CD1b, CD1c, CD1d, and CD1e. Based on their sequence similarity, the antigen-presenting CD1 members are classified into two groups: CD1a, CD1b, and CD1c in group I, and CD1d in group II. CD1e does not present antigens but is instead localized to lysosomes where it binds glycolipids and is involved in assisting the processing of the carbohydrate portion of glycolipids (de la Salle et al. 2005). Mice and rats, by contrast, have only a CD1d ortholog (Brigl and Brenner 2004; Silk et al. 2008a; Cohen et al. 2009). Similar to MHC class I proteins, CD1 molecules are type 1 integral membrane proteins consisting of α1, α2, and α3 extracellular domains, non-covalently associated with β_2-microglobulin (Brigl and Brenner 2004; Moody et al. 2005). However, CD1 proteins are relatively non-polymorphic with the exception of CD1e (Cohen et al. 2009; Salio et al. 2010).

The group I CD1 molecules present antigens to T lymphocytes that express diverse T cell antigen receptors (TCRs), and that appear to be similar in many ways to the general populations of T lymphocytes active in adaptive immunity. By contrast, in mice the majority of CD1d-reactive T cells express a semi-invariant TCR with an invariant α chain formed by a Vα14–Jα18 rearrangement in mice (Godfrey et al. 2004). Humans have populations with a homologous Vα24–Jα18 rearrangement (Porcelli et al. 1993; Dellabona et al. 1994), but it is much less certain that these cells constitute the majority population of CD1d-reactive cells. The invariant α chain is combined with a limited repertoire of β chains, Vβ8.2, Vβ2, and Vβ7 in mice, and Vβ11, a Vβ8 homolog, in humans (Gumperz and Brenner 2001; Godfrey and Berzins 2007). Furthermore, the majority of the CD1d-reactive T cell population with the invariant TCR α chain was characterized as co-expressing C-type lectin family natural killer (NK) cell receptors, therefore they are referred to as invariant natural killer T (*i*NKT) cells. Once tetramers of CD1d loaded with glycolipid antigens were developed, it became apparent that not all cells with the semi-invariant TCR express typical NK receptors, such as NK1.1 (Kronenberg 2005; Bendelac et al. 2007; Matsuda et al. 2008). Furthermore, several populations of T lymphocytes express NK receptors that do not have the invariant TCR, including some cells that recognize CD1d but with diverse TCRs and others that do not recognize CD1d. Therefore, CD1d-reactive NKT cells with a semi-invariant TCR are often referred to as type I NKT cells (Godfrey et al. 2004), to distinguish them from CD1d-reactive cells with more diverse TCRs, sometimes called type II NKT cells, and from cells with other specificities. However, here we will refer to these cells simply as *i*NKT cells.

*i*NKT cells represent a separate T lymphocyte subset with an innate-like or natural memory phenotype and behavior. As a result of their unique differentiation pathway

in the thymus, they acquire an antigen-experienced phenotype and they respond immediately to antigenic stimulation. There is a synergy between the response to weaker Ags, some of which are presumably self-ligands, and cytokines, particularly IL-12 (Brigl et al. 2003; Sada-Ovalle et al. 2008). However, *i*NKT cells also respond to stimulation with cytokines alone, such as the combination of IL-12 and IL-18, in the absence of TCR engagement (Nagarajan and Kronenberg 2007), thereby functioning in this context similarly to cytokine-activated NK cells. After stimulation of their TCR with lipid Ags presented by CD1d, *i*NKT cells rapidly secrete a large amount of cytokines and chemokines, and consequently they activate various immune cells. By this means, *i*NKT cells amplify innate immune responses and provide a bridge response between the earliest innate responder cells and a full adaptive immune response (Parekh et al. 2005; Matsuda et al. 2008).

In the last decade, the origin and structure of the lipid Ags that stimulate *i*NKT cells has been an intense area of research. Several endogenous or self as well as foreign Ags have been identified, and in some cases their likely physiological role in host immune defense has been analyzed. Characterization of synthetic Ags also has provided insight into the nature of the trimolecular interaction between lipid Ags, CD1d, and the TCR of *i*NKT cells, and the consequent immune response elicited from these cells. This review will concentrate on these *i*NKT ligands, including their origin, structure, intracellular processing, and presentation by CD1d, recognition by the invariant TCR and the consequences when they activate *i*NKT cells.

2 Synthetic Lipid Ags

The first and most well-known lipid Ag that can be recognized by the TCR of *i*NKT cells is α-galactosylceramide (αGalCer) (Fig. 2.1). αGalCer is a derivative of natural agelasphins, a class of closely related glycolipids that differ only for the lipid portion. These compounds were found in a screen of natural substances for anti-tumor activity that was carried out by the Kirin Pharmaceutical Company (Natori et al. 1994), and they were purified originally from the marine sponge *Agelas mauritanius*. αGalCer is a glycosphingolipid (GSL), a type of glycolipid with a ceramide constituting the lipid moiety. In αGalCer, the ceramide has a phytosphingosine base, meaning that the 18-carbon sphingosine is fully saturated. Kirin medicinal chemists modified the structure of the agelasphins by removing the acyl C2 hydroxyl group, which did not significantly affect anti-tumor activity, and by adding carbons to both the phytosphingosine to reach a C18 length and the acyl chain (= C26) to produce αGalCer, which they named KRN7000. In 1997, Kawano et al. showed that αGalCer was a potent ligand able to activate *i*NKT cells in a CD1d-dependent manner (Kawano et al. 1997).

A striking characteristic of αGalCer is that its hexose sugar has an α linkage between the ceramide moiety and carbohydrate head group. Mammalian cells have glycosphingolipids, but they can only synthesize ones with β-linked glycolipids. The α-anomeric conformation of the glycolipid is critical in most instances (see below) for

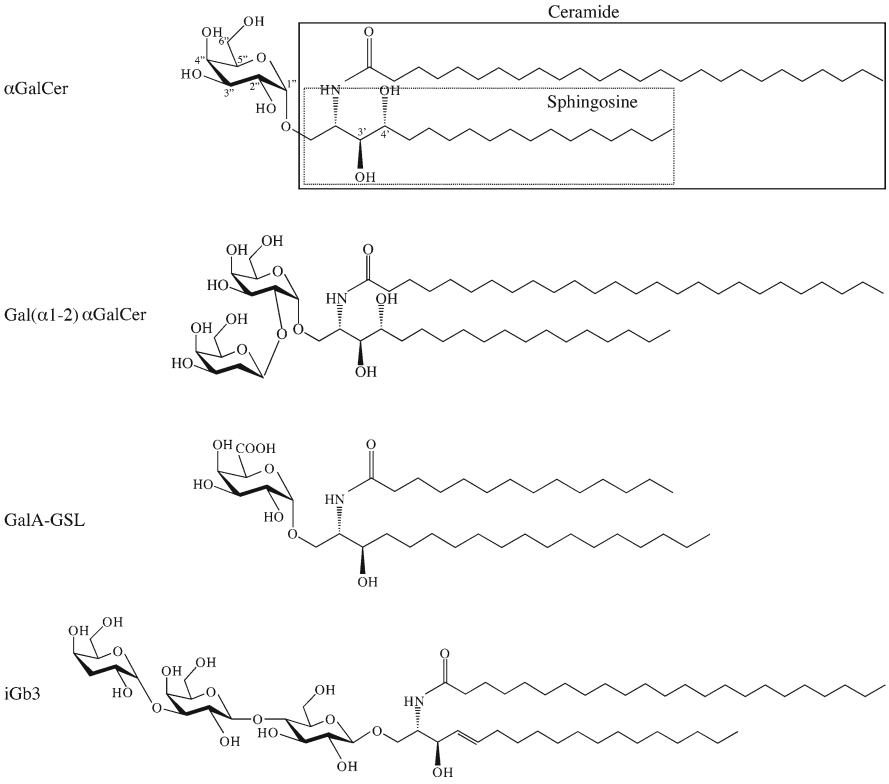

Fig. 2.1 Structures of some natural and synthetic GSL Ags that activate *i*NKT cells. Each of the Ags has a ceramide lipid (enclosed by the *larger box*) consisting of a sphingosine base (the lower of the two aliphatic carbon chains, enclosed by the *internal smaller box*) bound by a 1–1′ linkage to hexose sugar

antigenic activity, however, as substitution to a β-anomeric conformation (βGalCer) resulted in complete abrogation of *i*NKT cell stimulation in most studies. Figure 2.2 shows how a GSL with a β-linked sugar would present a very different epitope to a TCR when bound to CD1d. In experiments based on surface plasmon resonance measurements, there was no measurable affinity of the invariant TCR for CD1d loaded with βGalCer (Sidobre et al. 2004). In other studies, however, it was reported that βGalCer also could induce *i*NKT cell stimulation, although it was much less potent than its counterpart with an α-linked sugar (Ortaldo et al. 2004; Parekh et al. 2004). Furthermore, it is possible that a minute amount of αGalCer contaminated some βGalCer preparations. It was also shown that α-glucosylceramide, in which galactose was replaced by glucose, was less antigenic and substitution of galactose with α-linked mannose completely abrogated *i*NKT cell activation (Kawano et al. 1997), demonstrating a high degree of carbohydrate specificity for the *i*NKT cell TCR. Surprisingly, it has recently been shown that βManCer is an agonist for *i*NKT cells that causes a unique type of anti-tumor effector response in vivo (O'Konek et al. 2011).

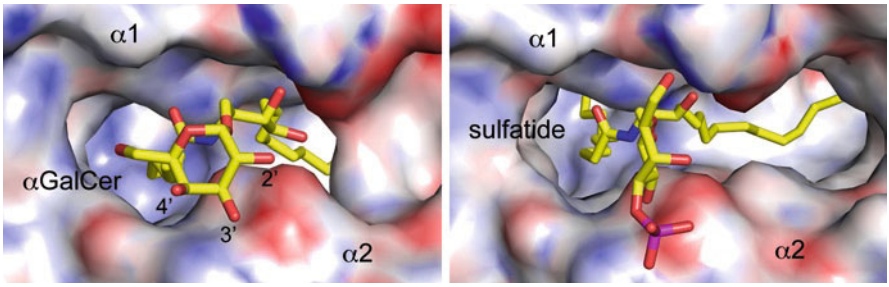

Fig. 2.2 *Top view* of the mouse CD1d binding groove in complex with αGalCer (*left*, PDB ID 1Z5L) or sulfatide (*right*, PDB ID 2AKR). The protein is shown as a molecular surface with electrostatic potential (electronegative in *red* and electropositive in *blue* from −30 to 30 kT/e). The ligands are shown in *yellow*. The hydroxyl groups on the protruding galactose sugar of αGalCer are indicated

More detailed structure–activity studies focusing on the sugar head group further demonstrated that the semi-invariant TCR could sense small modifications of the saccharide moiety. αGalCer analogs that were tested with any type of modification of the equatorial 2″ hydroxyl group (Fig. 2.1) of the galactose sugar, including 2″-amino, 2″-deoxy, or 2″-fluoro sugars, could not stimulate *i*NKT cells (Wu et al. 2005). The one exception is the disaccharide compound Gal(α1-2)αGalCer (Fig. 2.1), in which the second or outer galactose is α linked to the 2″ position of the inner galactose, that in turn is bound to the ceramide lipid. In this particular modification, the second galactose needs to be removed by lysosomal carbohydrate antigen processing to generate a potent antigen (Miyamoto et al. 2001; Prigozy et al. 2001; Wu et al. 2005). Chemical modifications at the 3″- and 4″-hydroxyl positions of the sugar affected antigenic potency to some extent, but these could still be recognized (Wu et al. 2005; Xing et al. 2005). Also, an additional galactose at the 4″-hydroxyl position required antigenic processing to remove this saccharide to generate αGalCer before it became antigenic to *i*NKT cells (Zhou et al. 2004). By contrast, modifications at the 6″-position could be well tolerated for binding to CD1d and interaction with the TCR, without requiring lysosomal processing to remove them (Prigozy et al. 2001; Zhou et al. 2004). Therefore, these studies show that a portion of the hexose sugar ring contributes to antigenic activity, in particular, the 2″ position, with the 3″ and 4″ also contributing. A synthetic ceramide antigen with a trihydroxyl threitol moiety, rather than a hexose sugar, also can be recognized by the invariant TCR although this structure is believed to mimic the central portion of the hexose sugar (Silk et al. 2008b).

In parallel with structure activity studies of the hexose sugar, careful studies on the influence of modifications of the ceramide on the antigenic activity of αGalCer were also carried out. Compared with the original GSLs identified from the marine sponge (*A. mauritanius*), αGalCer possessed a C4 hydroxyl group on the sphingosine base that is absent in the original GSLs, which enhanced the ability to stimulate *i*NKT cells (Brossay et al. 1998; Wu et al. 2005). Further analysis of the role of hydroxyl groups showed that removing both the 3′ and 4′-hydroxyl groups on the

phytosphingosine base could lead to the inactivation of the compound, and that the 3′-hydroxyl group likely was more important than the 4′-position hydroxyl (Morita et al. 1995; Brossay et al. 1998; Sakai et al. 1999; Miyamoto et al. 2001; Ndonye et al. 2005).

Variation in the length and saturation of the hydrocarbon chains gave surprising results, because although these portions of the molecule are buried in the CD1d groove, they have a surprising degree of influence not only on antigenic potency, but also on the quality of the immune response they elicit. The phytosphingosine of αGalCer could be truncated from 18 to 11 carbons without a catastrophic loss of antigenic activity, although this compound was not as effective as αGalCer in stimulating *i*NKT cell hybridomas. A fatty acid chain is required, demonstrated by the finding that an αGalCer variant with its C26 fatty acid substituted with an aniline ring was not able to stimulate *i*NKT hybridoma cells for IL-2 synthesis (Brossay et al. 1998). However, unexpectedly, an acyl chain with even only two carbons could still activate *i*NKT cells (Morita et al. 1995; Brossay et al. 1998). A recent report showed that a synthetic GSL with a cyclopropyl sphingosine (C13-15) was significantly diminished in its antigenic activity; it retained some potency (Kinjo et al. 2008).

Several αGalCer variants with modifications of the ceramide lipid enhanced in vivo production of IL-4, the prototypical Th2 cytokine. Yamamura and coworkers initiated this line of work by synthesizing an αGalCer analog with a phytosphingosine chain reduced from 18 to 9 carbons, and the N-acyl chain from 26 to 24 carbons. While it had been well known that *i*NKT cells stimulated by αGalCer immediately produced both T helper type 1 (Th1) and T helper type 2 (Th2) cytokines (Kawano et al. 1997), they found that OCH induced a Th2 biased cytokine profile, with a quick and predominant release of IL-4 and a diminished IFNγ response (Miyamoto et al. 2001; Oki et al. 2004). Further investigations showed that the induction of biased cytokine profile was not limited to the compounds with modified phytosphingosine moiety, but in fact was observed with other modifications of the CD1d-buried lipid. For example, it was shown that αGalCer analogs in which the C26:0 N-acyl chain was substituted with shorter, unsaturated fatty acids, similarly modified the outcome of *i*NKT cell activation in favor of IL-4 synthesis (Yu et al. 2005). An αGalCer analog that has two unsaturated bonds in a fatty acid chain consisting of 20 carbons (C20:2) showed an obvious tendency to induce more IL-4 relative to IFNγ, with only a small effect on antigenic potency. These findings are consistent with another report in which the authors found that truncation of either the phytosphingosine or N-acyl chain endowed GSLs with the ability to bias *i*NKT cell-induced responses in favor of Th2 cytokines (Goff et al. 2004).

A different type of modification of the ceramide lipid could induce increased in vivo production of the prototypical Th1 cytokine, IFNγ. By replacing the oxygen atom linking and the galactose sugar with ceramide with a methylene group, Tsuji et al. synthesized and tested a C-glycosidic form of αGalCer, named C-αGalCer. After intravenous injection, C-αGalCer induced as much IFNγ as αGalCer, but it

induced much less IL-4. Interestingly, by stimulating Th1 responses, C-αGalCer could be shown to induce more potent anti-malarial and anti-cancer responses (Schmieg et al. 2003; Yang et al. 2004).

What is the mechanism for alterations in the *i*NKT cell response caused by modifying the ceramide lipid? By analogy with altered peptide ligands, it was attractive to propose that a reduced TCR affinity and/or a shorter half-life of the glycolipid/CD1d complexes on the cell surface, contributing to a reduced duration of TCR signaling, would cause a Th2 skewed cytokine profile from *i*NKT cells. Consistent with this, it was reported that a stronger and/or longer stimulation of *i*NKT cells in vitro caused more IFNγ release through an NF-κB-mediated mechanism (Oki et al. 2004). Three findings, however, were not consistent with this direct mechanism. First, it was found that the immediate, in vivo response of *i*NKT cells to glycolipid antigen stimulation, when analyzed directly ex vivo, for example, by intracellular cytokine staining, was not Th2 skewed with a weaker antigenic stimulus (Matsuda et al. 2003; Stanic et al. 2003b; Sullivan et al. 2010). Second, the Th1 skewing Ag C-αGalCer is an even weaker antigen for the invariant TCR than OCH (Sullivan et al. 2010). Third, although glycolipid Ag-activated *i*NKT cells do immediately produce both IL-4 and IFNγ, in addition to other cytokines, after in vivo stimulation, most of the IFNγ produced results from the activation of NK cells stimulated downstream of *i*NKT cell activation (Carnaud et al. 1999; Eberl and MacDonald 2000; Matsuda et al. 2003; Sullivan et al. 2010), a phenomenon referred to as *trans* activation (Parekh et al. 2005). In fact the peak of this IFNγ synthesis, as measured by ELISA for serum cytokines, comes much later than the peak of IL-4 synthesis (Oki et al. 2004). Furthermore, C-αGalCer was shown to be more effective at this *trans* activation than OCH (Sullivan et al. 2010). The key explanatory factor for the ability of C-αGalCer for stimulating NK cell activation, thereby causing a Th1 cytokine skewing of the overall immune response, is apparently its pharmacokinetics, which allows it to carry out a more prolonged stimulation of *i*NKT cells. Therefore, although C-αGalCer–CD1d complexes have a lower affinity for the TCR, after C-αGalCer injection glycolipid plus CD1d complexes accumulate in vivo over time in APC. In fact, APC from mice injected 44 h earlier with C-αGalCer are as potent as APC from mice injected 44 h earlier with αGalCer, when tested ex vivo for their ability to stimulate *i*NKT cell hybridomas (Sullivan et al. 2010). Because C-αGalCer does not bind with more stability to CD1d, this accumulation of the compound in complex with CD1d must reflect its longer half-life in APC. By contrast, APC from mice injected even 17 h earlier with OCH no longer have stimulatory activity. Consistent with the importance of the biologic half-life of the glycolipid Ag, other studies showed that Th2 skewing compounds are less stable in cells due to lysosomal degradation (Bai et al. 2009). In fact, the synthetic chemist Richard Franck et al. designed C-αGalCer with the idea that replacement of the O-glycosidic bond would make the compound more stable. A portion of the CD1d molecules on the cell surface are found in lipid rafts (Lang et al. 2004; Park et al. 2005), and a recent paper reported that compounds that induce a Th2 cytokine skewing did not accumulate as glycolipid/CD1d complexes in the

raft portion of the plasma membrane (Im et al. 2009). It is not known, however, why the presence of glycolipid plus CD1d complexes in lipid rafts is correlated with an increased biologic half-life in APC, and moreover, why the *trans* activation of NK cells requires prolonged *i*NKT cell stimulation.

3 Natural Microbial Lipid Ags

Although αGalCer and similar compounds are useful tools for the study of *i*NKT cells, and αGalCer is a relatively high affinity antigen that is under development as an anti-cancer agent (Cui et al. 1997; Shimizu et al. 2007; Kunii et al. 2009; Motohashi et al. 2009), it was never regarded to be an important natural antigen for this population, given that the mammalian immune system is not selected in evolution for defense against marine sponges (Sandberg and Ljunggren 2005). The first report of a CD1d-dependent, microbial lipid antigen-mediated stimulation of *i*NKT cells was from the Schaible group in 2004 (Fischer et al. 2004). A series of lipid compounds, including phosphatidylinositoldimannoside (PIM_2), lipoarabinomannan, lipomannan, diacyltrehalose, trehalose monomycolate, and trehalose dimycolate, were isolated and purified from *Mycobacterium bovis*. Only PIM could bind to CD1d, but the binding was abolished by phospholipase A_2 (PLA_2), which degrades PIM to the lyso form by removing the fatty acid at the C2 position. CD1d loaded with PIM in a mouse B cell lymphoma line, A20, stimulated splenic *i*NKT cells to release IFNγ in a CD1d-specific manner. Furthermore, a PIM-loaded CD1d tetramer stained both mouse and human *i*NKT cell populations, although only a very small percentage of the *i*NKT cells were stained compared to αGalCer-loaded tetramer. Because *i*NKT cells differ for the complementarity determining region (CDR) 3 of the TCR β chain, it is possible that only a subset of this population, based upon particular CDR3β sequences, has PIM reactivity.

In 2005, three groups independently found the first bacterial source for a glycolipid Ag that could activate essentially the entire population of *i*NKT cells from mice and humans. The Ags are GSLs, meaning that they have a ceramide lipid, and they were obtained from *Sphingomonas* spp., which are nearly ubiquitous, environmental Gram-negative bacteria that lack lipopolysaccharide (LPS) (Kinjo et al. 2005; Mattner et al. 2005; Sriram et al. 2005). Representative of this class of antigens are GSL-1 from *Sphingomonas paucimobilis* and GSL-1′ from *Sphingomonas yanoikuyae*, which highly resemble αGalCer in structure, with a ceramide backbone employing an α-anomeric conformation of glycosidic bond. The major difference between GSL-1 and GSL-1′, when compared to αGalCer, is that the *Sphingomonas* compounds use glucuronic acid and galacturonic acid, respectively, as the saccharide group, instead of the simple galactose in αGalCer. In later studies, GSL-1′ has been referred to as GalA-GSL, standing for galacturonic acid (GalA)-containing glycosphingolipid (GSL) (Fig. 2.1). It is interesting that the hydroxyl group at the 2-carbon position in the fatty-acyl chain of GSL-1 and GSL-1′ also appears in the original agelasphins that αGalCer was derived from (Natori et al. 1994). Based on the overall structural similarity and the presence of *Sphingomonas* bacteria

in sea water, it is possible that the agelasphins were a component of some *Sphingomonas*-like bacteria that were associated with the marine sponge *A. mauritanius* (Tsuji 2006). GSL-1 and GSL-1′, and similar compounds found by others, have the ability to stimulate mouse and human *i*NKT cells both in vivo and in vitro, although these compounds are less potent than αGalCer (Kinjo et al. 2005; Mattner et al. 2005; Sriram et al. 2005), with a corresponding approximately 50-fold reduction in TCR affinity for the complex GalA–GSL with CD1d (Wang et al. 2010). Mice deficient for CD1d, or for the Jα18 segment required to form the invariant TCR, had reduced clearance of the bacteria at early times, although by day 10 the bacteria were cleared even in *i*NKT cell deficient mice, with no evidence for liver damage when moderate doses of bacteria were used.

Not only do different *Sphingomonas* strains differ in their GSL components, but also a single strain can vary its GSL synthesis according to the nutritional supplements and other environmental changes (Kawahara et al. 2000, 2001, 2006). Besides GSL Ags containing monosaccharide sugars, antigens with tri- or tetrasaccharides also have been tested for antigenic activity. For the most part, those GSL Ags with more complex carbohydrates were not highly stimulatory, due to a failure by APC to efficiently degrade the more complex sugar head groups to a simple monosaccharide form that could be recognized (Long et al. 2007; Kinjo et al. 2008). One variant, known as GSL-4A, with a tandem tetrasaccharide α-mannose(1–2) α-galactose(1–6)α-glucosamine(1–4)α-glucuronosyl(1–1)ceramide, was able to stimulate *i*NKT cells, albeit to a much lesser degree compared with the monosaccharide-containing GSL. Unexpectedly, GSL4A could be recognized directly by the TCR of *i*NKT cells after presentation by CD1d, without a requirement for lysosomal processing of its tetrasaccharide head group (Kinjo et al. 2008). Regardless, the varying antigenic activity of the *Sphingomonas* GSL components, combined with the ability of these bacteria to modulate their GSL biosynthesis, might indicate a strategy by which these microorganisms could evade the immune response mediated by *i*NKT cells (Kinjo et al. 2008).

Although *Sphingomonas* bacteria are not highly pathogenic, and in fact there is evidence suggesting they may be a commensal organism (Wei et al. 2010), they potentially could be involved in the pathogenesis of infectious or inflammatory diseases. It has been reported that exposure to *Sphingomonas* bacteria is involved in the causation of a mouse model of primary biliary cirrhosis, an autoimmune disease in which the bile ducts are damaged (Hsueh et al. 1998; Perola et al. 2002; Selmi et al. 2003; Mattner et al. 2008).

A second type of microbe known to have glycolipid Ags that activate most *i*NKT cells is *Borrelia burgdorferi*, a spirochete that causes Lyme disease, currently the most common vector-borne disease in the USA (Bacon et al. 2008). Depending on the inbred strain background, mice infected by this pathogen can develop chronic inflammation of varying severity in skin, joints, heart, and the nervous system (Yang et al. 1994). Infected CD1d-deficient mice developed increased arthritis, and the secretion of pathogen-specific IgM antibodies by MZB cells was impaired in the absence of CD1d, coinciding with an elevated pathogen burden in the blood (Kumar et al. 2000; Belperron et al. 2005) and in the joint itself (Lee et al. 2010). These results indicated CD1d is required to control *Borrelia* infection. As noted

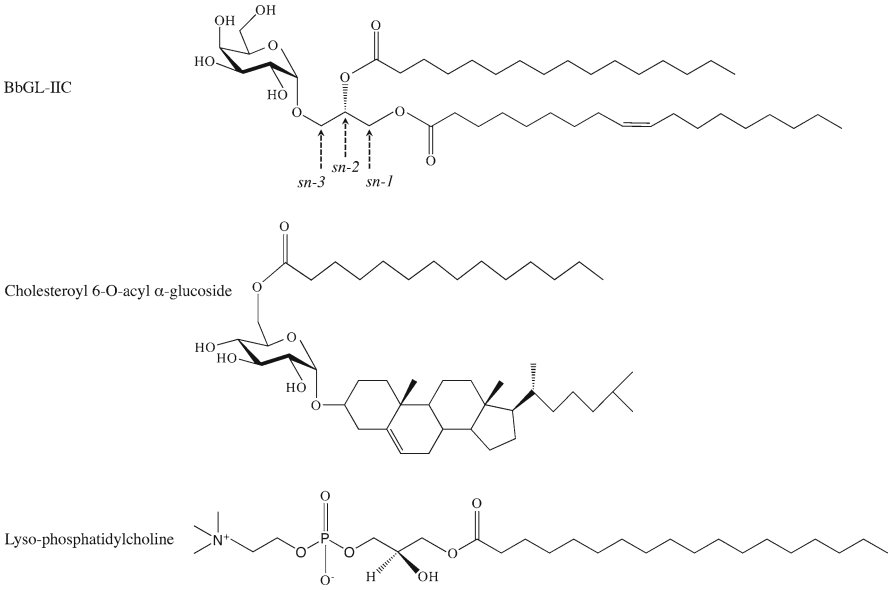

Fig. 2.3 Structures of synthetic and natural Ags that are not GSLs. *Top*: the *B. burgdorferi* glycosylated diacylglycerol Ag BbGL-IIc is depicted. *Middle*: cholesterol containing Ag from *H. pylori*. *Bottom*: lyso-phosphatidylcholine, a self-Ag for human *i*NKT cells

above, however, CD1d can stimulate cells in addition to *i*NKT cells, but in papers published recently, Jα18 knockout mice were shown to have defects in spirochete clearance and the prevention of arthritis when analyzed on the BALB/c background (Tupin et al. 2008), or carditis, when C57BL/6 mice were tested (Olson et al. 2009). These data indicate that *i*NKT cells have a critical role in the host defense against *Borrelia* pathogens.

Two abundant glycolipids were found in the cell wall of *B. burgdorferi*, and these are targets of an antibody response in infected individuals (Hossain et al. 2001). One of these is cholesteroyl 6-*O*-acyl-β-D-galactopyranoside, called *B. burgdorferi* glycolipid 1 (BbGL-I), and the other is 1,2-di-*O*-acyl-3-*O*-α-D-galactopyranosyl-sn-glycerol (BbGL-II) (Ben-Menachem et al. 2003). BbGL-II was found to stimulate *i*NKT cells in a CD1d-dependent manner (Kinjo et al. 2006). It consists of a D-galactose moiety linked to diacylglycerol (DAG) (Fig. 2.3). The structure of BbGL-II is distinct from that of the GSL Ags, because its lipid moiety is identical to a typical glycerophospholipid, instead of a sphingolipid, with a ceramide backbone. Strikingly, however, this DAG *i*NKT cell Ag also has an α-anomeric glycosidic bond linking the hydrophilic saccharide portion to the hydrophobic lipid.

In fact, purified BbGL-II is not a uniform entity, as it contained a mixture of at least five different fatty acids that in principle could be linked in different ways to the sn-1 and sn-2 positions of the glycerol. Kinjo et al. analyzed eight synthetic versions of BbGL-II that differ only with regard to their fatty acid composition, and

interestingly, only one of these, BbGL-IIc, with a C18:1 oleic acid in the sn-1 position and a C16:0 palmitic acid in the sn-2 position was highly antigenic for *i*NKT cells. Human CD1d presented different BbGL-II variants to *i*NKT cells, preferring those with more unsaturated bonds in the fatty acids. The structural basis of this difference is uncertain, but human CD1d has a bulky tryptophan at position 153 in the α2 helix, near the opening of the groove where the sugar protrudes, compared to glycine in the equivalent position for mouse CD1d. Mutagenesis studies show that this position influences the preferential presentation of DAG Ags with certain fatty acids (Wang et al. 2010), perhaps because of differences in the conformation of the CD1d molecule when opened for lipid loading.

Recently it was shown that a cholesterol-containing antigen from *Helicobacter pylori*, which causes stomach ulcers, also could activate *i*NKT cells (Chang et al. 2011). The antigen, cholesteroyl 6-*O*-acyl α-glucoside, could activate *i*NKT cells in vitro in a CD1d-dependent fashion, and could modulate the function of *i*NKT cells in vivo in favor of IFNγ production, which was protective for asthma in young mice exposed to this compound (Chang et al. 2011). It is not certain, however, how the cholesterol moiety participates in the binding to CD1d and/or interactions with the semi-invariant TCR.

4 Endogenous Lipid Ags

In a variety of contexts, *i*NKT cells exhibit CD1d-dependent activation in the apparent absence of microbial Ags. Therefore, these cells are believed to be self-reactive, and it was further assumed that there is a single self-Ag, or a limited set of closely related and relatively high affinity self-Ags that can activate the invariant TCR. This hypothesis remains unproven (Gapin 2010), however, and several types of self-Ags have been identified, including GSLs and antigens that are not GSLs (Pei et al. 2011). Furthermore, none of the self-Ags have been shown convincingly to be required absolutely for the differentiation or stimulation of *i*NKT cell populations. An early analysis by mass spectrometry of the cellular lipids bound to CD1d found glycosylphosphatidylinositol (GPI) to be a major ligand (Joyce et al. 1998). However, there was no evidence showing this ligand can stimulate *i*NKT cells physiologically. Therefore, GPI was suggested to function as a "spacer" by filling the groove of CD1d to prevent its collapse when the protein is synthesized in the endoplasmic reticulum, with true stimulatory Ags acquired later as CD1d traffics to and recycles through endosomes. Subsequent chemical analyses of the lipids bound to CD1d found a variety of species, including phosphatidyl inositol (PI), phosphatidyl choline (PC), and the lyso forms of these antigens, i.e., forms lacking one of the fatty acid chains (Cox et al. 2009; Yuan et al. 2009).

The first demonstration that a self-derived cellular lipid had a stimulatory effect on *i*NKT cells was reported by Gumperz et al. (2000). They found that plate-bound mouse CD1d molecules presented lipid Ags from an extract of tumor cells to an *i*NKT cell hybridoma. The lipid Ags were identified to be phosphatidylinositol (PI)

and its derivatives, with PI having the highest antigenic potency. However, this *i*NKT cell hybridoma did not respond to αGalCer, and therefore it apparently was not representative of the *i*NKT cell population. Similarly, it was reported that disialoganglioside GD3, another self-derived cellular lipid provided an Ag for a subset of *i*NKT cells. GD3 immunization was required, however, in order to detect a GD3-reactive *i*NKT cell population (Wu et al. 2003). In both of these cases, a subset of cells with a particular Vβ TCR, that defines a particular specificity, may have been detected.

Because GSLs provide such high potency exogenous Ags for *i*NKT cells, it is perhaps reasonable to suppose that self-Ags also are different types of GSLs. Evidence consistent with GSLs providing self-Ags has been reported in several papers, but these did not identify a particular structure (Paget et al. 2007; Salio et al. 2007). Paget et al. found CpG ODN, a TLR9 ligand, activated DCs to synthesize a charged α-linked GSL(s), and also to produce type I IFN, both of which were required to stimulate *i*NKT cells (Paget et al. 2007). Also, Salio et al. showed Toll-like receptor (TLR) ligands could induce increased GSL biosynthesis in Ag presenting cells, coincident with enhanced recognition of CD1d-associated endogenous lipids by *i*NKT cells. Recently it was shown that APC from mice deficient for α-galactosidase A had increased stimulatory activity for *i*NKT cells. α-Galactosidase A removes terminal galactose sugars from certain oligosaccharide-containing GSLs, and together with other experimental results, the authors concluded that this finding reflects the ability of α-galactosidase A to degrade a major self-Ag for *i*NKT cells (Darmoise et al. 2010).

Further identification of potentially important self-antigens was obtained by analyzing cells and mice deficient for enzymes important for GSL biosynthesis. In mammals, there are two GSL biosynthetic pathways: the major one synthesizes oligosaccharide-containing GSLs, including gangliosides, from β-glucosylceramide, and the minor one synthesizes GSLs from β-galactosylceramide. β-galactosylceramide deficiency did not cause a significant decrease in *i*NKT cell frequency (Stanic et al. 2003a). Because loss of ceramide glucosyltransferase is a lethal mutation, the effect of β-glucosylceramide deficiency was determined in the GM95 cell line, a mutant of the B16 melanoma deficient for this enzyme. GM95 transfectants with transient CD1d expression had defects in stimulating *i*NKT cells, while the control cells, which were rescued by introducing the cDNA encoding the wild-type enzyme, did not. This result indicated that β-glucosylceramide might be the biosynthetic precursor of the self-Ag, as β-glucosylceramide itself cannot stimulate *i*NKT cells directly. It is surprising, therefore, that stable CD1d transfectants of GM95 were able to stimulate *i*NKT cell autoreactivity (Pei et al. 2011).

An intriguing finding in the search for *i*NKT cell self-Ags was the identification of the GSL isoglobotrihexosyl ceramide (iGb3) as a putative self-Ag required not only for *i*NKT cell differentiation, but also for the stimulation of the autoreactive *i*NKT cells in the periphery (Zhou et al. 2004; Mattner et al. 2005). This identification was based in part on the finding that β-hexosaminidase b-deficient mice, a model of human Sandhoff's disease, have a profound reduction in *i*NKT cell number, while this was not found with several other GSL biosynthetic deficiencies. The conclusion

that iGb3 is the main thymic *i*NKT cell-selecting ligand subsequently has been challenged in different ways. Several reports have questioned if the defect in *i*NKT cells is related directly to Ag biosynthesis, as opposed to resulting from lipid storage disease, which would cause a more general defect in the lysosomal site of CD1d Ag loading (Gadola et al. 2006b; Schumann et al. 2007). Others have questioned if humans have a functional iGb3 synthase (Christiansen et al. 2008), and if mice have significant amounts of iGb3 in double positive thymocytes, the cells that positively select *i*NKT cells, or in the DCs that are most important for stimulating them in the periphery (Speak et al. 2007). The presence of iGb3 in these cell types remains controversial (Li et al. 2008). The most compelling evidence against iGb3 as the main positively selecting self-Ag, however, comes from the finding that *i*NKT cell number and function are normal in mice deficient for iGb3 synthase (Porubsky et al. 2007).

Regardless, iGb3 is a mammalian GSL, and it can stimulate *i*NKT cells. It consists of a ceramide, like all GSLs, and a trisaccharide head group, composed of two galactose rings and a glucose ring in tandem (Fig. 2.1). Consistent with other mammalian GSLs, but distinct from the microbial lipid Ags, the sugar portion and the lipid portion are linked with a β-anomeric conformation of the glycosidic bond. This raises the interesting question as to how this bulky trisaccharide is recognized by the invariant TCR, and with the β-glucose linked to the ceramide drastically out of position, which of the three sugars participates most in TCR recognition. The study of chemical analogies of iGb3 indicates that the terminal galactose of the trisaccharide is the most important for TCR recognition (Chen et al. 2007a), consistent with a model (Zajonc et al. 2008) in which the third sugar is squashed down into a position similar to the lipid-proximal sugar in monosaccharide-containing antigens.

Just as exogenous lipid Ags are not limited to GSLs, for example, DAG-containing Ags also have been found (Kinjo et al. 2006), a similar diversity in lipid structure has also been reported for the putative self-Ags. As mentioned above, a glycerophospholipid, PI, also is able to stimulate an *i*NKT cell hybridoma (Gumperz et al. 2000), but it is not the universal *i*NKT cell ligand. A recent study reported the surprising finding, however, that lyso-phosphatidylcholine (lyso-PC), eluted along with several other lipids from human CD1d, could stimulate the majority of human *i*NKT cell clones tested in a CD1d-dependent fashion (Cox et al. 2009; Fox et al. 2009). Lyso-PC, different from other phosphoacylglycerols like PI (Fig. 2.3), only contains a single hydrophobic acyl chain, yet is the most active compound compared to all of the others that were eluted from human CD1d. It is unknown why a glycerol Ag with a single fatty acid chain is more potent than one with two acyl chains, while the opposite is true for GSLs. Moreover, we found that lyso-PC could not stimulate mouse *i*NKT cells, although the data suggested that the self-Ags in some cell types could not be GSLs (Pei et al. 2011). This difference between mouse and human *i*NKT cell self-Ags is not surprising, however, as previously it was shown that presentation of the self-Ag for mouse *i*NKT cells required lysosomal localization of mouse CD1d, while a similar requirement was not found for human *i*NKT cell responses to self-Ag (Chen et al. 2007b). These data suggest that the two self-Ag recognition systems could be quite different, although this is curious considering the conservation of the invariant TCR, and its well-conserved recognition of microbial Ags.

5 Structural Basis of Lipid Ag Recognition

In structure, CD1d is closely related to the MHC class I antigen presenting molecules, as it consists of a β_2m light chain and a heavy chain with Ag-binding α1 and α2 domains attached to a more membrane proximal immunoglobulin-like α3 domain. To the C terminal side of the α3 domain there is a *trans*membrane sequence and a short cytoplasmic tail (Brigl and Brenner 2004; Cohen et al. 2009). High-resolution three-dimensional structures of liganded CD1d, with and without the *i*NKT cell TCR bound, have provided important insights into the structural basis for lipid Ag presentation and recognition (Gadola et al. 2006a; Kjer-Nielsen et al. 2006; Borg et al. 2007; Pellicci et al. 2009). These have been complemented by site-directed mutagenesis studies of CD1d (Burdin et al. 2000; Kamada et al. 2001) and the TCR (Scott-Browne et al. 2007; Mallevaey et al. 2009). Compared with MHC class I or II molecules, the Ag binding groove of CD1d is deeper, narrower, and more hydrophobic. It is composed of two connected pockets, A′ and F′, that accommodate the hydrocarbon chains of lipids. The A′ pocket is deeply buried and bigger with the capacity of accommodating up to a 26-carbon long hydrocarbon chain. The hydrocarbon chains must wind around a central pole formed by amino acids Cys12 and Phe70, and they do this in either a clockwise or counterclockwise direction, depending on the Ag bound. The F′ pocket is smaller, and it can accommodate 18 carbon chains, or even longer, in a more or less linear orientation. The F′ pocket is also more accessible to the solvent, at least when the TCR is not bound. A lipid Ag is accommodated in the CD1d groove with the hydrophobic lipid moiety anchored in the two pockets and the saccharide head group protruding from an opening in the surface of the CD1d protein. Therefore, the *i*NKT cell TCR contacts mainly the exposed carbohydrate moiety, and, to a lesser degree, the hydrophilic portion of lipid backbone, as well as amino acids on the top of CD1d.

The α-anomeric linkage of the sugar is a key factor that affects the recognition of lipid Ags by the invariant TCR. The crystal structures of α-linked GSL–CD1d complexes show that the carbohydrate portions of the Ag adopt a parallel orientation to the top of CD1d surface. The β-anomeric conformation renders the saccharide head group in a perpendicular orientation to the top of CD1d, and therefore pointing up toward the TCR, likely interfering with the TCR interaction. As noted above, iGb3 and βManCer must constitute exceptions. For iGb3, perhaps the highly exposed trihexosyl head group is squashed by the invariant TCR to lie flat between the TCR and CD1d surface (Zajonc et al. 2005, 2008; Wu et al. 2006; Yin et al. 2009). While αManCer, with the mannose sugar having a 2′′ OH in the axial position, is not antigenic, it is possible that when bound to CD1d the β-linked sugar orients this hydroxyl in a more optimal position for contacting the CDR3α region of the TCR, similar to the equatorial OH found in GSL Ags containing α-linked galactose or glucose.

Hydrogen bonds formed between CD1d and the hydrophilic portions of the glycolipid Ag, including the carbohydrate, and for GSL Ags the 3′ hydroxyl of the sphingosine, are important for orienting the Ag for TCR recognition. All the GSL Ags adopt a similar mode of interaction with CD1d, with the N-acyl chain in the

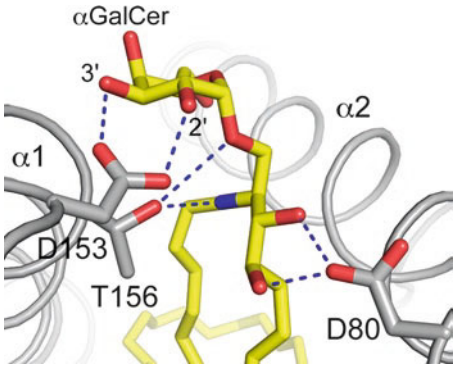

Fig. 2.4 Binding of GSL ligands in the mouse CD1d binding groove. Details of the interaction of αGalCer (ligand in *yellow*, PDB ID 1Z5L) with residues on the α1 and α2 helices of mouse CD1d are depicted. H bonds that stabilize the bound Ag are shown as *blue dashed lines*

A′ pocket and the sphingosine in F′ pocket. If the acyl chain is too short to fill the A′ pocket, a spacer lipid, apparently is recruited to occupy the remainder of the pocket (Zajonc and Kronenberg 2007, 2009; Zajonc and Wilson 2007). However, when the sphingosine is short, as for OCH, the F′ pocket tends to collapse at least partially, therefore leading to subtle structural changes at the CD1d surface above this pocket, and consequently affecting recognition by the *i*NKT TCR (McCarthy et al. 2007). The fixed mode of GSL Ag binding is based on the planar N-amide of the ceramide and hydrogen bonds between Asp80 of the α1 helix with the sphingosine hydroxyls, and Asp153 of the α2 helix with the 2′ and 3′ hydroxyls of the galactose sugar (Fig. 2.4).

DAG-containing Ags have a lipid backbone that is less rigid than the ceramide backbone of GSLs, and their different structure leads to two possible binding orientations to mouse CD1d for Ags in this category. Phosphatidylcholine (PC) is bound by CD1d with the sn-1 fatty acid in the F′ pocket and sn-2 fatty acid in the A′ pocket (Giabbai et al. 2005), while PIM_2 was bound in opposite orientation (Zajonc et al. 2006), which, as a result, will lead to a slight difference in head group positioning for the two lipids above the CD1d surface. For DAG containing glycolipid Ags with sugar head groups, the H bonding pattern is different from the GSLs. The galactose of BbGL-IIc is tilted up and away from the α2 helix of mouse CD1d, thereby losing the H bonds with Asp153, and there is a single H-bond between the galactose and Arg79 of mouse CD1d, a bond not observed with any GSL Ags.

A structural comparison of αGalCer to the two bacterial Ags, *S. yanoikuyae* GalA-GSL and *B. burgdorferi* BbGL-IIC, bound to mouse CD1d, with and without the TCR, is highly instructive of the principles governing synthetic and bacterial glycolipid Ag recognition. When αGalCer is bound to mouse CD1d, the sugar is protruding in the same orientation before and after TCR engagement, and the roof over the F′ pocket is closed. Therefore, the antigen/CD1d complex is correctly oriented and no accommodation is required when the TCR binds. For GalA–GSL complexes with CD1d, which have a weaker affinity for the invariant TCR, the sugar is oriented correctly when the GSL Ag binds CD1d without the TCR, but the roof over the F′ pocket is not closed. Closure does occur, however, in the complex

with the TCR. Therefore, in this case mouse CD1d accommodates the TCR by conformational change above the F′ pocket. BbGL-IIc complexed with mouse CD1d is nearly tenfold weaker in binding affinity compared to GalA-GSL, and therefore very substantially weaker, approximately 500-fold weaker, than αGalCer. In this case, the sugar is rotated 60° counterclockwise from the optimal position when bound to mouse CD1d without the TCR, and the roof over the F′ pocket also is not closed. Remarkably, when the invariant TCR is bound, the trimolecular complex that includes BbGL-IIc is quite similar to the one with αGalCer. Therefore, binding of the TCR involves not only conformational changes over the F′ pocket of mouse CD1d but also movement of the galactose sugar by 60° in the clockwise direction.

These findings also shed some light on the mysterious selectivity for particular fatty acids for the recognition of the DAG-containing glycolipid Ags from *B. burgdorferi*. Although BbGL-IIf is not antigenic for mouse *i*NKT cells, it also binds to mouse CD1d (Wang et al. 2010). However, the different possible binding modes for the DAG-containing compounds causes the loss of antigenic activity in this case. For the antigenic BbGL-IIc, the C18:1 fatty acid linked to the sn-1 position of the glycerol is bound to the A′ pocket of mouse CD1d. For the non-antigenic BbGL-IIf, it is the sn-2 fatty acid, also C18:1, that is bound in the A′ pocket. While we do not know why the A′ pocket strongly prefers a C18:1 fatty acid over both the C16:0 alternative in BbGL-IIc, or the C18:2 in BbGL-IIf, the difference in orientation of the glycerol backbone causes the sugar in BbGL-IIf to be rotated a further 60° counterclockwise compared to the complex of BbGL-IIc with mouse CD1d, or 120° compared to αGalCer complexes with mouse CD1d. This is apparently too far out of the optimal position for effective accommodation and invariant TCR binding. We have less information about how an Ag like BbGL-IIf is presented effectively by human CD1d, but as noted above, the evidence suggests that the presence of Trp153 in human CD1d compared to Gly155 in mouse CD1d is in part responsible.

The reciprocal cross-species reactivity of mouse and human *i*NKT TCRs (Brossay et al. 1998) and high-resolution crystal structures of mouse and human CD1d-glycolipid-TCR tri-molecular complexes show that the invariant TCR binds in a similar pattern to diverse antigens, reflecting the high degree of evolutionary conservation in their interactions. The main contacts of the TCR with CD1d antigen presenting molecule involve amino acids in CDR3α with a contribution of amino acids in CDR2β, particularly Tyr50β. These interactions principally involve amino acids above the F′ pocket. However, because of variability in the TCR β chain, both with regard to the presence of diverse CDR3β regions, and the use in mice of three predominant Vβ segments, as noted above the TCR of *i*NKT cells is in effect semi-invariant. It is therefore not surprising that differences in the Vβ regions affect the interactions of the TCR with mouse CD1d–αGalCer complexes (Borg et al. 2007; Pellicci et al. 2009). For example, compared with the Vβ8.2 chain in the invariant TCR, structural analysis of a Vβ7-containing invariant TCR showed that this β chain contributed to a greater extent to interactions with the CD1d–αGalCer complex (Pellicci et al. 2009). Furthermore, the presence of Vβ7 resulted in an altered interface between the α chain and CD1d, despite sharing of identical, invariant Vα chains. The structural and mutagenesis studies also show that CDR3β

sequences can participate in the trimolecular interaction to varying extents, thereby influencing TCR avidity (Mallevaey et al. 2009). These alterations also provide a structural basis not only for variability in avidity for common antigens for the entire population, but they also provide a rationale for the presence of subspecificities within the population, such as described for GD3 and perhaps other Ags.

6 Conclusion/Summary

Antigens for *i*NKT cells come from diverse sources, including synthetic Ags related to αGalCer, microbial Ags, and endogenous or self-Ags. Although much has been learned about the types of glycolipid Ags that are recognized by *i*NKT cells, how they bind to CD1d, and how the complexes interact with the TCR to achieve a conserved binding mode, the picture remains incomplete.

Considerable effort has been placed on the testing of synthetic Ags related to αGalCer, a highly potent Ag that was the first one discovered. Significant variations on the basic αGalCer structure, which alter either the sugar and/or the stereochemistry of its linkage to ceramide, the sphingosine base, or the fatty acid, can still be recognized by *i*NKT cells, and in some cases provide Ags equal to, or even greater in potency, than αGalCer (Schmieg et al. 2003). Variants of αGalCer can engender highly different cytokine responses, however, and while this involves a network of cellular responses initiated downstream of the initial *i*NKT cell activation, the molecular and cellular mechanisms for cytokine skewing by glycolipid Ags remain to be fully determined.

Microbial Ags that activate a large fraction of *i*NKT cells have been identified from three types of bacteria, and in two cases they share the common features of having a lipid with two acyl chains and an α-linked hexose sugar. Although the lipids are buried in the CD1d groove, studies have shown how the lipids can determine antigenic potency by influencing the orientation of the sugar protruding from the roof of CD1d. Furthermore, it has been demonstrated that these two types of microbes, *Sphingomonas* spp. and *B. burgdorferi*, produce mixtures of antigenic and non-antigenic glycolipids, which could constitute an immune evasion mechanism. For the third type of bacteria, *H. pylori*, the Ag has a very different structure that includes a cholesterol moiety derived from the eukaryotic host. The underlying biochemistry of the interactions between the *H. pylori*, Ag, CD1d, and the TCR is not known. Furthermore, although there is detailed knowledge of the structure of the Ags from these few species, the number of different types of bacteria having such Ags, the diversity of their structures, and the relationship of the antigen-specific response to the clearance of the bacterial infection all require further investigation.

Like the synthetic and microbial Ags, the self-Ags also have diverse structures. iGb3 clearly is a GSL self-Ag that activates *i*NKT cells, but it is highly unlikely that is required or the major self-Ag, and data from several groups suggest there could be other GSL self-Ags. In humans, an entirely different structure, lyso-PC, has been described as a self-Ag that activates many *i*NKT cells. Lyso-PC does not

activate mouse *i*NKT cells, however, which makes it difficult to show that this Ag is required for *i*NKT cell differentiation. Moreover, it would be surprising if mouse and human *i*NKT cells depended on entirely different self-Ags, considering the high degree of interspecies conservation of the *i*NKT cell TCR. Additionally, it remains uncertain if there is a single, major self-glycolipid required for the selection and/or activation of *i*NKT cells, or alternatively, if there are collections of relatively low affinity glycolipids bound to CD1d collectively that are sufficient for activation.

How is it that the semi-invariant TCR can recognize so many different types of Ags? Part of the answer lies in the extraordinarily high affinity of the TCR interaction with αGalCer–CD1d complexes, measured in some studies to be as low as 11 nM. It is therefore not difficult to measure an antigenic response to compounds with an affinity that is 500 times weaker than αGalCer, thereby allowing for considerable structural variation. Additionally, unlike for MHC class I and class II antigen presenting molecules, the roof over the CD1d groove is mostly closed, especially when the TCR is bound, and therefore many of the important contacts between the semi-invariant TCR and the glycolipid–CD1d complex involve CD1d. These stereotypical contacts, involving mostly CDR3α and CDR2β, dictate a particular binding mode, with the TCR oriented parallel to the CD1d α helices. Given the importance of contacts with CD1d, it is therefore possible that lipid-containing Ags with different structures can provide the marginal boost to TCR affinity that would allow for TCR engagement and *i*NKT cell activation.

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Chapter 3
Invariant NKT Cell-Based Vaccine Strategies

John-Paul Jukes, Jonathan D. Silk, Mariolina Salio, and Vincenzo Cerundolo

Abstract The success of vaccination strategies depends on the efficient generation of appropriate antigen-specific T- and B-cell responses. The unique position of invariant natural killer T (iNKT) cells at the interface of the innate and adaptive immune systems and their ability to direct the maturation of dendritic cells and B cells offers the possibility of harnessing them to "jump-start" the antigen-specific immune response to both microbial pathogen and tumor antigens. In this chapter, we explore the development of pharmacological agents that when used in vaccination strategies as adjuvants to antigenic proteins are able to activate iNKT cells which then augment antigen-specific T- and B-cell responses. In addition, we consider the future directions and challenges in translating these findings from experimental data obtained in mice to use in the clinic.

1 Introduction

Advances in molecular technology have permitted the design of highly specific synthetic protein vaccines that stimulate a more focused immune response repertoire than is possible with traditional vaccines based on live attenuated pathogens or inactivated whole organisms. Compounds that mimic the signals involved in stimulation of an antigen-specific T-cell response to infectious disease and tumors have significant potential as adjuvants for vaccination strategies. To achieve this, a detailed understanding of the cells and signaling pathways involved in the immune response to natural infection is required. Both T- and B-cell responses are modulated by dendritic cells (DC), which are themselves activated by a complex network

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of signals initiated by interactions with the pathogen, through receptors for pathogen-associated molecular patterns, such as the Toll-like receptors (TLRs), and ligation of CD40 by other antigen-responsive cells. Understanding that invariant natural killer T (iNKT) cells express CD40L constitutively (Vincent et al. 2002) and hence modulate DC and B-cell activity has resulted in their investigation as a means of harnessing the immune system to develop more effective vaccination strategies than those currently available. The use of specific agonists to activate iNKT cells, so that they "jump-start" the immune response, is an attractive adjuvant therapy for vaccine development (Cerundolo et al. 2009).

In this chapter, we explore the notion that iNKT cell adjuvants can be used as a means of boosting the immune response to infectious diseases and to tumors.

2 Activation of iNKT Cells

iNKT cells are a unique subset of T cells that expresses an antigen-specific T-cell receptor (TCR) composed of an invariant α-chain (Vα14-Jα18 in mice and Vα24-Jα18 in man) paired with a restricted repertoire of β chains (Vβ2, Vβ7 and Vβ8.2 in mice, Vβ11 in man). iNKT cells account for approximately 10% of the total T-cell population in the liver, and <1% in the spleen, lymph nodes and blood in mice (with slightly lower frequencies reported in humans) (Hammond et al. 1999; Ishihara et al. 1999). Unlike conventional T cells that recognize protein-derived antigens presented by major histocompatibility complex (MHC) class I and class II molecules, the TCRs on iNKT cells recognize either exogenous or endogenous lipid ligands presented in the context of the nonpolymorphic MHC class I-like CD1d molecules (Balk et al. 1989; Macdonald 2007). Development of iNKT cells branches off from the pathway of mainstream T-cell development, and leads to the acquisition of a memory/activated phenotype which together with their high frequency confers on iNKT cells the ability to rapidly secrete large amounts of cytokines in response to TCR engagement (Matsuda et al. 2008; Godfrey et al. 2010).

Activation of iNKT cells occurs in response to a number of endogenous and exogenous lipid ligands. Exogenous lipids derived from *Sphingomonas capsulata* and *Borrelia burgdorferi* are recognized directly by the iNKT TCR when they are bound to CD1d (Kinjo et al. 2005, 2006; Mattner et al. 2005; Wang et al. 2010) independently of TLR-mediated activation of antigen-presenting cells or the secretion of interleukin-12 (IL-12). Indeed, the cell wall of gram-negative *S. capsulata* lacks lipopolysaccharide but is rich in α-linked glycosphingolipids that have a number of different structures due to variability in the sequence of the α-linked sugar, the acyl chain and sphingosine base of the ceramide lipid (Kinjo et al. 2008). Thus the cell wall consists of a combination of immunogenic (e.g., α-glucuronosylceramide) and nonimmunogenic glycosphingolipids (e.g., tetrasaccharide-containing glycosphingolipids) (Kinjo et al. 2005; Mattner et al. 2005), the balance of which is proposed to determine the immune response to infection and may also represent a mechanism of immune evasion. Similarly *B. burgdorferi* lipid antigens that are recognized by

iNKT cells are galactosyl diacylglycerols that vary in length and degree of saturation (Kinjo et al. 2006). In addition, the surface glycol-conjugate lypophosphoglycan of *Leishmania donovani* (Amprey et al. 2004), the phosphatidylinositol tetramannoside (PIM-4) of *Mycobacterium leprae* (Fischer et al. 2004) and the ubiquitous α-proteobacterium *Novosphingobium aromaticivorans* (Mattner et al. 2008) have been shown to activate iNKT cells. $CD1d^+$ macrophages infected with *Mycobacterium tuberculosis* are able to activate iNKT cells and in doing so aid bacterial clearance both in vitro and in vivo (Sada-Ovalle et al. 2008). In some circumstances, for example during infection with *Salmonella enterica*, iNKT cells can be activated indirectly by stimulatory cytokines (e.g., IL-12 and type-1 interferons) released by DCs subsequent to TLR signaling (Brigl et al. 2003; Paget et al. 2007; Salio et al. 2007). In this case, the cytokines serve to amplify the weak activating signals that are generated by the ligation of endogenous (self) glycolipid ligands (Cox et al. 2009; Fox et al. 2009; Salio and Cerundolo 2009; Yuan et al. 2009). In addition, antigen-presenting cell (APC)-derived cytokines can activate iNKT cells in the absence of TCR recognition and engagement of lipid–CD1d complexes (Nagarajan and Kronenberg 2007).

iNKT cells are highly conserved between mice and humans – cells from both species are stimulated by CD1d-bound α-galactosylceramide (α-GalCer – the archetypal iNKT cell agonist) and because of their restricted TCR diversity respond at high frequency to lipid ligands. Of significance to the development of vaccine adjuvants, iNKT cells can be activated by pharmacological compounds that bind to CD1d molecules and are recognized by the iNKT TCR. α-GalCer (KRN7000) (Kawano et al. 1997) is a lipid compound derived from the marine sponge *Agelas mauritianus* and has a 26-carbon alkyl chain with a shorter sphingosine chain occupying the two hydrophobic channels, A′ and F′ of CD1d, respectively, leaving the polar head exposed for recognition by the TCR (for detailed crystallographic studies see Zeng et al. 1997; Koch et al. 2005; Zajonc et al. 2005 and Chap. 10 in this volume). The glycolipid head group of α-GalCer is engaged by the iNKT TCR α-chain, while the TCR β-chain stabilizes this interaction by binding to the CD1d molecule (Borg et al. 2007; Scott-Browne et al. 2007; Mallevaey et al. 2009; Pellicci et al. 2009). Responsiveness to α-GalCer broadly discriminates between the two main subsets of NKT cells. Type I or iNKT cells, the subject of this review, are responsive to α-GalCer, whereas type II or noninvariant NKT (non-iNKT) cells do not respond to α-GalCer and are less well characterized, although a subset of type II NKT cells has been shown to be reactive to sulfatides presented by CD1d (Halder et al. 2007).

Activation of iNKT cells by α-GalCer initially results in the rapid production of both Th1 and Th2 type cytokines [such as IL-2, -3, -4, -6, -9, -10, -17, -21, GM-CSF and tumor necrosis factor (TNF)-α] but within 24 h this becomes a predominantly Th1-type profile (IFN-γ and diminished IL-4) (Coquet et al. 2008; Matsuda et al. 2008), which is due to sustained IFN-γ secretion by NK cells activated in trans by iNKT cells. It is the presence of substantial pools of preformed cytokine mRNA (e.g., IL-4 mRNA) in iNKT cells that allows them to achieve the initial rapid release of cytokines following activation (Matsuda et al. 2003).

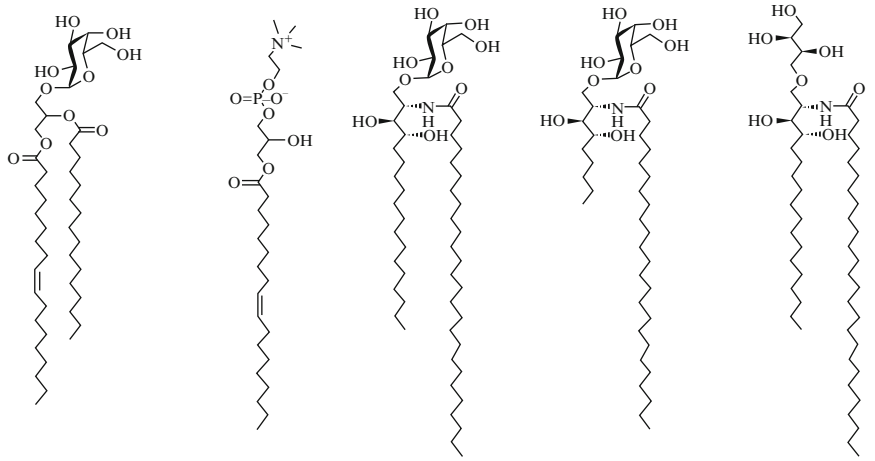

Fig. 3.1 Examples of iNKT cell agonists. iNKT cell agonists from the spirochete *B. burgdorferi* (BbGL-II) and synthetic compound α-galactosylceramide (α-GalCer). Analogs of α-GalCer have been synthesized that preferentially promote Th2-like cytokines, following iNKT cell activation (OCH) or reduce activation-induced DC lysis but also have adjuvant properties (threitolceramide; ThrCer)

While synthetic iNKT cell agonists such as α-GalCer evoke stronger and faster iNKT cell activation than microbial proteins or TLR agonists (Kinjo et al. 2005, 2006; Kim et al. 2008), there are limitations of using such compounds to activate iNKT cells. Overstimulation of iNKT cells can induce energy (a state in which iNKT cells become unresponsive to subsequent α-GalCer injections) and lysis of DC that are loaded with α-GalCer, following TCR engagement (Parekh et al. 2005; Uldrich et al. 2005; McCarthy et al. 2007; Silk et al. 2008). Thus, in recent years several synthetic analogs of α-GalCer have been synthesized that activate iNKT cells both in vitro and in vivo (Savage et al. 2006; McCarthy et al. 2007; Reddy et al. 2009; Li et al. 2010; O'Konek et al. 2011). Examples of endogenous, exogenous, and synthetic iNKT cell ligands are illustrated in Fig. 3.1.

3 iNKT Cells at the Interface Between Innate and Adaptive Immunity

iNKT cells occupy a unique position at the interface between the innate and adaptive arms of the immune response. Activation of iNKT cells enhances adaptive immune responses by upregulation of DC and B-cell maturation via the co-stimulatory molecule CD40 ligand (CD40L/CD154), and downstream activation of NK cells

through the production of IFN-γ (Vincent et al. 2002; Fujii et al. 2003; Galli et al. 2003; Hermans et al. 2003).

In early studies, Vincent et al. (2002) showed that in vitro the presence of CD1d-restricted iNKT cell clones was essential for DC maturation – a crucial step in effective T-cell activation. Support for these observations was subsequently provided in vivo by ourselves and others: iNKT cells are able to induce DC maturation rapidly in vivo (i.e., upregulation of CD40, 70, 80, 86, MHC class II) following activation with α-GalCer, a process that requires CD40–CD40L for optimal DC–iNKT cell interaction (Fujii et al. 2003; Hermans et al. 2003; Taraban et al. 2008). In addition, it was shown that following DC presentation of α-GalCer, iNKT TCR ligation was characterized by the rapid production of TNF-α – a requirement for the upregulation of the co-stimulatory molecules, CD80/86 (Fujii et al. 2004). These findings were confirmed by the demonstration that in the absence of iNKT cells recombinant TNF-α evoked similar upregulation of CD80/86 (Fujii et al. 2004). The failure of α-GalCer to elicit T-cell immunity when injected into $CD40^{-/-}$ or $CD40L^{-/-}$ mice confirmed that CD40 is required for DC production of IL-12 and the development of T-cell immunity (Hermans et al. 2003; Fujii et al. 2004). Subsequently, we showed that following iNKT cell activation soluble mediators (such as type-1 and -γ interferons) are required for the maturation of both DC and B cells (Hermans et al. 2003; Silk et al. 2004). It is now apparent that several co-stimulatory molecules are able to regulate iNKT cell activation, for example PD-1, OX40, 4-1BB, and ICOS (Zaini et al. 2007; Akbari et al. 2008; Chang et al. 2008; Kim et al. 2008; Taraban et al. 2008; Wang et al. 2009).

iNKT cells have been shown to be important mediators of cancer immunosurveillance. Initial investigations showed that prolonged survival of mice with melanoma could be realized by injecting α-GalCer (Morita et al. 1995). Later work confirmed the role of iNKT cells by the demonstration that mice deficient in NKT cells ($CD1d^{-/-}$) are more susceptible to methylcholathrene-induced tumors than are wild-type mice (Smyth et al. 2000) although the protective effect may be indirect as tumors lacking CD1d can be rejected in wild-type mice (Crowe et al. 2002).

Although it is still not fully understood how different subsets of iNKT cells control tumor growth, one possibility is by modulation of cells with suppressive capabilities. Recently, we have shown that neutrophils ($CD11b^{+}CD15^{+}$) are differentiated in the presence of systemic serum amyloid A-1 (SAA-1) to an anti-inflammatory IL-10-producing phenotype (De Santo et al. 2010). SAA-1 is a protein secreted in response to tissue injury and infection and is also found in plasma and the primary tumor of melanoma patients, in which levels correlate not only with tumor progression but also with the differentiation of IL-10-secreting neutrophils (De Santo et al. 2010). Critically, activation of iNKT cells through presentation of α-GalCer or SAA-1-induced natural lipid ligands by neutrophils is sufficient to limit IL-10 production by enhancing IL-12 production, through CD1d- and CD40L-dependent mechanisms (De Santo et al. 2010).

In addition, we have shown that the suppressive activity of myeloid derived suppressor cells (MDSC; $CD11b^{+}Gr1^{+}$) generated during influenza A infection can be

overcome by activation of iNKT cells (De Santo et al. 2008) and that survival of influenza A-infected Jα281$^{-/-}$ mice could be prolonged by the adoptive transfer of iNKT cells which limited MDSC suppressive activity by reducing their release of nitric oxide synthase and arginase 1 (De Santo et al. 2008). By extending these findings to patients infected with influenza A virus, we demonstrated that myeloid cells with suppressive activity were present in the peripheral blood, and that their suppressive activity was reduced by iNKT cell activation (De Santo et al. 2008).

Not only are iNKT cells pivotal in the generation of potent antigen-specific CD4$^+$ and CD8$^+$ T-cell responses (Fujii et al. 2003; Hermans et al. 2003; Stober et al. 2003; Silk et al. 2004), they also play an important role in the promotion of antigen-specific B-cell differentiation. Upon recognition and internalization of extracellular antigen by the B-cell receptor (Batista and Harwood 2009) and subsequent processing and presentation on MHC molecules, cognate T-cell help is recruited to maximize B-cell activation (Lanzavecchia 1985). In an analogous manner, activation of iNKT cells with glycolipid antigens presented by CD1d molecules on B cells can induce maturation, augment antibody titers, and promote the expansion of the B-cell memory pool (Galli et al. 2003, 2007). Significantly, in the absence of CD4$^+$ T cells, activation of iNKT cells by α-GalCer can provide the necessary assistance for maximal activation of B cells (Galli et al. 2007). It has been shown that iNKT B-cell activation is dependent on CD1d and occurs not only in the presence of α-GalCer-loaded CD1d on B cells, but also in the absence of α-GalCer, indicating that endogenous ligand may also be recognized in this context (Galli et al. 2003). In addition, CD1d-dependent iNKT B-cell activation can be mediated indirectly by modulation of DC activity (Tonti et al. 2009).

iNKT cell-dependent B-cell responses in vivo have recently been exploited by ourselves and others by targeting B-cell receptor uptake of CD1d-restricted antigens (Barral et al. 2008; Leadbetter et al. 2008). In both cases, iNKT cell activation facilitated higher titers of specific IgM and early class-switched antibodies, providing additional rationale for the use of iNKT cell ligands as adjuvant therapy in vaccination strategies.

Little is presently known about the sites of iNKT cell activation in vivo. Recently, we and others have elucidated the mechanism of iNKT cell activation in hepatic lymph nodes during infection with *B. burgdorferi* (Lee et al. 2010) or in a resting state within lymph nodes using state-of-the-art microscopy (Barral et al. 2010). Following infection with *B. burgdorferi*, Kupffer cells were shown to activate iNKT cells by the capture of blood borne *B. burgdorferi*, inducing CXCR3-dependent clustering of iNKT cells and forming stable contacts with iNKT cells via CD1d (Lee et al. 2010). Importantly, absence of iNKT cells led to reduced serum levels of IFN-γ and dissemination of the pathogen to the tissue (Lee et al. 2010). In the absence of infection, we have shown that following injection of silica microspheres coated with liposomes containing α-GalCer, iNKT cells were rapidly activated and localized to the subcapsular sinus where CD169$^+$ macrophages were able to internalize α-GalCer and present it to iNKT cells on CD1d (Barral et al. 2010). Importantly, depletion of such macrophages using clodronate liposomes limited iNKT cell expansion and cytokine production within the lymph nodes (Barral et al. 2010). Interestingly, CD169$^+$ macrophages have recently been shown to have a

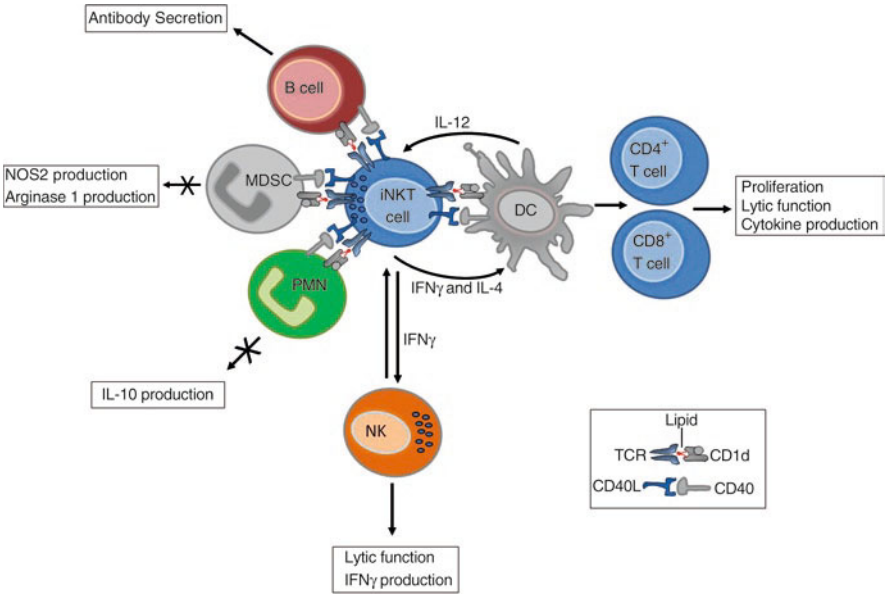

Fig. 3.2 iNKT cells can interact with and modulate the function of cells of the immune system. iNKT cells can directly and indirectly influence cells of the immune system, such as NK and T cells. These interactions are bidirectional, as iNKT cells receive signals from antigen-presenting cells (APCs) and vice versa. Signals can be received through cell-surface receptors, such as T-cell receptors (TCRs) recognizing glycolipid–CD1d complexes, co-stimulatory receptors, such as CD40, CD27 and OX40 recognizing their ligands CD40L, CD70 and OX40L, respectively, as well as through soluble mediators, such as cytokines. Activated iNKT cells inhibit the function of myeloid-derived suppressor cells (MDSCs), which suppress immune responses with nitric oxide synthase 2 (NOS2) and arginase 1

pivotal role in the uptake of dead tumor cells that accumulate in tumor draining lymph nodes, and cross-present tumor antigens to $CD8^+$ T cells (Asano et al. 2011). Critically, depletion of $CD169^+$ macrophages resulted in a failure to prime tumor-specific $CD8^+$ T cells and increased tumor burden (Asano et al. 2011). It remains to be shown whether iNKT cells have a role in promoting $CD169^+$ macrophage uptake of tumor antigen or if iNKT agonists could be used to increase the efficiency of tumor-specific $CD8^+$ T-cell priming. However, recently Semmling et al. (2010) have shown that iNKT cells can influence naive $CD8^+$ T-cell recruitment during cross-priming. The cross-presentation of extracellular antigens by $CD8\alpha^+$ DC to $CD8^+$ T cells requires "licensing" by $CD4^+$ T helper cells (Bevan 2006). In addition to these interactions, Semmling et al. (2010) were able to demonstrate that activation of iNKT cells with microbial and synthetic glycolipids was able to influence cross-priming of naive $CD8^+$ T cells as $CD8\alpha^+$DC preferentially produce the chemokine CCL17 and attract $CCR4^+$ naive $CD8^+$ T cells. In contrast, licensing of $CD8\alpha^+$ DC by $CD4^+$ T helper cells resulted in the recruitment of $CCR5^+$ naive $CD8^+$ T cells, demonstrating two alternative chemokine pathways to promote naive $CD8^+$ T-cell priming (Semmling et al. 2010). A summary of the ability of iNKT cells to modulate the function of immune cells is shown in Fig. 3.2.

4 iNKT Cell-Based Vaccination Strategies

The seminal observation that iNKT cell activation could be used as an adjuvant to enhance vaccination strategies was made by Gonzalez-Aseguinolaza et al. Mice were administered either irradiated sporozoites (g-spz) or a recombinant adenoviruses expressing a malarial antigen (AdpgCS) with or without α-GalCer. Only those mice co-administered α-GalCer were protected against disease at sub-optimal doses of g-spz/AdpgCS (Gonzalez-Aseguinolaza et al. 2002). The following year, we and others confirmed that iNKT cells could function as an efficient adjuvant therapy by administering α-GalCer in combination with the model antigen ovalbumin (OVA) (Fujii et al. 2003; Hermans et al. 2003). A single injection of DC loaded with both α-GalCer and OVA increased the in vivo expansion of OVA-specific T cells compared to mice given either OVA or α-GalCer alone (Fujii et al. 2003). Likewise, DC ($CD11c^+$) purified 4 h after injection of mice with α-GalCer and OVA elicited longer OVA-specific $CD8^+$ (OT-1) and $CD4^+$ (OT-II) T-cell responses in vitro compared to DC from mice given OVA or α-GalCer alone (Fujii et al. 2003). In addition, injection of DC loaded with α-GalCer/OVA or co-injection of α-GalCer and OVA generated an OVA-specific T-cell expansion that induced a potent anti-tumor response against the OVA-expressing EG7 tumor cell line (Fujii et al. 2003; Hermans et al. 2003). Several key observations were made in the course of these studies illustrating that iNKT cell-mediated DC maturation is dependent on the interaction of CD40 with its ligand CD40L (Fujii et al. 2003; Hermans et al. 2003) and that successful expansion of OVA-specific $CD4^+$ and $CD8^+$ T cells required presentation of both α-GalCer and OVA by the same DC (Hermans et al. 2003).

Activation of iNKT cells with α-GalCer promotes the production of both Th1- and Th2-type cytokines. It was therefore of interest whether activation of iNKT cells with agonists that promote a Th2-like cytokine response (e.g., the sphingosine-truncated α-GalCer analog OCH; see Fig. 3.1) (Miyamoto et al. 2001) would alter downstream T-cell polarization when administered together with OVA. We found that OCH and α-GalCer both induced DC maturation in vivo and led to similar anti-tumor immune responses through the expansion of OVA-specific $CD8^+$ T cells illustrating that it is the provision of the CD40L signal, and not the cytokine profile that is responsible for initiating DC maturation and the consequent cytotoxic T lymphocyte (CTL) expansion (Silk et al. 2004). Importantly, this mode of CTL expansion has been shown not only for model antigens such as OVA but also for NY-ESO-1, an important human tumor antigen (Silk et al. 2004) and for the influenza antigen (PR8). A single intranasal immunization of α-GalCer together with inactivated PR8 is effective in promoting long-term clearance of influenza virus (Youn et al. 2007). The use of DC loaded with fluorescently labeled α-GalCer and PR8 revealed that iNKT cells proliferate following interaction with DC presenting α-GalCer in nasal mucosa tissue (Kamijuku et al. 2008). Recruitment of iNKT cells is dependent on the interaction of CXCR6 with its ligand CXCL16 as $CXCL16^{-/-}$ mice have reduced numbers of iNKT cells in nasal mucosa-associated tissue following immunization (Kamijuku et al. 2008). Interaction of CXCL16 with CXCR6 has previously been

identified as an important mechanism in regulation of iNKT cell trafficking to sites of inflammation (Jiang et al. 2005; Cullen et al. 2009). In addition to these insights, co-administration of α-GalCer and inactivated influenza A virus has been shown to promote enhanced innate immunity (Ho et al. 2008) and increase the survival of long-lived $CD8^+$ memory T cells by influencing the upregulation of pro-survival genes (i.e., *bcl-2*) (Guillonneau et al. 2009).

While rapid maturation of DC by iNKT cells is clearly beneficial in the case of soluble antigens it may detrimental to the delivery of antigen encoded by plasmid DNA, as time is required for the expression of the antigens. Indeed, the delivery of plasmid DNA and α-GalCer do not appear to enhance antigen-specific immune responses (unpublished observations). In spite of this, it has recently been shown that plasmid DNA encoding HIV-1 Gag when delivered intramuscularly with α-GalCer can support expansion of Gag-specific immune responses (Huang et al. 2008).

In the bid to optimize vaccination strategies using iNKT cell ligands, several routes of delivery have been investigated using mouse models. Fujii et al. (2002) compared the activation of iNKT cells following delivery of DC loaded with α-GalCer by the intravenous (i.v.) and subcutaneous (s.c.) routes. In contrast to sustained and rapid activation of iNKT cells following i.v. delivery, s.c. administration failed to activate iNKT cells effectively (Fujii et al. 2002). We have shown that the oral administration of α-GalCer and OVA could expand OVA-specific $CD8^+$ T cells although the dose of α-GalCer and OVA was significantly greater in comparison to that used for the i.v. route (Silk et al. 2004). Other protocols have used irradiated tumor cells, either loaded or not with α-GalCer, as a source of antigen by the s.c. or i.v. route. Administration of irradiated tumor cells in the absence of α-GalCer showed limited anti-tumor immunity, while tumor cells loaded with α-GalCer and injected i.v. promoted adaptive immunity that lasted for up to 2 months postinjection (Liu et al. 2005). Similarly, the use of tumor cells loaded with α-GalCer has shown that both NK and iNKT cells are activated, leading not only to the eradication of tumor cells but also to the cross-presentation of tumor antigens, and resulting in long-lived (6–12 months) $CD4^+$ and $CD8^+$ T-cell reactivity in multiple mouse tumor models (Shimizu et al. 2007a, b). Recently this approach has been used successfully in combination with anti-CD25 monoclonal antibody treatment, to deplete regulatory T cells and aid effective anti-tumor T-cell responses (Petersen et al. 2010). To date, intranasal (i.n.) delivery of vaccine has been successful both in tumors (using α-GalCer with OVA) and influenza virus infection (α-GalCer with PR8 virus) (Ko et al. 2005; Kamijuku et al. 2008) with no reported central nervous system adverse side effects (Youn et al. 2007).

5 Harnessing iNKT Cells in the Clinic

In recent years, a limited number of clinical trials have used injections of α-GalCer or of DCs pulsed with α-GalCer to enhance anti-tumor immune responses (Giaccone et al. 2002; Nieda et al. 2004; Chang et al. 2005; Ishikawa et al. 2005; Uchida et al. 2008).

Intravenous injection of α-GalCer in patients with solid tumors was well tolerated and iNKT cell activation and cytokine production were detectable, although the degree of responsiveness to α-GalCer correlated with patient number of iNKT cells prevaccination (Giaccone et al. 2002). As DC pulsed with α-GalCer have been shown to induce less iNKT cell anergy in animal models than injection of α-GalCer alone, probably due in part to the presentation of α-GalCer by nonprofessional APC, a number of clinical studies have used autologous DCs pulsed with α-GalCer in patients with metastatic tumors (Nieda et al. 2004), nonsmall cell lung cancer (Ishikawa et al. 2005; Motohashi et al. 2009), myeloma (Chang et al. 2005) and head and neck cancers (Uchida et al. 2008; Kunii et al. 2009). Following i.v. (Nieda et al. 2004; Chang et al. 2005; Ishikawa et al. 2005) or i.n. (Uchida et al. 2008) delivery of DCs loaded with α-GalCer, iNKT cells expanded and in one of the i.v. studies the numbers of iNKT cells remained elevated at 6 months postvaccination (Chang et al. 2005). Interestingly, the immunization of melanoma patients with DC pulsed with α-GalCer who had persistent cytomegalovirus (CMV) led to an increase in CMV-specific $CD8^+$ T cells suggesting that iNKT cell activation can expand antigen-specific T-cell responses in humans, confirming the results obtained in animal models (Chang et al. 2005).

6 The Future of iNKT Cell-Based Vaccination Strategies

The ability of iNKT cells to bridge innate and adaptive immunity has been recognized over the past decade, and the use of synthetic iNKT cell ligands has been exploited in the development of protocols to enhance vaccination strategies in animal models. Despite these advances, there remain several challenges for successful translation to the clinical setting.

Numerous synthetic iNKT cell ligands have been described (McCarthy et al. 2007; Reddy et al. 2009; Li et al. 2010; O'Konek et al. 2011), but the main challenge for the future is to allow patients to be vaccinated repeatedly without the induction of iNKT cell anergy. Potent ligands, such as α-GalCer, could be replaced by weaker agonists, such as the recently described nonglycosidic iNKT cell agonist, threitolceramide (ThrCer) (Silk et al. 2008). ThrCer has many advantages over α-GalCer as activation of mouse and human iNKT cells and maturation of DCs is weaker than that stimulated by α-GalCer. In addition, ThrCer induces less DC lysis than α-GalCer while maintaining comparable anti-tumor properties (Silk et al. 2008).

The next generation of iNKT cell agonists should be capable of selectively targeting DC or antigen-specific B-cell populations, an aim that could potentially be achieved by attaching the iNKT cell agonist directly to the protein antigen (Leadbetter et al. 2008), binding the agonist to cell type-specific antibodies, or coupling it to microspheres (Barral et al. 2008, 2010).

The ability of iNKT cells to induce rapid DC maturation upon activation, and potentiate antigen-specific T and B cells in animal models offers an exciting possibility to enhance the efficiency of vaccines. The challenge for the future is to translate iNKT cell-based vaccination strategies to the clinic.

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Chapter 4
Immune Regulation of Tumor Immunity by NKT Cells

Jessica J. O'Konek, Jay A. Berzofsky, and Masaki Terabe

Abstract While NKT cells comprise only a very small percentage of lymphoctyes, they play very important roles in many disease settings, including cancer. NKT cells can be subdivided into at least two groups that play opposing roles in cancer and also counterregulate each other. While type I NKT cells can promote strong antitumor immunity, type II NKT cells suppress antitumor immune responses and play more of a regulatory role, similar to Tregs and MDSCs. The balance between type I and type II NKT cells can determine whether immune responses to tumors will be activated, resulting in tumor elimination, or will be suppressed, allowing the tumor to grow. Understanding the interactions between NKT cells may aid in the development of new immunotherapies for cancer which can shift the balance of this immunoregulatory axis towards immune activation and tumor killing.

1 Definition of NKT Cells

Natural killer T (NKT) cells are a unique population of lymphocytes that possess phenotypic and functional characteristics of both NK cells and T cells. NKT cells are true T cells, as they express T cell receptors; however, they also express NK lineage markers (Godfrey et al. 2004). NKT cells are uniquely equipped to link the innate and adaptive immune systems. While NKT cells comprise only a small percentage of lymphocytes (1–2% of mouse splenocytes and 0.01–2% of human peripheral blood mononuclear cells), they play vital roles in many different disease settings, including autoimmunity, infection, and cancer.

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Unlike conventional T cells that recognize peptide antigens presented by MHC molecules, NKT cells are defined by their ability to recognize lipid antigens presented by the nonclassical MHC-like molecule CD1d. Once NKT cells are activated, they rapidly produce many cytokines, such as IFN-γ, IL-4, IL-13, and IL-17. NKT cells constitutively contain mRNA for IFN-γ and IL-4, suggesting that this preformed message allows NKT cells to respond very quickly following stimulation (Matsuda et al. 2003; Stetson et al. 2003). NKT cells also stimulate cytokine production of other cells, such as IL-12 from CD1d-expressing Dendritic cells (DCs). Because the balance of cytokines determines which immune effector cells are activated, NKT cells utilize cytokine production to steer the adaptive immune response in the desired direction.

2 Immune Regulation by Type I NKT Cells

NKT cells can be further subdivided into two subsets, type I and type II. Type I NKT cells (or iNKT cells) express a semi-invariant TCR α chain (Vα14Jα18 in mice and Vα24Jα18 in humans), which preferentially pairs with Vβ2, 7, and 8.2 in mice and Vβ11 in humans (Imai et al. 1986; Koseki et al. 1989; Porcelli et al. 1993; Dellabona et al. 1994; Lantz and Bendelac 1994; Makino et al. 1995; Godfrey et al. 2004). Type I NKT cells often express other surface markers such as CD44, CD69, and NK1.1; however, these markers cannot be used to define type I NKT cells, as none of them are expressed on all type I NKT cells (Chiu et al. 1999; Kronenberg 2005; McNab et al. 2007; Matsuda et al. 2000). In mice, type I NKT cells are $CD4^+$ or $CD4^-CD8^-$, and some human NKT cells also express CD8αα or CD8αβ (Bendelac et al. 1994; Gadola et al. 2002). Type I NKT cells are often identified by staining with CD1d tetramers loaded with the prototypical type I NKT cell antigen α-galactosylceramide (α-GalCer) (Benlagha et al. 2000; Karadimitris et al. 2001; Matsuda et al. 2000).

Although type I NKT cells have a very limited TCR repertoire, they recognize a wide variety of lipid antigens (Brutkiewicz 2006; Behar and Porcelli 2007; Tupin et al. 2007). The most extensively studied antigen for type I NKT cells is α-GalCer, a glycolipid originally isolated from a marine sponge. α-GalCer stimulation induces type I NKT cells to produce copious amounts of both Th1 (IFN-γ) and Th2 (IL-4, IL-13) cytokines. α-GalCer induces strong tumor protection in mice through mechanisms requiring IFN-γ and IL-12 (Cui et al. 1997; Fuji et al. 2000; Chiodoni et al. 2001; Hayakawa et al. 2002; Smyth et al. 2002). Effector cells that have been demonstrated to be involved in type I NKT cell-mediated tumor killing include activated $CD4^+$ and $CD8^+$ T cells (Nakagawa et al. 2004; Osada et al. 2004; Hong et al. 2006) as well as IFN-γ-activated NK cells (Hayakawa et al. 2001a; Smyth et al. 2001, 2002). The mechanisms by which NKT cells induce tumor immunity will be discussed in greater detail later in this chapter.

The structure of antigens plays a significant role in the cytokine profile induced by activated type I NKT cells. NKT antigens are discussed in greater detail in the chapter by Pei and Kronenberg. Some structure–function studies of type I NKT cell antigens have focused on alterations of the sugar moiety of glycosylceramides

as well as the linkage of the sugar head group to the ceramide tails. Modifications of the sugar moiety also alter the ability of the antigen to stimulate type I NKT cells. For example, α-GalCer is much more potent than α-glucosylceramide, while α-mannosylceramide failed to induce significant proliferation of type I NKT cells (Kawano et al. 1997). Differences in proliferation induced by these glycosylceramides with different sugar moieties were attributed to the affinity of the glycolipid/CD1d complex for the TCR (Sidobre et al. 2002). Far fewer studies have characterized β-linked glycosylceramides. It has been shown that β-galactosylceramide is able to bind the TCR on type I NKT cells, but it induces downregulation of TCR expression without causing cytokine release or effector cell activation (Ortaldo et al. 2004). Other reports of β-linked glycosylceramides demonstrated only weak induction of cytokines. Historically, β-linked glycosylceramides have not been shown to induce significant antitumor immune responses, although some activity against autoimmune diseases has been reported (Ortaldo et al. 2004; Parekh et al. 2004; Zigmond et al. 2007, 2009). The lysosomal glycolipid isoglobotrihexosylceramide (iGb3) is also β-linked and has been shown to be a weak endogenous agonist for type I NKT cells; however, it has not been shown to have antitumor activity (Zhou et al. 2004). We have recently characterized a novel β-linked glycosylceramide, β-mannosylceramide (β-ManCer), which induces significant antitumor immunity (O'Konek et al. 2011). The mechanism by which this type I NKT cell agonist protects against tumors will be discussed in greater detail later in this section.

Other antigens for type I NKT cells have been described, such as lyso-phospholipids which can activate type I NKT cells to produce cytokines and nonglycosidic antigens, such as threitolceramide, which stimulate type I NKT cells to mature DCs and prime antigen-specific T and B cells (Silk et al. 2008; Fox et al. 2009). Type I NKT cells can also be stimulated with lipids from Borrelia, Ehrlichia, and Sphingomonas microorganisms, suggesting a role for type I NKT cells in host defense (Kinjo et al. 2005; Mattner et al. 2005; Wu et al. 2005; Brutkiewicz 2006; Tupin et al. 2007). The effect of these agonists in the context of tumor immunity has not been reported.

Far fewer endogenous tumor antigens have been discovered. Phosphatidyl inositol, which is expressed by murine tumors, could activate type I NKT cell hybridomas (Gumperz et al. 2000). Another phospholipid, palmitoyl-oleoyl-*sn*-glycero-3-phosphoethanolamine, purified from tumor cells also activated type I NKT cells (Rauch et al. 2003). GD3, a ganglioside expressed in human melanoma, also induced cytokine production by mouse NKT cells (Wu et al. 2003). It is likely that many more endogenous antigens for type I NKT cells exist and will be discovered in the future. The wide variety of antigens which can stimulate type I NKT cells demonstrates that despite very limited TCR usage, type I NKT cells are able to distinguish many different antigen structures to initiate the appropriate immune reaction.

The majority of what is known about the mechanism by which type I NKT cells protect against tumor formation is in the context of type I NKT cell activation by α-GalCer (Fig. 4.1). α-GalCer was originally characterized for its antitumor effect and has been shown to induce strong protection against tumors in many different mouse models of transplantable tumors (Kawano et al. 1998; Fuji et al. 2000; Chiodoni et al. 2001; Hayakawa et al. 2004; Osada et al. 2004; Ambrosino et al. 2007).

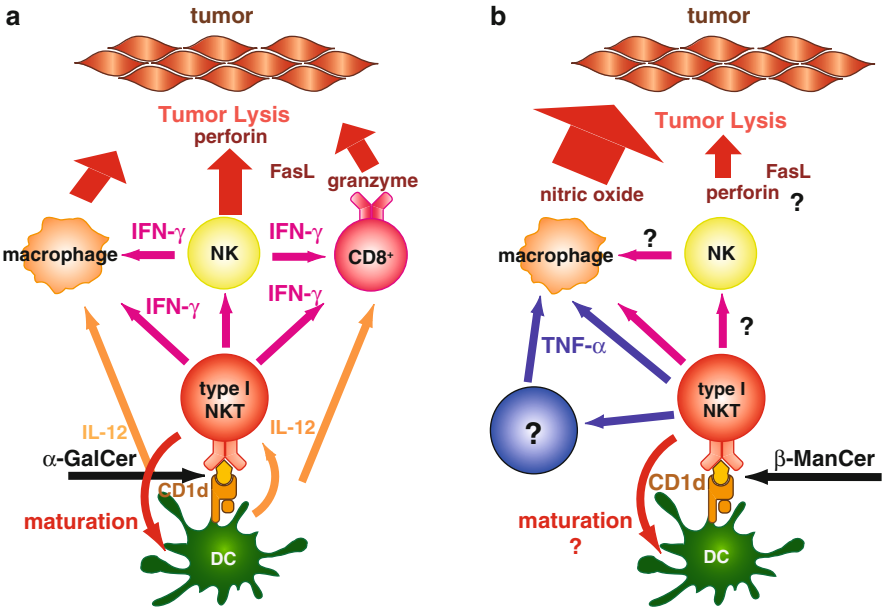

Fig. 4.1 Two mechanisms of type I NKT cell-induced tumor immunity. (**a**) When type I NKT cells are activated by α-GalCer presented by dendritic cells (DCs), they rapidly produce many cytokines. IFN-γ is the critical cytokine for the induction of antitumor immunity, as it is necessary for the activation of macrophages, NK, and $CD8^+$ T cells which all can contribute to tumor lysis. Activated type I NKT cells also induce maturation of the DCs, which produce IL-12 which enhances the antitumor response. (**b**) In contrast, IFN-γ is not required for the induction of tumor immunity upon activation of type I NKT cells with β-ManCer. Instead TNF-α and nitric oxide synthase (NOS) are the key mediators of tumor protection. TNF-α, likely produced by NKT cells or another intermediary cell type, induces upregulation of NOS activity in macrophages to induce tumor lysis

α-GalCer can also protect against oncogene or chemically induced tumors (Hayakawa et al. 2003). While it has been demonstrated that α-GalCer-activated NKT cells can directly lyse tumor cells in vitro (Kawano et al. 1998), the major role of NKT cells in antitumor immune responses is the activation of effector cells, such as NK cells and $CD8^+$ T cells, which ultimately kill tumor cells (Smyth et al. 2000). In most situations, IFN-γ is necessary for α-GalCer-induced antitumor immunity (Kawano et al. 1998; Fuji et al. 2000; Hayakawa et al. 2001a; Smyth et al. 2002; Ambrosino et al. 2007; Terabe and Berzofsky 2008). For example, in a model of liver and lung metastasis, the sequential production of IFN-γ from NKT cells followed by NK cells was necessary for α-GalCer's antitumor effects (Smyth et al. 2002). The c-glycosidic analog of α-GalCer, which induces a more Th1-skewed cytokine profile with more IFN-γ production, induces stronger protection against tumors compared with α-GalCer (Schmieg et al. 2003), further suggesting the importance of IFN-γ in type I NKT-mediated tumor protection with these antigens.

It has been reported that cytokine secretion by type I NKT cells requires CD28-CD80/CD86 co-stimulation (Hayakawa et al. 2001b). Stimulation with α-GalCer also induces IL-12 production by APCs, and this response is due to the

upregulation of co-stimulatory molecules CD40 on DCs and CD40 ligand on type I NKT cells (Fujii et al. 2004). In some situations, IL-12 can substitute for α-GalCer and induce tumor protection (Takeda et al. 2000). Several other mechanisms have been implicated in NKT cell-mediated cytotoxicity against tumors, including TNF-related apoptosis-inducing ligand (TRAIL), Fas ligand, perforin, and granzyme (Kawano et al. 1999; Nieda et al. 2001; Gumperz et al. 2002).

NKT cells can prevent tumor formation, even in the absence of the addition of an exogenous antigen. Jα18KO mice, which are deficient for type I NKT cells, are more susceptible to tumor growth in some transplantable tumor models (Ambrosino et al. 2007). Jα18KO mice also have a higher incidence of developing tumors following treatment with the carcinogen methylcholanthrene (Smyth et al. 2000, 2001; Crowe et al. 2002; Nishikawa et al. 2003). Loss of type I NKT cells increases the onset of spontaneous tumorigenesis in $p53^{+/-}$ mice and transgenic adenocarcinoma of the mouse prostate (TRAMP) mice (Swann et al. 2009; Bellone et al. 2010). These studies suggest a role for type I NKT cells in immunosurveillance which can suppress spontaneous tumor formation.

We have recently discovered a new mechanism by which type I NKT cells can induce tumor elimination. Activation of type I NKT cells with β-ManCer induces significant protection against tumors in the absence of IFN-γ through a nitric oxide- and TNF-α-dependent mechanism (O'Konek et al. 2011; Fig. 4.1). The discovery of an additional mechanism by which NKT cells can protect against tumors suggests that our understanding of how NKT cells function is limited by the antigens used to stimulate the cells. As new antigens are discovered, new mechanisms of NKT-mediated tumor protection may also be revealed.

While α-GalCer has shown a lot of promise in preclinical studies, clinical trials with α-GalCer (KRN7000) have yet to be overwhelmingly successful. A few possible explanations have been suggested. First, the frequency of type I NKT cells is much lower in humans (0.01–2% peripheral blood mononuclear cells), and advanced cancer often correlates with reduced number and function of type I NKT cells (Tahir et al. 2001; Giaccone et al. 2002; Dhodapkar et al. 2003; Kronenberg 2005). Second, α-GalCer induces strong anergy of type I NKT cells, at least in mice where it has been demonstrated that following injection of α-GalCer, type I NKT cells could not be restimulated for at least 1 month (Fujii et al. 2002). Third, humans but not mice have antibodies against α-linked galactose (Galili et al. 1987, 1988; Yoshimura et al. 2001). Since β-ManCer induces type I NKT cell-mediated tumor protection through a mechanism different from that of α-GalCer and contains a β-linked mannose sugar and not an α-linked galactose sugar, it may have the potential to work better in human cancer patients than α-GalCer.

3 Immune Suppression by Type II NKT Cells

Far less is known about type II NKT cells than type I NKT cells. Because there are no good markers for type II NKT cells, they are defined as NKT cells which are not type I, meaning they do not express the semi-invariant TCR or recognize α-GalCer.

Type II NKT cells are also defined by their ability to recognize antigens presented by CD1d; however unlike type I NKT cells, type II NKT cells utilize a diverse TCR repertoire (Cardell et al. 1995; Chiu et al. 1999; Godfrey et al. 2004). Type II NKT cells recognize different glycolipid antigens from those which stimulate type I NKT cells. Sulfatide is the prototypical antigen for type II NKT cells (Jahng et al. 2004; Zajonc et al. 2005). Lysosulfatide, a sulfatide analog lacking a fatty acid chain, is a more potent activator of type II NKT cells (Blomqvist et al. 2009). Because it has been reported that some type II NKT cell hybridomas do not recognize sulfatide (Park et al. 2001; Jahng et al. 2004), it is likely that type II NKT cells may be further subdivided based on the antigens they recognize. While it has been reported that at least a subset of type II NKT cells can be identified by staining with CD1d-tetramers loaded with sulfatide, these tetramers are not as easily prepared or as commonly used as α-GalCer-loaded CD1d-tetramers, and they may exclude type II NKT cells which are not reactive to sulfatide. A recent study characterized the TCR repertoire of CD1d-sulfatide tetramer reactive cells and found preferential usage of Vα3/Vα1-Jα7/Jα9 and Vβ8.1/Vβ3.1-Jβ2.7 (Arrenberg et al. 2010). Characterization of type II NKT cells to define markers specifically expressed on these cells will be crucial for furthering our understanding of this cell population.

Type II NKT cells are involved in immune suppressive mechanisms and play opposing roles to type I NKT cells. For example, type II NKT cells have been implicated in suppressing autoimmunity. Non-obese diabetic (NOD) mice, which overexpress a TCR from a type II NKT cell clone, do not develop diabetes (Duarte et al. 2004). Type II NKT cells also can suppress experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis (Jahng et al. 2004). Type II NKT cells have been implicated in protection from graft-versus-host disease following bone marrow transplantation. It was demonstrated that type II NKT cells in donor bone marrow could prevent graft-versus-host disease through two mechanisms: IFN-γ production inducing Fas-mediated apoptosis of T cells and IL-4 production skewing T cells towards a Th2 phenotype (Kim et al. 2007). In tumor models, type II NKT cells can suppress antitumor immunity and promote tumor growth (Moodycliffe et al. 2000; Terabe et al. 2000, 2003a; Renukaradhya et al. 2008). Some tumor cell lines which grow well in wild-type mice and Jα18KO mice, which lack type I NKT cells, fail to grow in CD1dKO mice, which lack all NKT cells, indicating that amongst NKT cells, type II are sufficient for the suppression of tumor immunity (Terabe et al. 2005). Also, antibody blockade of CD1d inhibits tumor growth, presumably by inhibiting type II NKT cells (Terabe et al. 2005; Teng et al. 2009a, b). When type II NKT cells are stimulated with sulfatide, they can suppress tumor immune surveillance and enhance tumor growth (Ambrosino et al. 2007). Taken together, these data suggest a role for type II NKT cells in suppressing immune responses that can induce tumor rejection.

Type II NKT cells have also been identified in humans. Type II NKT cells isolated from human bone marrow could suppress mixed lymphocyte reactions through the secretion of Th2 cytokines (Exley et al. 2001). In patients with multiple myeloma, an increase in type II NKT cells that are specific for lysophosphatidylcholine have been identified (Chang et al. 2008). With the fact that the frequency of type II NKT cells is higher in humans than in mice, it may be possible that type II

NKT cells play an important role in the regulation of tumor immunity in humans (Kenna et al. 2003), and in general may play similar immunosuppressive roles in humans as has been described in mice.

A mechanism by which type II NKT cells can suppress tumor immunity has also been characterized. In the 15-12RM fibrosarcoma model where subcutaneous tumors spontaneously regress and then recur, it was demonstrated that $CD4^+$ T cells suppress the immunosurveillance by $CD8^+$ CTLs (Matsui et al. 1999). It was subsequently found that these suppressive $CD4^+$ T cells were CD1d-restricted Vα14Jα18 negative NKT cells, or type II NKT cells. In order to exert their suppressive functions, type II NKT cells produce IL-13 which bind to receptors on $CD11b^+Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSCs). When TNF-α also binds to its receptor on the MDSCs, IL-13Rα2 is upregulated on the surface of the MDSC. This receptor then binds to IL-13 secreted by type II NKT cells which activates AP-1 to induce TGF-β production (Terabe et al. 2003a; Fichtner-Feigl et al. 2005, 2008). The TGF-β then inhibits the activation of cytotoxic $CD8^+$ T cells, thereby inhibiting tumor lysis. In this model, type II NKT cells suppress antitumor responses in the absence of exogenous stimulation, suggesting that they are recognizing tumor-derived glycolipids. Work to identify these endogenous NKT antigens is in progress.

Antibody blockade of TGF-β inhibited tumor growth and synergized with vaccines to kill tumors (Terabe et al. 2003b, 2009; Takaku et al. 2010). This prompted a phase I clinical trial in metastatic malignant melanoma patients of a human monoclonal anti-TGF-β antibody GC1008, which demonstrated safety and showed some antitumor activity (Morris et al. 2008). Phase II clinical trials are in preparation to further investigate the therapeutic activity of the antibody as well as the efficacy in combination with tumor vaccines.

In some tumor models, tumor growth is similarly increased in mice which lack type I NKT cells (Jα18KO) as mice which lack all NKT cells (CD1dKO) as compared with wild-type mice, suggesting that the lack of type I NKT cells is responsible for increased tumor growth (Swann et al. 2009). Interestingly, other tumors which have similar or increased growth in Jα18KO mice compared to wild-type fail to grow in CD1dKO mice, suggesting that type II NKT cells suppress tumor immunity in these models (Terabe et al. 2005; Ambrosino et al. 2007; Renukaradhya et al. 2008). These studies indicate that in some tumor models, type I NKT cells predominate, whereas in other models type II NKT cells play a stronger role. Studies are in progress to determine what influences which type of NKT cell dominates. Regardless of which type of NKT cells seem to be more important in the regulation of these tumors, it is clear that NKT cells play critical roles in regulation of tumor immunity.

4 Counterregulation of Type I and Type II NKT Cells

The immune system is a complex network of many different cell types which often regulate the function of one another. Consistent with this theme, not only do type I and type II NKT cells play opposing roles, but they also regulate each other (Ambrosino et al. 2007, 2008; Halder et al. 2007; Terabe and Berzofsky 2007, 2008;

Berzofsky and Terabe 2008). Type II NKT cells activated in vitro with sulfatide could suppress the proliferation of type I NKT cells activated by α-GalCer (Ambrosino et al. 2007). This interaction also occurred in vivo, as sulfatide suppressed the protection induced by α-GalCer against subcutaneous tumors and lung metastases (Ambrosino et al. 2007). Additionally, when type I NKT cells are absent, type II NKT cells appear to be even more suppressive, as tumor growth is enhanced further in Jα18KO mice. This suggests that type I NKT cells can inhibit or reduce the suppressive effects of type II NKT cells. The mechanism by which type II NKT cells suppress type I NKT cells is still unclear, and studies to characterize this interaction are currently in progress. There is some evidence to suggest that this suppression is mediated by cell-to-cell contact as opposed to soluble factors, as transfer of conditioned media from sulfatide-activated NKT cells could not suppress the proliferation of α-GalCer-activated type I NKT cells (Ambrosino et al. 2007). The interaction between type I and type II NKT cells has also been shown in a concanavalin A-induced hepatitis model, where the disease pathogenesis is mediated by type I NKT cells. In this model, activation of type II NKT cells by sulfatide induced anergy of type I NKT cells through IL-12 and macrophage inflammatory protein 2 (MIP-2) production by tolerized plasmacytoid DCs (Halder et al. 2007). The discovery of this interaction between type I and type II NKT cells defined a new immunoregulatory axis (Ambrosino et al. 2007). Antitumor immunotherapy strategies targeted at NKT cells have a greater chance of success if they can exploit this interaction and shift the balance of this axis towards activating type I NKT cells while suppressing type II NKT cells.

5 Interactions Between NKT Cells and Other Cell Types

Not only can NKT cells regulate each other, but they also interact with many other cell types (Fig. 4.2). Type I NKT cells can induce maturation of DCs through CD40/CD40L interaction (Fujii et al. 2004) and activate effector cells such as $CD8^+$ T cells and NK cells (Carnaud et al. 1999; Toura et al. 1999; Eberl and MacDonald 2000; Smyth et al. 2001; Fujii et al. 2002, 2003, 2004; Hong et al. 2009). The enhancement of $CD8^+$ T cell responses by α-GalCer activated type I NKT cells was shown to be mediated by upregulation of CD70 expression on DCs which allows for interaction with CD27 on $CD8^+$ T cells (Taraban et al. 2008). We have recently found that activated type II NKT cells could suppress antigen-specific proliferation of $CD4^+$ T cells (unpublished observations). It also has been reported that both $CD4^+$ and $CD8^+$ T cell responses are weaker in CD1dKO mice, suggesting that NKT cells can enhance these immune responses (Hong et al. 2009; Shin et al. 2010).

MDSCs are immature cells of myeloid lineage that can suppress immune responses through the production of arginase, nitric oxide, and TGF-β (Gabrilovich 2004; Bronte and Zanovello 2005). MDSCs, which are defined as Gr-$1^+$$CD11b^+$, accumulate with increasing tumor burden in both mouse tumor models and human cancer patients (Pak et al. 1995; Almand et al. 2001; Schmielau and Finn 2001; Gabrilovich 2004; Bronte and Zanovello 2005). As described earlier, TGF-β production by MDSCs can be stimulated by IL-13 produced by type II NKT cells,

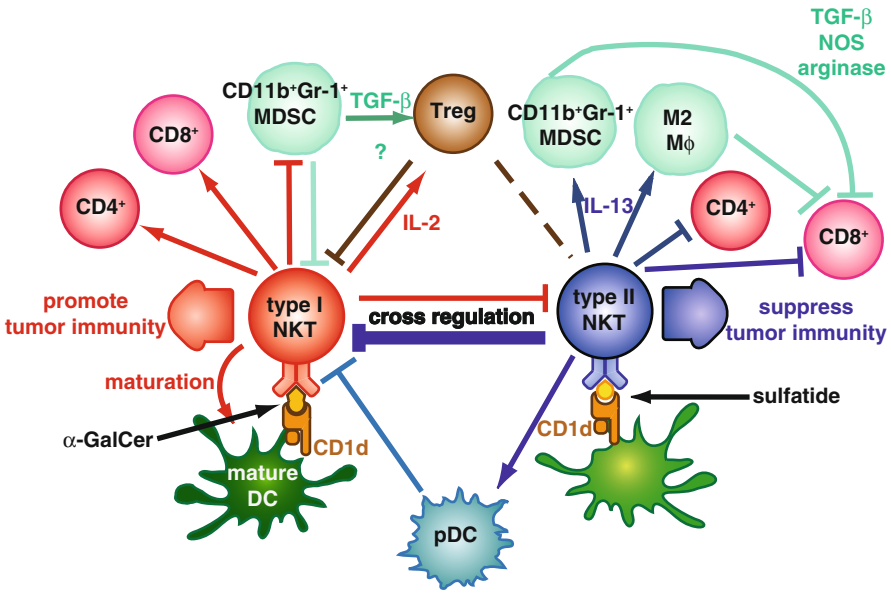

Fig. 4.2 Interactions between NKT cells and other cell types. Type I and type II NKT cells not only have opposing functions in the regulation of tumor immunity, but they also counterregulate one another. Activation of type I NKT cells induces tumor immunity, while activation of type II NKT cells (by sulfatide or other tumor-derived glycolipids) suppresses tumor immunity. In some settings, activated type II NKT cells can suppress the activation and function of type I NKT cells. Additionally, type II NKT cells activated by sulfatide can activate plasmacytoid DCs (pDCs) to induce anergy of type I NKT cells, thus inhibiting their function. Activated type I NKT cells promote the function of $CD4^+$ and $CD8^+$ effector T cells and inhibit $CD11b^+Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSCs). Both Tregs and MDSCs can suppress the function of type I NKT cells. In contrast, type II NKT cells inhibit $CD4^+$ and $CD8^+$ T cells. IL-13 production by type II NKT cells enhances the suppressive function of MDSCs and M2 macrophages which also inhibit $CD8^+$ T cell function. The interaction between type I and type II NKT cells defines an immunoregulatory axis, and manipulation of the balance of this axis towards the induction of tumor immunity may increase the efficacy of immunotherapies for cancer

along with TNF-α (Terabe et al. 2003a; Fichtner-Feigl et al. 2005, 2008). Arginase expression in MDSCs can also be induced by IL-13 (Gallina et al. 2006). IL-13 produced by type II NKT cells could activate tumor-associated macrophages, which are considered to be a subset of MDSCs (Sinha et al. 2005).

Not only can type II NKT cells activate MDSCs, but MDSCs can also suppress type I NKT cells. In a B16 melanoma model where α-GalCer is unable to induce significant antitumor immunity, inhibition of MDSCs by retinoic acid allowed α-GalCer to protect against tumors (Yanagisawa et al. 2006). During influenza infection in both mice and humans, activated type I NKT cells can reduce the ability of MDSCs to express suppressive molecules, such as nitric oxide and arginase (De Santo et al. 2008). Type I NKT cells isolated from human neuroblastoma tumors could directly kill tumor-associated macrophages (Song et al. 2009). Taken together, these studies suggest a role for activation of MDSCs by type II NKT cells and suppression of MDSCs by type I NKT cells.

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) have been well characterized for their ability to suppress various types of immune responses (Sakaguchi 2004). Some evidence suggests that interactions between Tregs and NKT cells likely exist. α-GalCer-mediated prevention of diabetes in NOD mice was dependent on the presence of Tregs (Ly et al. 2006). In a mouse model of the autoimmune disease myasthenia gravis, α-GalCer prevented disease development through activation of type I NKT cells which secreted IL-2 and increased the number of Tregs in the animal (Liu et al. 2005). Activation of human type I NKT cells with α-GalCer also could promote proliferation of Tregs through IL-2 (Jiang et al. 2005). These data suggest that IL-2 produced by activated type I NKT cells sustain the survival and proliferation of Tregs. Conversely, in a mouse model of lung metastasis, an increase in Tregs reduced the number of type I NKT cells in the lungs, and this was associated with increased tumor burden (Nishikawa et al. 2003). Cytokine production and proliferation of human type I NKT cells activated by α-GalCer could also be suppressed by Tregs (Azuma et al. 2003). While these studies suggest that crosstalk occurs between Tregs and NKT cells, further characterization is needed to fully understand these interactions. Like MDSCs and Tregs, type II NKT cells are very important regulatory cells and can even shape the immune environment by influencing the development and function of Tregs and MDSCs.

6 Conclusions

While NKT cells represent a very small percentage of lymphocytes, they play very important roles in the immune network. NKT cells have characteristics of the innate immune system as they are activated early in immune responses; however, they are also part of the adaptive immune system as they are activated by specific antigens. These cells are specially poised to bridge the gap between innate and adaptive immunity and have the ability to steer the immune response in the desired direction. NKT cells are both positive and negative regulators in the immune network, with type II NKT cells playing similar regulatory roles as MDSCs and Tregs. The balance of the immunoregulatory axis that exists between type I and type II NKT cells can control whether immune activation or suppression occurs and may strongly influence the subsequent adaptive immune responses. Manipulation of this balance may be a critical component of immunotherapy for cancer as well as autoimmune and infectious diseases.

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Chapter 5
The Regulation of CD1d$^+$ and CD1d$^-$ Tumors by NKT Cells

The Roles of NKT Cells in Regulating CD1d$^+$ and CD1d$^-$ Tumor Immunity

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Abstract Natural killer T (NKT) cells are T cells that also express NK cell markers. Unlike conventional T cells that recognize peptide antigens, NKT cells recognize lipids. CD1d, a MHC class I-like molecule, presents lipid antigens to NKT cells. Upon activation, NKT cells secrete both Th1 and Th2 cytokines, linking the innate and adaptive immune response. Due to the heterogeneity of NKT cells, their roles in the immune system are mixed and controversial. Here, we summarize the current knowledge of NKT cells as antitumor effector cells, in the control of both CD1d$^+$ and CD1d$^-$ tumors as model systems. Overall, NKT cells exhibit a remarkable broad range of antitumor activities and show great potential for antitumor immunotherapy.

1 Introduction

Natural killer T (NKT) cells were first identified as a subset of T lymphocytes that express markers until then only found on NK cells (Fowlkes et al. 1987; Koseki et al. 1989, 1990, 1991; Lantz and Bendelac 1994). The development of this subset of T cells was then found to be β_2-microglobulin-dependent (Bendelac et al. 1994; Bix et al. 1993; Ohteki and MacDonald 1994). Subsequently, it was found that these cells are reactive to the major histocompatibility complex (MHC) class I-like molecule, CD1d (Bendelac et al. 1995). Although "NKT" is the most accepted term for this subset of T cells, it is also well-acknowledged that a more accurate definition of this subset of T cells should be "CD1d-dependent natural killer-like T cells". Like conventional T cells, NKT cells also develop in the thymus (reviewed by Godfrey et al. 2004). Based on T-cell receptor (TCR) usage, NKT cells can be divided into

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type I (invariant NKT; iNKT) and type II (other CD1d-restricted) NKT cells. Most NKT cells are $CD4^+$ or double negative (DN, $CD4^-CD8^-$), but some are $CD8^+$ ($\alpha\alpha$ heterodimer) (Benlagha et al. 2000; Gumperz et al. 2002; Matsuda et al. 2000). Due to the heterogeneity of NKT cells, their roles in antitumor immunity and response to microbes are varied. These details are discussed in Sect. 3.

As indicated above, NKT cells are activated by CD1d, which is structurally similar to the MHC class I molecule (Zeng et al. 1997). Unlike MHC class I molecules that present short peptide antigens to $CD8^+$ T cells, CD1d presents lipid antigens to NKT cells (Brutkiewicz 2006). Like MHC class I molecules, the CD1d heavy chain is composed of three extracellular domains ($\alpha1$, $\alpha2$, and $\alpha3$) and a transmembrane domain; however, CD1d has a comparatively short cytoplasmic tail. When the CD1d molecule is synthesized in the endoplasmic reticulum (ER), the $\alpha1$ and $\alpha2$ domains form a hydrophobic groove, where a self-lipid antigen is loaded. These self-lipids, including phosphatidylinositol (PI) and phosphatidylcholine (PC), generally cannot activate NKT cells. Instead, they likely play an analogous role to the invariant chain-derived CLIP peptide for MHC class II molecules (reviewed by Brutkiewicz 2006). Microsomal triglyceride transfer protein (MTP), a protein involved in lipoprotein assembly (Gordon et al. 1994), has been shown to be important for CD1d function. It is likely required for the loading of self-lipids into the hydrophobic groove formed by the $\alpha1$ and $\alpha2$ domains of CD1d in the ER (Brozovic et al. 2004; Dougan et al. 2005). Either before or after lipid loading, the CD1d heavy chain binds to β_2m and is then transported to the Golgi, where the glycans on CD1d are processed and modified before being transported to the plasma membrane (Kawana et al. 2007; Kim et al. 1999). From the surface, due to the YXXZ motif (Y is tyrosine, X is any amino acid, and Z is a bulky hydrophobic residue), an important binding motif for clathrin-mediated endocytosis in its cytoplasmic tail (Bonifacino and Traub 2003; Lawton et al. 2005), surface CD1d is internalized and traffics through compartments of the endocytic pathway (Brutkiewicz et al. 2003; Moody and Porcelli 2003). CD1d-mediated lipid antigen presentation requires lipid exchange in late endocytic compartments and re-expression on the cell surface to activate NKT cells (Jayawardena-Wolf et al. 2001; Roberts et al. 2002). Sphingolipid activator proteins (SAPs), especially saposin B, may facilitate lipid binding to CD1d (or other CD1 molecules) throughout the endocytic pathway (Kang and Cresswell 2004; Winau et al. 2004; Yuan et al. 2007; Zhou et al. 2004). Among the lipid antigens that activate NKT cells, some are natural cellular ligands, such as isoglobotrihexosylceramide (iGb3), whereas others are microbial lipids such as α-glucuronosylceramide (GSL-1) from *Sphingomonas* and *Ehrlichia* (Kinjo et al. 2005; Mattner et al. 2005; Sriram et al. 2005). α-Galactosylceramide (α-GalCer), a glycolipid extracted from marine sponges, was the first identified CD1d-restricted, iNKT cell activating lipid (Kawano et al. 1997), and rapidly activates iNKT cells both in vitro and in vivo (Brutkiewicz 2006; Kawano et al. 1997). Currently, α-GalCer and its analogs are being extensively studied for their ability to stimulate iNKT cells and their potential application in antitumor immunotherapy (Teng et al. 2007; Tsuji 2006).

Upon activation by CD1d-expressing antigen-presenting cells (APCs), NKT cells secrete both Th1 (e.g., IFN-γ, GM-CSF) and Th2 (e.g., IL-4, IL-5, IL-13)

cytokines, suggesting that NKT cells are important in linking innate and adaptive immunity (Yoshimoto and Paul 1994). Activated NKT cells also exhibit cytolytic activity against CD1d$^+$ cells. Reduced NKT cell numbers and impaired CD1d-mediated activation of NKT cells have been observed in cancer patients (Lynch et al. 2009; Dhodapkar et al. 2003; Spanoudakis et al. 2009; Tahir et al. 2001; van der Vliet et al. 2008), suggesting that iNKT cells are critical players in antitumor immunity.

2 Different Subsets of NKT Cells

2.1 Type I NKT Cells

This subset of NKT cells was the first and has been the most studied since the discovery of α-GalCer (Burdin et al. 1998; Gumperz et al. 2000; Kawano et al. 1997). These cells express an invariant TCR α-chain rearrangement (Vα14Jα18 in mice and Vα24Jα18 in humans) and limited TCR β-chain usage (Vβ8.2, Vβ7 and Vβ2 in mice; Vβ11 in humans). They have been called invariant (iNKT), canonical, or type I NKT cells (Bendelac et al. 1997; Godfrey et al. 2004). Invariant NKT cells are present in high numbers in the livers of mice (5–30% of liver mononuclear cells), with a lower frequency in other organs (Kronenberg and Gapin 2002). In humans, the levels of iNKT cells are much lower: only ~1% of liver mononuclear cells and <0.1% of peripheral blood mononuclear cells (Karadimitris et al. 2001; Kenna et al. 2003). However, a recent report has suggested that iNKT cells do accumulate in the human omentum (~10% of lymphocytes) (Lynch et al. 2009).

As indicated above, iNKT cells recognize α-GalCer-loaded CD1d and become activated to secrete both Th1 and Th2 cytokines. The injection of α-GalCer to both mice and humans will cause a cytokine "storm", anergy, and a reduction in iNKT cells (Giaccone et al. 2002; Osman et al. 2000; Parekh et al. 2005). However, iNKT cells can be cultured and specifically expanded in vitro in the presence of α-GalCer (Exley et al. 1997; Watarai et al. 2008).

There are several murine models commonly used for studying NKT cell function in vivo (Kronenberg and Gapin 2002). These include the NKT cell-deficient CD1d$^{-/-}$ and Jα18$^{-/-}$ mice. In CD1d$^{-/-}$ mice, due to the lack of CD1d molecules, neither type I nor type II NKT cells are present. However, in Jα18$^{-/-}$ mice, in which the Jα gene used in the TCR α chain rearrangement in iNKT cells is deleted, only type I NKT cells are absent (but type II NKT cells are still present). To further study type I NKT cells, Vα14 transgenic mice were generated, which possess predominantly iNKT cells (Bendelac et al. 1996). These mice have been useful for studying the development of iNKT cells as well as their function in the immune system.

SLAM-associated protein (SAP) is an adaptor protein expressed in T cells and NK cells. SAP$^{-/-}$ mice are devoid of iNKT cells (Pasquier et al. 2005). Patients with SAP-deficient X-linked lymphoproliferative disorder show a fatal defective immune

response to an Epstein–Barr virus (EBV) infection (Gaspar et al. 2002). These patients also have defective iNKT cell development, but normal conventional T cell and NK cell development. This was the first case that associated a genetic loss of iNKT cells with an immunodeficiency in humans, suggesting iNKT cell may play a pivotal role in an EBV infection (Pasquier et al. 2005). Recently, it was reported that a homozygous missense mutation in a gene encoding the IL-2-inducible T-cell kinase also caused a deficiency in iNKT cells. As a consequence, a dysfunctional immune response to therapy-resistant EBV occurred and resulted in the death of this patient (Huck et al. 2009).

2.2 Type II NKT Cells

As mentioned above, α-GalCer is a glycolipid ligand for type I NKT cells. The majority of α-GalCer/CD1d tetramer-positive human T cells express an invariant TCR (Vα24 Jα18) that is homologous to mouse iNKT cells; however, a small fraction of those T cells do not express Vα24 (Gadola et al. 2002). These Vα24$^-$ T cells use a diverse TCR Vα and Vβ repertoire, and are therefore considered to be type II NKT cells. The function of these cells is similar to iNKT cells, but they have much lower affinity for the CD1d/α-GalCer monomer than do iNKT cells (Gadola et al. 2002). It is generally believed that there are considerably more type II NKT cells than type I NKT cells in humans (Exley et al. 2001). There are also type II NKT cells that are CD1d-restricted but not reactive to α-GalCer. In a transgenic mouse model of hepatitis B virus (HBV), the occurrence of acute hepatitis is mediated by NKT cells that are not CD1d/α-GalCer tetramer$^+$ (Baron et al. 2002). As expected, these NKT cells are CD1d-dependent but do not express the invariant Vα14 TCR (Baron et al. 2002; Vilarinho et al. 2007). In ulcerative colitis patients, an elevated subpopulation of CD4$^+$CD161$^+$ T cells that do not express Vα24 is able to respond to CD1d-mediated activation and produce IL-13, resulting in inflammation (Fuss et al. 2004). Due to the paucity of glycolipid ligands identified for type II NKT cells, it is difficult to expand them ex vivo. As a result, studies focused on the development and function of type II NKT cells are limited. A self-antigen, sulfatide, is one of the few known ligands for type II NKT cells (Arrenberg et al.; Halder et al. 2007; Zajonc et al. 2005). NKT cells that bind to a CD1d/sulfatide tetramer are present in the thymus, spleen, and liver (Jahng et al. 2004). Because NKT cell development is thymus-dependent and the maturation of these cells is subject to negative selection, similar to MHC class I- and II-restricted T cells, mice that lack sulfatide show an approximate tenfold increase in CD1d/sulfatide tetramer$^+$ T cells (Jahng et al. 2004). These cells may be useful as intervention strategies in human autoimmune demyelinating diseases (Jahng et al. 2004). A number of investigators have also been using CD1$^{-/-}$mice and Jα18$^{-/-}$ mice to study the roles of type II NKT cells in both antitumor and antimicrobial immune responses (De Santo et al. 2008; Renukaradhya et al. 2008; Terabe et al. 2005).

2.3 *CD4$^+$, CD8$^+$, or DN NKT Cells*

Originally, it was believed that NKT cells are either CD4$^+$ or DN (CD4$^-$CD8$^-$) (Kronenberg and Gapin 2002), but more recent work has shown that human NKT cells can also be CD8$^+$ ($\alpha\alpha$) (Gumperz et al. 2002). A very small population of CD8$^+$ iNKT cells (<0.01% of total splenic T cells) has also been reported to be present in both Vα14 transgenic and wild type mice (Lee et al. 2009). These CD8$^+$ NKT cells are cytolytic, produce pro-inflammatory cytokines, and can directly kill tumor cells (Gadola et al. 2002; Yuling et al. 2009). CD4$^+$ NKT cells, on the other hand, show less lytic activity and may be immunoregulatory cells responsible for inhibiting tumor-specific CTL growth (Bricard et al. 2009; Osada et al. 2005). As a result, one could consider using CD4$^-$ (DN and/or CD8$^+$) NKT cells in antitumor immunotherapy.

2.4 *IL-17-Producing NKT Cells*

The presence of IL-17-producing NKT cells in the thymus and liver has recently been reported (Coquet et al. 2008; Michel et al. 2008). The majority of these cells are CD4$^-$NK1.1$^-$, usually considered to be at an immature stage of development (Bendelac et al. 1994; Gapin et al. 2001). No report to date has found these IL-17-producing NKT cells in tumor models. Rather, they are probably involved in leukocyte infiltration or hyperactivity in the airways (Michel et al. 2007; Pichavant et al. 2008).

3 The Regulation of Tumor Immunity by Type I NKT and Type II NKT Cells

Type I NKT cells are best known for their antitumor activities in both mice and humans (Tables 5.1 and 5.2). Jα18-deficient mice, in which type I (but not type II) NKT cells are absent, show enhanced sensitivity to tumor metastases as compared to wild type mice (Renukaradhya et al. 2008; Swann et al. 2009; Terabe et al. 2005). A number of patients with a variety of tumors have been found to possess fewer circulating NKT cells than healthy volunteers (Giaccone et al. 2002; Kawano et al. 1999; Lynch et al. 2009; Tahir et al. 2001). α-GalCer has shown antitumor activity in tumor-bearing mice, but there has been limited success so far in humans (Tables 5.1 and 5.2). The mechanism(s) of the antitumor activity mediated by type I NKT cells is discussed in more detail in Sect. 4.

In contrast to type I NKT cells, Terabe et al. (2005) reported that type II NKT cells suppress tumor immunosurveillance and this inhibition can be reversed by an anti-CD1d antibody, suggesting CD1d-dependence. Our laboratory observed a similar phenomenon in a model using CD1d$^+$ tumors (Renukaradhya et al. 2008).

Table 5.1 Regulation of tumor growth by NKT cells in preclinical/clinical models

NKT subset involved	Method of administration or model system	Tumor type	Effects on tumor growth in vivo	References
Type I NKT	Adoptive transfer of $CD8^+$ NKT	EBV-associated lymphoma and carcinoma	EBV-induced $CD8^+$ NKT cells suppress tumorigenesis	Yuling et al. (2009)
	N/A	Colorectal carcinoma	NKT cells are depleted in cancer patients' omentum	Lynch et al. (2009)
	CD1d or Jα18 knockout mice	Sarcomas and hematopoietic cancers caused by a p53 loss	NKT cells suppress the onset of the tumors	Swann et al. (2009)
	Immunization with tumor extracts and α-GalCer; CD1d or Jα18 knockout mice; i.v. αGalCer/sCD1d-anti HER2 fusion	B16 melanoma cells	Tumor cells loaded with α-GalCer induce NKT cell-dependent resistance to tumor implantation in mice; NKT cells induce antitumor immunity through $CD4^+$ T cells and IFN-γ; optimization of a tumor-specific $CD8^+$ T-cell response	Hong et al. (2006, 2009), Shimizu et al. (2007), Stirnemann et al. (2008)
	Adoptive transfer of NKT cells	NS0 lymphoma	Showed protection against a B-cell lymphoma	Renukaradhya et al. (2008)
	i.p. injection of CD1d antibody, α-GalCer and its analogs	Experimental mouse breast and renal tumors	NKT cells, in combination with anti-DR5/anti-4-1-BB, induce tumor rejection	Teng et al. (2007)
	Intratumoral vaccination of α-GalCer-loaded DCs	Murine pancreatic carcinoma	Expansion of IFN-γ-producing NKT cells	Nagaraj et al. (2006)
	CD1d or Jα18 knockout mice	Murine T-cell lymphoma	iNKT cells play a negative role in the host's antitumor immune response	Renukaradhya et al. (2006)
	i.v. injection of NKT cells	Methylcholanthrene (MCA)-induced sarcomas B16F10 melanoma	Rejection of tumors primarily mediated by $CD4^-$ NKT cells, not $CD4^+$ NKT cells	Crowe et al. (2005)
	α -GalCer/IL-21 combination treatment	B16F10 melanoma	Enhanced antitumor activity	Smyth et al. (2005)

Type II NKT	CD1d or Jα18 knockout mice	Sarcomas and hematopoietic cancers caused by a p53 loss	Probably not critical in regulating the tumors	Swann et al. (2009)
	CD1d or Jα18 knockout mice	NS0 lymphoma	Inhibiting antitumor activity	Renukaradhya et al. (2008)
	CD1d or Jα18 knockout mice	Mouse 15-12RM fibrosarcoma	Type II NKT cells reduced antitumor immunosurveillance	Terabe et al. (2005)
NK1.1+ and CD3+ NKT	i.v. adoptive transfer of NKT cells activated by IL-12 and IL-18	ALC or MC57X tumor model in mice	Rejection of tumor growth	Baxevanis et al. (2003)

Table 5.2 The antitumor effects of iNKT cells in clinical trials

Method of administration	Tumor type	Effects on tumor growth in vivo	References
i.v. injection of α-GalCer-pulsed, IL-2/GM-CSF-cultured PBMCs	Advanced and recurrent nonsmall-cell lung cancer	Increased IFN-γ-producing cells following α-GalCer stimulation in PBMCs associated with significantly prolonged median survival time	Motohashi et al. (2009)
Intra-arterial infusion of activated Vα24$^+$ NKT cells and submucosal injection of α-GalCer-pulsed APC	Recurrent head and neck carcinoma	Together with α-GalCer-pulsed DCs, NKT cells exhibited antitumor activity	Kunii et al. (2009)
Nasal submucosa injection of α-GalCer-loaded APCs	Unresectable or recurrent head and neck cancer	α-GalCer-loaded APCs administration was safe and induced antitumor activity in some patients	Uchida et al. (2008)
i.v. injection of activated Vα24$^+$ NKT cells expanded in vitro	Non-small cell lung cancer	No partial or complete response	Motohashi et al. (2006)
Intravenous injection of monocyte-derived mature DCs loaded with α-GalCer	Myeloma, renal cell cancer	Expanded NKT cells, elevated serum levels of IL-12 p40 and IFN-gamma inducible protein-10. Increased memory CD8$^+$ T cells	Chang et al. (2005)
i.v. injection of α-GalCer-loaded DCs	Lung cancer	No severe adverse effects were observed. No partial or complete response. Elevated levels of Vα24$^+$ NKT cells observed in one case	Ishikawa et al. (2005)
KRN7000, i.v.	Advanced solid tumors	No toxicity observed. Biological effects were observed in patients with high pretreatment NKT cell numbers. No clinical response was found	Giaccone et al. (2002)

In our study, we analyzed the roles for type I and type II NKT cells in the host's innate antitumor response against a murine B-cell lymphoma model in vivo. Although tumor-bearing CD1d$^{-/-}$ mice (lacking both type I and type II NKT cells) showed comparable tumor resistance as wild type mice, there was greater tumor growth in Jα18$^{-/-}$ mice, suggesting that type II NKT cells inhibit type I NKT cell-dependent antitumor activity (Renukaradhya et al. 2008). Thus, these reports demonstrate that type I and type II NKT cells can play distinct and counteracting roles in antitumor immunity (reviewed in Terabe and Berzofsky 2007).

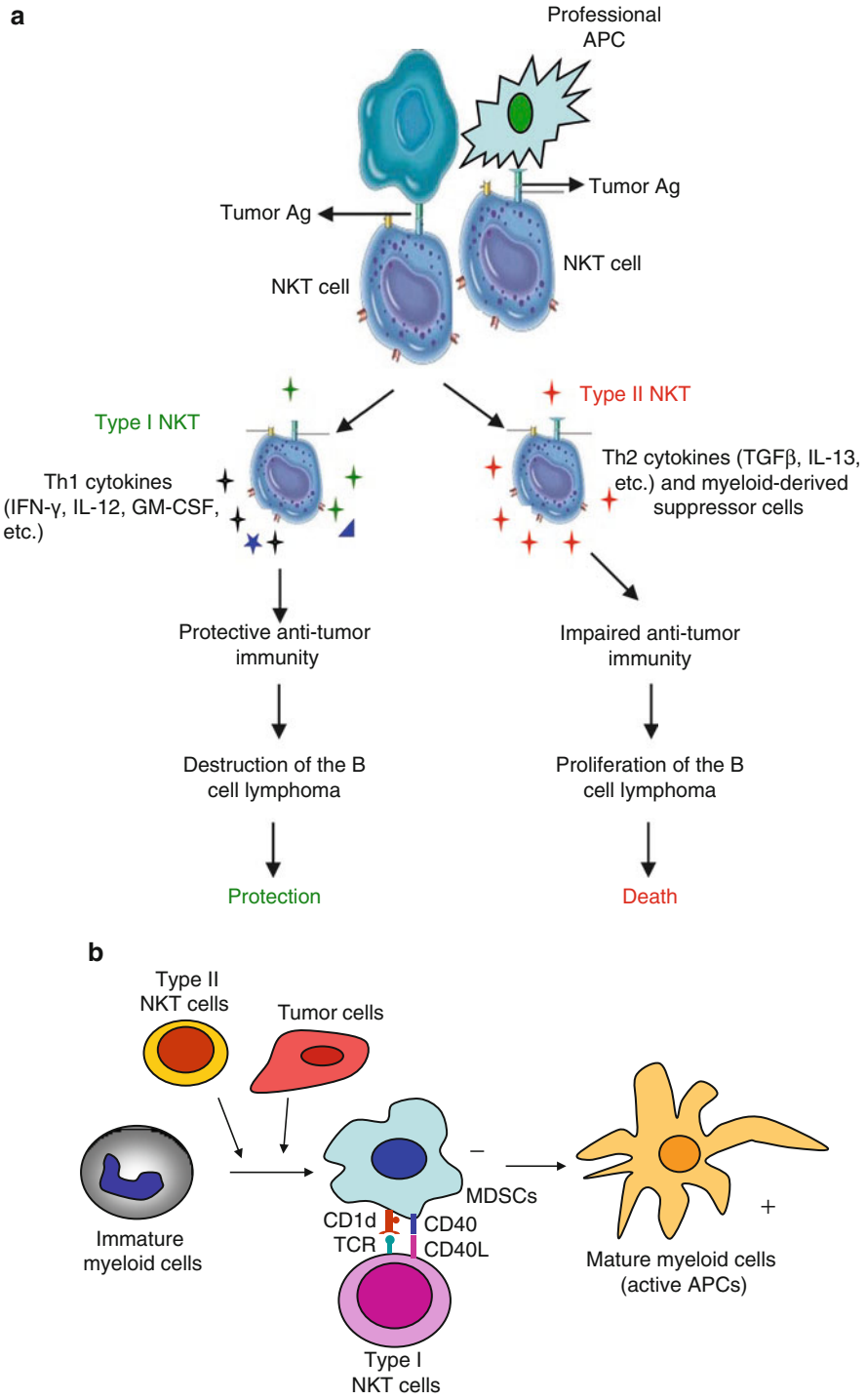
A potential reason behind the disparate roles of type I and type II NKT cells in antitumor immunity may be their different effects on the development of myeloid-derived suppressor cells (MDSCs). We and others have shown that type II NKT cells promote the accumulation of MDSCs in tumor-bearing mice (Fig. 5.1a) (Renukaradhya et al. 2008; Terabe et al. 2003). MDSCs are a heterogeneous population of immature myeloid cells that expand in cancer, inflammation and infectious diseases, and impair T-cell immunity (Gabrilovich and Nagaraj 2009). On the other hand, activated type I NKT cells can facilitate the maturation of MDSCs to become activated APCs and enhance CTL immunity (De Santo et al. 2008; Ko et al. 2009). The interaction of NKT and MDSCs is believed to be both CD1d- and CD40-dependent (De Santo et al. 2008). Thus, type II NKT cells together with tumor tissues promote the development of myeloid cells into immunosuppressive MDSCs, and thereby reduce the host's own antitumor immunosurveillance. In contrast, type I NKT cells activate and mediate the differentiation and maturation of MDSCs to become mature APCs and enhance antitumor activities (Fig. 5.1b).

In a CD1d$^+$ T-cell lymphoma mouse model, we found that iNKT cells can actually facilitate tumor growth, probably by suppressing the activity of tumor-specific CTL (Renukaradhya et al. 2006). CD1d-expressing T cells, such as human Jurkat cells, are poor APCs in presenting endogenous antigens to NKT cells (unpublished data). These data suggest that iNKT cells are unable to be activated by at least some CD1d$^+$ T lymphomas and thereby become immunosuppressive.

4 Antitumor Activity of NKT Cells

4.1 CD1d-Postive Tumor Cells

A variety of tumor cell types express CD1d on the surface, particularly those of hematopoietic origin (Metelitsa et al. 2003). Some of these can efficiently activate NKT cells (Nicol et al. 2000; Shimizu et al. 2007; Stirnemann et al. 2008), whereas others cannot (Sriram et al. 2002). The latter case is probably due to a down-regulation of endogenous CD1d function in tumor cells. Ectopic expression of CD1d in tumor cells can make them more susceptible to iNKT cell-mediated antitumor activity (Renukaradhya et al. 2006, 2008). These data suggest that the CD1d-mediated activation of iNKT cells provides strong protection against tumor progression.


a
Professional APC
Tumor Ag
Tumor Ag
NKT cell
NKT cell
Type I NKT
Type II NKT
Th1 cytokines (IFN-γ, IL-12, GM-CSF, etc.)
Th2 cytokines (TGFβ, IL-13, etc.) and myeloid-derived suppressor cells
Protective anti-tumor immunity
Impaired anti-tumor immunity
Destruction of the B cell lymphoma
Proliferation of the B cell lymphoma
Protection
Death
b
Type II NKT cells
Tumor cells
Immature myeloid cells
CD1d
TCR
CD40
CD40L
MDSCs
Mature myeloid cells (active APCs)
Type I NKT cells

There are three mechanisms by which iNKT cells can display antitumor activity against CD1d$^+$ tumors: (1) Direct cytolytic activity against CD1d$^+$ tumor cells; (2) Secretion of pro-inflammatory cytokines (e.g., IFN-γ, TNF-α) that activate NK cells and/or CTL that in turn directly kill tumor cells; (3) Activation of APCs to initiate adaptive antitumor immunity.

4.1.1 Direct Cytolytic Activity Against CD1d$^+$ Tumor Cells

Activated iNKT cells express perforin (Gumperz et al. 2002) and Fas ligand on their surface (Gumperz et al. 2002; Nakagawa et al. 2001), which can directly kill tumor cells. CD1d expression on myelomonocytic leukemias provides a target for cytolytic NKT cells (Metelitsa et al. 2003). Although CD56$^+$ NKT cells are more efficient killer cells than those that are CD56$^-$, in the presence of α-GalCer, the cytolytic activity by both subsets of NKT cells is approximately the same. The majority of NKT cell-mediated cytotoxicity is dependent on the perforin/Granzyme B pathway, whereas the remainder is through TNF-α, Fas/FasL, or TRAIL pathways (Metelitsa et al. 2003). Invariant NKT cells also enhance antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by NK cells, although the mechanism is not well-understood (Moreno et al. 2008a). Haraguchi et al. (2006) also observed that in vitro cytolysis of EL-4 mouse T lymphoma cells by Vα24$^+$ NKT cells and the in vivo eradication of these cells are dependent on the level of CD1d expression on the tumor cell surface. These data all suggest that the direct killing of CD1d$^+$ tumor cells by iNKT cells is very important for mediating their antitumor activity (Fig. 5.2a).

4.1.2 Cytokine Secretion Can Activate NK Cells and CTL-Mediated Cytolysis

Although iNKT cells may act like cytotoxic T cells, it is believed that the most important function of iNKT cells is to secrete cytokines; thus, regulating the immune system. Activated iNKT cells secrete both Th1 and Th2 cytokines. Stimulating iNKT cells in vivo by α-GalCer injection results in NK activation. However, in iNKT cell-deficient mice, α-GalCer is unable to activate NK cells, suggesting that NK

Fig. 5.1 (**a**) Schematic illustration of how type I NKT cells can be protective (and type II NKT cells suppressive) in the antitumor responses against a B-cell lymphoma. Type I NKT cells induce the production of Th1 cytokines and cause protection. Type II NKT cells trigger the secretion of Th2 cytokines, like TGF-β and IL-13, and induce the accumulation of myeloid-derived suppressor cells, impairing antitumor activity. (**b**) Schematic illustration of how type I and type II NKT cells regulate the maturation of myeloid cells. Myeloid-derived suppressor cells (MDSCs) are immunosuppressive and inhibit antitumor activity. Mature myeloid cells are active antigen-presenting cells (APCs) that can enhance antitumor activity. Type II NKT cells as well as tumor cells promote the accumulation of MDSCs but type I NKT cells induce the differentiation and maturation of MDSCs to become active APCs. "+": up-regulates antitumor activity; "–": down-regulates antitumor activity

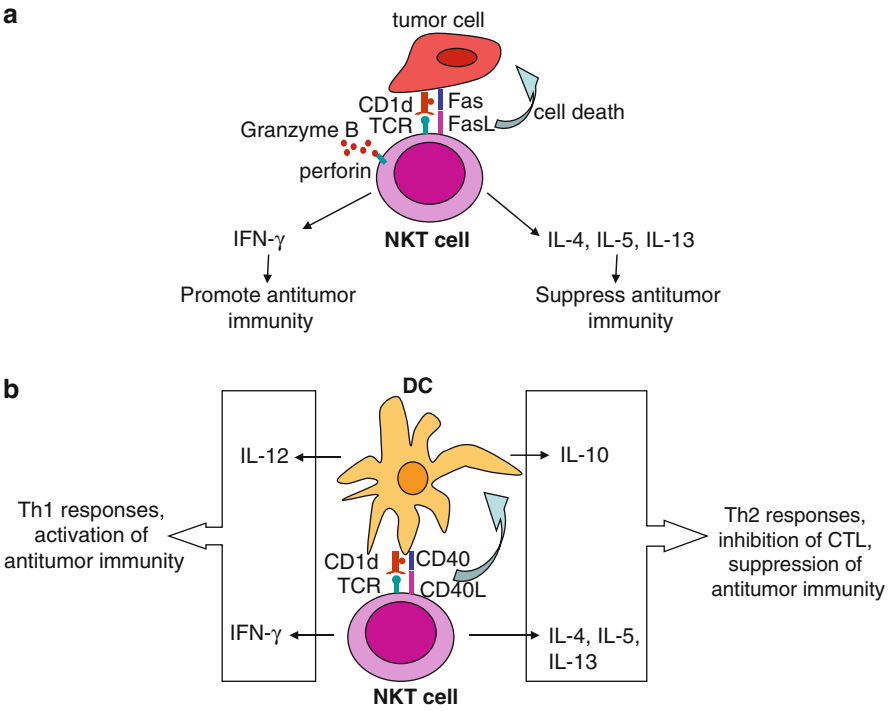


Fig. 5.2 Regulation of tumor immunity by NKT cells. (**a**) NKT cells can be activated by CD1d-expressing tumor cells and secrete Th1 cytokines to enhance antitumor immunity, or secrete Th2 cytokines to suppress antitumor immunity. Activated NKT cells can also kill CD1d+ tumor cells directly, via Fas/FasL interactions and/or through perforin/granzyme release. (**b**) In CD1d+and CD1d– tumors, NKT cells can also be activated by CD1d-expressing DCs, producing both Th1 and Th2 cytokines. CD1d/NKT cell interactions also modulate the development and maturation of DCs to secrete either Th1 cytokines (e.g., IL-12) to promote the antitumor immune response, or Th2 cytokines (e.g., IL-10) to inhibit antitumor activity

activation by α-GalCer is through indirect activation of iNKT cells (Brutkiewicz and Sriram 2002; Carnaud et al. 1999; Eberl and MacDonald 2000). Invariant NKT cells strongly enhance tumor-specific $CD8^+$ CTL activity and this is dependent on iNKT-derived IFN-γ (Moreno et al. 2008b; Nakamura et al. 2003). Similarly, in a skin graft model, iNKT cells have been shown to increase the rejection of OVA-expressing grafts by promoting OVA-specific $CD8^+$ T cells through the local production of IFN-γ (Mattarollo et al. 2010).

4.1.3 Activation of APCs to Initiate Adaptive Antitumor Immunity

Activated NKT cells can also regulate the differentiation and maturation of DC to secrete different cytokines during the host's antitumor immune response (Fig. 5.2b). As discussed above in Sect. 3, one of the proposed antitumor mechanisms of type I

(but not type II) NKT cells is stimulating the differentiation and maturation of MDSCs into mature APCs. Human DCs exposed to TLR ligands and activated by iNKT cells have increased expression of maturation markers (Hermans et al. 2007). Cytokines secreted by activated NKT cells elevate IL-12 (but inhibit IL-23) production by DCs, suggesting that iNKT cells modify the balance between IL-12 and IL-23 to enhance IL-12-dependent cell-mediated immunity, while they suppress IL-23-dependent inflammation (Uemura et al. 2009). Fujii et al. (2003) showed that injection of α-GalCer to mice induced the maturation of splenic DCs with increased co-stimulatory molecules and MHC class II expression on their surface. Not surprisingly, this process is iNKT cell-dependent, but it is MyD88-independent. Activated iNKT cells can also induce an increase in DC migration from skin to draining lymph nodes, as well as elevate T-cell priming and immune responses (Gorbachev and Fairchild 2006). Finally, when α-GalCer was administered with (or relatively soon following) ovalbumin (OVA) to mice, enhanced CD4$^+$ and CD8$^+$ T cell responses were observed. This effect was dependent on the interaction between iNKT cells and CD1d molecules, and also required CD40 signaling. Importantly, the enhancement of T-cell responses by iNKT cells resisted challenge with OVA-expressing tumors (Hermans et al. 2003).

4.2 CD1d-Negative Tumors and NKT Cells

Although some tumor cells are CD1d$^+$, most that have been studied in the context of NKT cells are CD1d$^-$. Thus, NKT cells are clearly important in these CD1d$^-$ tumor model systems. NKT cells can be activated by DCs, macrophages, or other CD1d-expressing cells. As discussed in Sect. 4.1, activated NKT cells play roles in both innate and adaptive antitumor immunity (Fig. 5.2b). Terabe et al. (2005) have very nicely shown that type I NKT cells protect (and type II NKT cells aggravate) CD1d$^-$ tumor occurrence in mice in the 15-12RM, CT26-L5, and CT26 tumor model systems. We have made a similar observation with a CD1d$^+$ tumor (Renukaradhya et al. 2008). This suggests that both type I and type II NKT cells can play similar roles (positive and negative, respectively) in the host's response against both CD1d$^+$ and CD1d$^-$ tumors.

Neuroblastomas are also CD1d$^-$ tumors. In this system, it has been reported that iNKT cells infiltrate into tumor tissue, which is associated with a lack of *MYCN* oncogene amplification (Song et al. 2007). Interestingly, patients with these tumors have a better prognosis than those with *MYCN*-amplified tumors (Metelitsa et al. 2004). Thus, iNKT cells are equally important in both CD1d$^+$ and CD1d$^-$ tumors. One potential mechanism by iNKT cells against CD1d$^-$ tumors may be through cytolysis of CD1d$^+$ tumor-associated monocytes/macrophages (TAM) (Song et al. 2009). TAMs can promote tumor cell proliferation and suppress antitumor adaptive immunity (Solinas et al. 2009). These cells may cross-present tumor-derived glycolipids to activate NKT cells (Song et al. 2009).

5 Evasion from NKT Cell-Mediated Antitumor Innate Immunity

5.1 Down-Regulation of CD1d Expression

Because NKT cells mediate both innate and adaptive antitumor immune responses and play important roles in immunosurveillance, tumor cells develop multiple means to escape from NKT-cell-mediated immune responses. Down-regulation of MHC class I and II molecules from the surface of tumor cells is a common mechanism for immune evasion (Andersen et al. 1993; Coleman and Stanley 1994; Seliger et al. 2000; Zheng et al. 1999). Similarly, some CD1d-expressing tumor cells have shown reduced surface levels of CD1d that correlate with the stage of tumor progression, as has been reported in multiple myeloma (Spanoudakis et al. 2009). Further, a high level of TGF-β produced by tumor tissues can impair the host's antitumor immune response, as it has been shown TGF-β inhibits CD1d expression in DCs (Ronger-Savle et al. 2005). Of related importance, our laboratory has observed that TGF-β inhibits CD1d-mediated antigen presentation under conditions whereby CD1d levels are not altered (unpublished data). Patients with malignant lymphoma have reduced CD1d expression on $CD14^+$ cells (Imataki et al. 2008). Although circulating myeloid DCs from advanced cancer patients normally express CD1d on their surface, they are impaired in their ability to activate NKT cells, resulting mainly in the production of Th2 cytokines. This is probably due to elevated levels of TGF-β and defective IL-12 production from DCs (van der Vliet et al. 2008).

5.2 Shedding Lipids (or Other Substances) That Can Block CD1d-Mediated Activation of NKT Cells

Another mechanism of immune evasion by tumors is the production of inhibitory substances that can block functional CD1d/NKT cell interactions. Neoplastic transformation of cells is associated with changes in cell surface glycolipid composition (Hakomori 1996). The altered glycolipids are often shed into the microenvironment and cause suppression of immunosurveillance. L5178Y-R, a murine T-cell lymphoma, sheds the tumor-associated glycolipid, gangliotriaosylceramide (Gg3Cer) into the environment (Young et al. 1986). Although L5178Y cells express CD1d on cell surface, they are unable to stimulate NKT cells (Sriram et al. 2002). We found that Gg3Cer shed by the tumor cells inhibited CD1d-mediated activation of iNKT cells (Sriram et al. 2002). When lipid shedding was blocked by the use of a glucosylceramide synthesis inhibitor, L5178Y cells were then able to be recognized as iNKT cells (Sriram et al. 2002). Ovarian cancer-associated ascites also contain inhibitory substances (likely lipids) that specifically inhibit NKT cell activation by CD1d, but these factors do not inhibit MHC class I-mediated activation of $CD8^+$

T cells (Webb et al. 2008). In addition, NKT cells from advanced multiple myeloma patients show a deficiency in the CD1d-dependent production of IFN-γ, although their NKT cells are still functional after stimulation with α-GalCer-pulsed DCs. This suggests that some tumor-derived glycolipids may block the function of NKT cells in vivo (Dhodapkar et al. 2003).

5.3 Abnormal Signal Transduction Pathways in Tumor Cells (or Tumor-Associated APCs) Result in Reduced NKT Activation

The endocytic trafficking of membrane-associated molecules is regulated by several cell signaling pathways. It has been reported that extracellular Ca^{2+} enhances dynamin- and V-ATPase-dependent endocytosis and suppresses the activity of the plasma membrane V-ATPase, resulting in bone resorption inhibition in osteoclasts (Sakai et al. 2010). The Wnt signaling pathway also increases the recycling of functional GABAA receptors on hippocampal neurons through the activation of calcium/calmodulin-dependent kinase II (CaMKII) (Cuitino et al. 2010). As discussed in the introduction, CD1d molecules need to be transported into endocytic compartments for loading with proper lipid antigens before being re-expressed on the cell surface and activating NKT cells. We have shown that CD1d-mediated antigen presentation is regulated by the activation of several cell signaling pathways (Brutkiewicz et al. 2007; Renukaradhya et al. 2005). For example, the mitogen-activated protein kinase (MAPK) p38 inhibits (but ERK1/2 promotes) antigen presentation by CD1d (Renukaradhya et al. 2005). Furthermore, protein kinase C δ is critical for antigen presentation by both CD1d and MHC class II (Brutkiewicz et al. 2007). Thus, it is possible that abnormal cell signaling in CD1d$^+$ tumor cells blocks CD1d-mediated antigen presentation and thereby reduces NKT cell function in response to these tumors. Indeed, tumor occurrence has been reported to be associated with abnormally upregulated activation of Ras/MAPK pathways in breast cancer (Mittal et al. 2009). An oncogene, 14-3-3γ, induces oncogenic transformation of NIH3T3 cells in SCID mice by activating MAPK and PI3K signaling pathways (Radhakrishnan and Martinez 2010). These studies are consistent with the idea that abnormal activation of cell signaling pathways in tumor cells inhibits CD1d-mediated activation of NKT cells and results in immune evasion by the tumor.

5.4 Disruption of TCR-Signaling in NKT Cells?

As mentioned in Sect. 2.1, patients with SAP-deficient X-linked lymphoproliferative disorder and SAP$^{-/-}$ mice have very few iNKT cells (Pasquier et al. 2005), suggesting that SAP plays a critical role in NKT cell development. SAP is an important

cytoplasmic adaptor molecule as it binds to tyrosine residues present in the cytoplasmic domain of the signaling lymphocytic activation molecule (SLAM), and promotes ligand-specific tyrosine phosphorylation for controlling different cellular functions, including the production of Th2 cytokines in $CD4^+$ T cells (Cannons et al. 2004), as well as cytotoxicity mediated by NK and $CD8^+$ T cells (Benoit et al. 2000; Dupre et al. 2005). It also binds to Fyn, a Src family tyrosine kinase, and recruits it to SLAM receptors (Chan et al. 2003). The interactions between SAP and Fyn are very important for NKT cell development. In $Fyn^{-/-}$ mice, NKT cells are also selectively impaired (Eberl et al. 1999). Based on these findings, one may speculate that tumor immune evasion may cause a TCR signaling defect in NKT cells, resulting in the dysfunctional NKT cells sometimes found in cancer patients. However, no report has yet linked tumor metastasis to impaired TCR signaling, and most NKT cells in cancer patients have been shown to be functionally normal after they are stimulated by APCs from healthy donors (Dhodapkar et al. 2003; Kawano et al. 1999).

6 Conclusions and Future Directions

Although mice have served as important models to study NKT cells and the knowledge obtained from mice is generally applicable to humans, there do seem to be fewer type I (and probably more type II) NKT cells in humans. However, due to their special roles in bridging the innate and adaptive immune responses, NKT cell participation in antitumor immunity is critical. Type I NKT cells have shown some promising results in antitumor therapy, but such studies are in their early stages. By contrast, type II NKT cells are believed to suppress antitumor immunity; thus, there is a clear need to understand this latter subpopulation of NKT cells in greater detail. However, only a few reagents have been developed to date that will help us study the basic biology of type II NKT cells and how they suppress antitumor immune responses. Therefore, an important area of future study will be to explore the roles of type II NKT cells not only in antitumor immunity, but immunoregulation in general.

We have discussed how NKT cells are involved in antitumor activity and additional potential mechanisms whereby they perform these functions. NKT cells can directly kill $CD1d^+$ tumor cells as they have cytolytic activity. They can also indirectly increase the cytolytic activity of NK cells and CTL against tumor cells. NKT cells also modulate the differentiation and maturation of APCs and induce adaptive antitumor immunity. Numerous reports have shown CD1d-mediated activation of NKT cells is down-regulated in both $CD1d^+$ and $CD1d^-$ tumors. Further, tumors can impair CD1d-mediated NKT activation by blocking CD1d/NKT interactions. By better understanding the overall roles played by NKT cells in antitumor immunity, we will be better equipped to develop these cells as an additional weapon in the arsenal used to fight against a wide variety of cancers.

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Chapter 6
DC-Based Immunotherapy Targeting NKT Cells

Shin-ichiro Fujii and Kanako Shimizu

Abstract A primary focus of tumor immunotherapy research is to modify the immune system so that it becomes immunized and not tolerized to the presentation of antigens by or from tumor cells. Dendritic cells (DCs) are the logical target for the development of tumor immunotherapies, because the DCs instruct the ensuing immune response. For invariant iNKT cell targeted therapy, the glycolipid NKT cell ligand, α-galactosylceramide is loaded on DCs. This treatment resulted in increased numbers of IFN-γ producing cells, and this correlated with antitumor effects. On the other hand, activated iNKT cells demonstrate an adjuvant effect by inducing the maturation of endogenous DCs, thereby linking innate and adaptive immunity. Cross-presentation to T cells is key to this linkage and depends on the $CD8a^{+}$DC subset and the expression of CD40, CD70, and CCL17 molecules on DCs. Based on these adjuvant mechanisms, we will introduce here new concepts about iNKT cell-targeted adjuvant therapies.

1 Introduction

The different types of immune cells, such as macrophages, dendritic cells (DCs), and a variety of lymphocytes act not only by mediating their own distinctive protective functions, but also by interacting with each other to optimize the subsequent response against tumors. In this chapter, we will review what has been learned about the interaction between invariant natural killer T (iNKT) cells and DCs. In particular, we will focus on iNKT cell activation by DCs and reciprocally, licensing of conventional DCs by iNKT cells. From this point view, our discussion will primarily extend to therapeutic approaches to cancer.

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iNKT cells have several features that make them a unique type of T cell. They all express a nearly invariant T cell receptor encoded by Vα14Jα18 in mice and Vα24Jα18 in humans and can rapidly produce both Th1 and Th2-type cytokines after ligand stimulation (Taniguchi et al. 2003; Godfrey et al. 2004; Bendelac et al. 2007; Terabe and Berzofsky 2008). Identification of an exogenous glycolipid ligand, α-galactosylceramide (α-GalCer), has spearheaded iNKT cell research. α-GalCer is widely used as a synthetic ligand for activating iNKT cells and is presented to them by the monomorphic, HLA-class I-like molecule, CD1d. Because iNKT cells have the potential to produce and quickly release large quantities of cytokines such as interferon-γ (IFN-γ), they have been studied for their potential role in the initiation of protective immune responses against tumors.

In an immune response against pathogens, one of the first cells to be activated is the DC of the innate immune system. DCs are classified into two major groups, conventional and plasmacytoid DCs. Diverse subsets of DCs in various tissues throughout the body carry out multiple functions, both in the steady state and during an ongoing immune response. However, it has been reported that when compared to conventional myeloid DCs, plasmacytoid DCs neither express CD1d nor induce cytokine production by iNKT cells (Liu 2005; Montoya et al. 2006). Therefore, in this chapter we will focus only on the conventional DCs.

DCs can directly sense pathogen components via toll-like receptors (TLRs), and other pattern recognition receptors. Their response to this recognition is complex, but includes up-regulation of surface co-stimulatory molecules, for T cells, secretion of cytokines and chemokines, enhanced antigen presentation, and migration to secondary lymphoid tissues. DC maturation, first proposed as a critical step in immunogenicity (Inaba and Steinman 1986), is now accepted as a crucial component in the induction of most adaptive immune responses. The term "maturation" in this case refers to an intricate differentiation process whereby DCs respond rapidly to environmental stimuli. Therefore, the type of stimulus determines the program of DC differentiation and the nature of the subsequent host immune response.

Mature DCs are the most powerful antigen presenting cells (APCs) in terms of their ability to directly activate iNKT cells (Kawano et al. 1997; Fujii et al. 2002). On the other hand, administration of the iNKT ligand α-GalCer brings about DC maturation, resulting in enhanced protective immune responses, a phenomenon that has been termed the NKT cell adjuvant effect (Fujii et al. 2007, 2010). The main difference in this case between TLR ligands and iNKT cell ligands is the ligand-mediated form of DC maturation. Microbial constituents (DNA and RNA) directly mature DCs by binding TLRs on DCs in a process dependent on TRIF or MyD88 pathways. In contrast, natural or synthetic iNKT cell ligands cannot induce maturation of DCs directly, instead it is not until these ligands are presented on the CD1d molecule on DCs that iNKT cells can induce maturation of DCs through cell-to-cell contact via a MyD88 independent pathway (Munz et al. 2005; Ishii and Akira 2007).

We will here summarize an identified molecular mechanism by which the interaction of DCs and iNKT cells can link the two arms of the immune system, innate and acquired immunity and then discuss potential therapeutic strategies based on this information.

2 iNKT Cell Targeting Therapy Using Ex Vivo α-GalCer-Loaded DC

DCs are not only well known as professional APCs for T cell immunity, but have been identified as important APCs for innate lymphocytes.

Conventional DCs can directly activate iNKT cells via cytokine release, i.e., type I IFN or IL-12 and IL-18, together with an endogenous, but so far unidentified iNKT cell ligand on DCs (Paget et al. 2007; Tupin et al. 2007). Stimulation of DCs with various TLR ligands has been shown to modulate their lipid biosynthetic pathways to enhance recognition of CD1d-associated lipids by the iNKT cell TCR (Salio et al. 2007). CpG-ODN stimulated DCs enhanced the expression of endogenous glycolipids and their presentation by CD1d, resulting in activation of the iNKT cells (Paget et al. 2007). However, it appears that DC loaded with synthetic iNKT cell ligand such as α-GalCer, α-C-GalCer, or α-carba-GalCer are even more potent activators of iNKT cells (Fujii et al. 2002, 2006; Schmieg et al. 2005; Tashiro et al. 2010). Mature DCs express high levels of major histocompatibility complex (MHC) class II and co-stimulatory molecules, such as CD80 and CD40. These co-stimulatory molecules on DCs may be necessary for iNKT cell proliferation, rather than for cytokine production (Uldrich et al. 2005). In addition, mature DCs have the potential to produce IL-12. Soluble IL-12 can directly stimulate iNKT cells through the IL-12 receptor even in the absence of α-GalCer (Shin et al. 2001). On the other hand, recognition of α-GalCer induces activation of iNKT cells efficiently even in the absence of IL-12. Therefore, iNKT cells can be fully activated via the integrated effects of α-GalCer and IL-12 produced by DCs (Kitamura et al. 1999; Toura et al. 1999).

In a study aimed at enhancing iNKT cell response, it was found that the injection of α-GalCer-loaded DCs (DC/Gal) that had been developed ex vivo from progenitors has a great advantage over simple injection of free α-GalCer (Fujii et al. 2002). The iNKT cells from DC/Gal-treated mice have an altered cytokine profile, secreting much more IFN-γ than IL-4. A single dose injection of DC/Gal generated a prolonged burst of IFN-γ secretion by iNKT cells. In contrast, the IFN-γ response to soluble α-GalCer was rapid, but short lived. Taken together, these studies demonstrated that α-GalCer has different functional effects, depending on whether it is administered as a free glycolipid or in association with CD1d on DCs.

The efficiency of in vivo versus in vitro loading of α-GalCer for inducing sufficient iNKT cell activation has also been examined (Fujii et al. 2007; Shimizu et al. 2007a). Several hours were required for the in vitro loading of DCs and even longer on other cells. For example, macrophages and B cells express CD1d and act as potent APCs for iNKT cells, however their capacity to directly activate iNKT cells is weaker than DCs. Even DCs need at least 12 h to activate iNKT cells in vitro, due to the stability of CD1d–glycolipid complex on APCs (Fujii et al. 2006; McCarthy et al. 2007; Salio et al. 2010). The importance of the biological stability inCD1d-mediated antigen presentation may be supported by further analysis of glycolipid presentation in which the stability of CD1d–lipid complexes depends on biochemical properties of the binding lipids, i.e., process of lipid displacement and its regulation by lipid

structure and acidic pH. Particularly, antigen loading onto CD1d is enhanced by low pH (4.5–5.5) of the late endosomes or lysosomes (Bai et al. 2009; Salio et al. 2010).

Ligands inducing a strong Th1-cytokine response preferentially load onto CD1d molecules in the endolysosomal compartment and are raft associated (Peng et al. 2007; Im et al. 2009). This was the predominant response of DCs, resulting in sustained IFN-γ production compared to that of other cells. On the other hand, the induction of anergic responses by free α-GalCer could be explained not only by its capture by non-DCs, but also by the activity of other molecules, e.g., IL-10 secretion by DCs (Kojo et al. 2005) and PD-1/PD-L during the iNKT-DC interaction (Parekh et al. 2009).

The antitumor effects of DC/Gal therapy were mediated by the activation of both iNKT cells and NK cells, and the number of these IFN-γ producing innate lymphocytes appeared to positively correlate with the antitumor effects in DC/Gal treated mice. In lung tumor models, it has been shown that IFN-γ together with IL-12 and/or other cytokines produced by DCs leads to in vivo activation of NK and iNKT cells (Hayakawa et al. 2001). There appears to be a complex synergy here, IL-12 from DCs in addition to IFN-γ produced by iNKT cells can up-regulate IL-12R expression on NK cells, which then enhances NK cell-mediated IFN-γ production (Kitamura et al. 1999).

Clinical studies have demonstrated the safety and efficacy of iNKT cell therapy (Nieda et al. 2004; Chang et al. 2005; Motohashi and Nakayama 2009). iNKT cell activation induced by α-GalCer-pulsed immature DCs provoked NK and T cell expansion in patients with metastatic malignancies (Nieda et al. 2004). Chang et al. (2005) demonstrated that mature α-GalCer-pulsed DCs induced a >100-fold expansion of iNKT cells, which was sustained for up to 6 months after treatment. Particularly, Motohashi et al. (2009) in clinical studies using α-GalCer-pulsed IL-2/GM-CSF-cultured PBMCs demonstrated that lung cancer patients receiving this therapy had prolonged stable disease with increased median survival time (MST). The number of IFN-γ producing iNKT and NK cells increased more than twofold after this therapy. Thus, similar to the findings in mouse models, IFN-γ production by iNKT and NK cells seems to be a good marker for the efficacy of this cell therapy.

3 NKT Cells License Conventional DCs In Vivo

3.1 The In Vivo Adjuvant Effect of Activated iNKT Cells: From Tolerance to T Cell Immunity

DCs in lymphoid tissues in the normal steady state are functionally immature, because they are able to capture antigens but are incapable of initiating T cell-mediated immunity (Hawiger et al. 2001). Thus, for effective antitumor therapy, this in vivo T cell tolerance must be converted to protective immunity. It should prove fruitful to harness innate lymphocyte-mediated DC maturation for immunotherapy, because the innate lymphocytes themselves have direct antitumor activity and moreover they can mobilize an adaptive immune response via DCs.

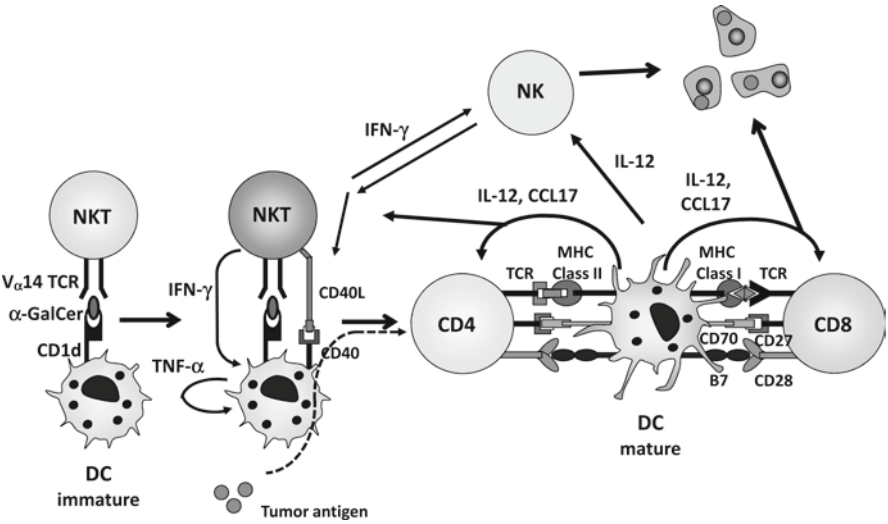

Fig. 6.1 α-GalCer can link innate and adaptive immunity via full maturation of DCs. Upon administration of α-GalCer, this iNKT cell ligand can be loaded onto immature DCs and present antigen to iNKT cells initiating their maturation, which includes the production of IL-12 and TNF-α and elevated expression of co-stimulatory molecules. Up-regulation of co-stimulatory molecules, such as CD40, CD80, and CD86 is generally initiated within 4 h, however up-regulation of CD70 on DCs can only be seen ~40 h after administration of α-GalCer, thus it represents a later phase of DC maturation. The IL-12 leads to the activation of additional innate NK cells. When antigen and α-GalCer are co-administered to mice, the DCs respond to CD40L on NKT cells to fully mature and induce strong antigen-specific $CD4^+$ and $CD8^+$ T cell responses (adaptive immunity). In this response, DCs attaining full maturation express CCL17 to recruit both T cells and iNKT cells

iNKT cells have the ability to license conventional DCs in vivo. α-GalCer cannot directly induce the maturation of DCs in vitro and immature DCs cannot activate T cells. Importantly, however, DCs can be activated in vivo by administration of α-GalCer (Shimizu et al. 2007a; Fujii 2008). Thus, in the initial step in the iNKT cell–DC interaction, α-GalCer presented on immature DCs in vivo activates iNKT cells, which then can initiate DC maturation (Fig. 6.1).

In terms of adaptive T cell responses, the fact that α-GalCer could serve as an adjuvant for $CD8^+$ T cells was first shown in a malaria vaccine model by co-administration of α-GalCer and irradiated mouse malaria (*P. yoelii*) sporozoites (Gonzalez-Aseguinolaza et al. 2002). Subsequently, we demonstrated a fully mature DC-mediated induction of T cell immunity (Fujii et al. 2003; Hermans et al. 2003). When ovalbumin (OVA) within dying cells was presented as cell-associated antigen to a subset of DCs, tolerance was induced (Liu et al. 2002). In contrast, we and others showed that OVA-specific T cell responses developed in immunized mice when α-GalCer was co-administered together with soluble or cell-associated OVA antigen (Fujii et al. 2003; Hermans et al. 2003). Importantly in terms of developing therapeutic strategies, the mice became resistant to challenge with an OVA-expressing tumor. To verify

that DCs play a crucial role in this system, DCs from mice injected with antigen plus α-GalCer were transferred to naïve mice and the results confirmed the DCs-mediated adaptive T cell immunity. Hermans et al. (2003) also described the importance of timing, i.e., the glycolipid needed to be given at the same time as or close to the OVA injection, because DCs show reduced uptake of antigens after maturation. Therefore, the timing of DC maturation and antigen delivery to DCs will be crucial for induction of effective T cell immunity.

Confirming the role for iNKT cells, α-GalCer-induced DC maturation was not observed in Jα18$^{-/-}$ iNKT deficient mice, and these mice given antigen plus α-GalCer failed to generate a T cell response. We were able to determine how many cells are required for this adjuvant effect, and found that 2×10^5 cells is the minimum number of syngeneic iNKT cells but not allogeneic NKT cells in an adoptive transfer model of iNKT cells to Jα18$^{-/-}$ mice before being given OVA antigen plus α-GalCer (unpublished data).

3.2 The Mechanism of NKT Cell-Induced DC Maturation

iNKT cells activated by α-GalCer can clearly serve as a DC maturation stimulus in vivo. Since one logical mechanism for this adjuvant activity would be to mature antigen capturing DCs, we examined the effects of α-GalCer on splenic DC functions (Fujii et al. 2003).

iNKT cell-mediated maturation of DCs was first studied in terms of inflammatory cytokines and CD40-CD40L signaling (Fujii et al. 2004). TNF-α and IFN-γ are initially secreted and detectable in the serum during the early phase after immunization. The TNF-α is derived from CD11c$^+$DCs and IFN-γ is derived from iNKT cells and CD11c$^+$NK cells which are different from the IFN-γ-producing killer DCs (IK-DCs) (Fujii et al. 2006). In an in vivo study, where TNF-α and IFN-γ signaling was blocked in immunized mice, the co-stimulatory molecules were not up-regulated on DCs, suggesting a requirement for these cytokines *for phenotypic maturation of DCs*. On the other hand, in α-GalCer-treated CD40$^{-/-}$ and CD40L$^{-/-}$ mice, expression of co-stimulatory molecules (CD80, CD86, and others) on DCs was up-regulated to the same extent as in α-GalCer-treated WT mice (Fujii et al. 2004). The findings have also been confirmed by Matsuda et al., who showed that IFN-γ production from iNKT cells in CD40$^{-/-}$mice could be detected at 2 h after α-GalCer administration (Matsuda et al. 2003). These findings indicate that CD40 signaling is not crucial *for iNKT cell activation and phenotypic maturation of DCs*, but instead functions to augment the activation of iNKT cells.

There are some factors that determine how effectively a given immunization protocol links the stimulation of the innate immune system with the development of a lasting adaptive immune response. Antigen concentration and levels of CD1d expression on glycolipid-loaded cells as well as the function of host DCs are all important (Fujii 2008), as was shown by the fact that DC-deficient mice as well as TAP$^{-/-}$ mice were unable to develop T cell immunity after immunization (Fujii et al.

2004; Shimizu et al. 2007b). Other studies showing that $CD40^{-/-}$ and $CD80/86^{-/-}$ mice did not develop T cell immunity after immunization demonstrate the importance of endogenously expressed co-stimulatory molecules on DCs (Shimizu et al. 2007b). Thus, some features of DC maturation, such as up-regulation of co-stimulatory molecules, can be induced by proinflammatory cytokines, but cytokines alone seem insufficient for the induction of adaptive immunity in vivo. Additional changes can be imparted to the DCs by CD40 ligation, which contributes to the generation of both $CD4^+$ and $CD8^+$ T cell immunity (Fig. 6.1). The magnitude of the innate immune response generated by all these factors can be directly correlated with the subsequent adaptive immune response. Particularly, CD40L expression on iNKT cells correlates with T cell immunity (Fujii et al. 2006).

Following their maturation in situ by either TLR ligand or anti-CD40 antibody stimulation, conventional DCs express CD70 (Bullock and Yagita 2005; Sanchez et al. 2007). It has been recently appreciated that these CD70-expressing licensed DCs can induce a $CD8^+$T cell response. The receptor for CD70, CD27, is *constitutively* expressed on naïve $CD4^+$ and $CD8^+$ T cells in mice and humans, indicating its important role at the DC interface during T-cell priming (Hendriks et al. 2000; Borst et al. 2005). CD27/CD70 interactions promote clonal expansion of primed $CD4^+$ and $CD8^+$ T cells at effector sites, to a large extent by supporting T-cell survival (Hendriks et al. 2003; Rowley and Al-Shamkhani 2004). Two recent papers reported that CD70 expressing $DEC205^+$DCs in spleen induce an IL-12-independent IFN-γ pathway for $CD4^+$T cell response (Soares et al. 2007), while CD70 expressing $CD103^-CD11b^{hi}$DC cross-primed $CD8^+$ T cells in an influenza infection model (Ballesteros-Tato et al. 2010). During the iNKT cell-mediated DC maturation, up-regulation of co-stimulatory molecules, such as CD40, CD80, and CD86, is generally initiated within 4 h after administration of α-GalCer, however up-regulation of CD70 on DCs is not seen ~40 h later (Fujii et al. 2009). Interestingly, up-regulation of CD70 has been demonstrated to play a crucial role in the adjuvant effect of iNKT cells for T cell responses (Taraban et al. 2008). Thus, the CD40L signal from iNKT cells might lead to the expression of CD70 on DCs for T cell immunity (Fig. 6.1).

It is now clear that mutual interactions between DC and T cells mediated by CD40L-CD40, CD80/CD86-CD28, and CD70-CD27, and perhaps other unknown receptor/ligand pairs are crucial for T cell response.

3.3 Activated iNKT Cell-Mediated DC Cross-Presentation to T Cells

After antigen capture by DCs, it is internalized in phagosome and endosomes. The DCs can then present exogenous antigen in association with MHC class I by a process unique to DCs, called "cross-presentation" (Cresswell et al. 2005; Amigorena and Savina 2010). Cross-presentation has been generally thought to be mediated by two pathways, i.e., cytosolic pathway (TAP dependent and proteasome dependent) and the vacuolar pathway. Indeed, macrophages and neutrophils have extensive phagocytic

or endocytic activity. However, they cannot cross-present antigen, because incoming antigen was degraded or MHC class I epitopes were destroyed, due to the highly acidic conditions in their phagosomes and endosomes. In DCs, by contrast, the pH in phagosome and endosomes is less acidic, and internalized proteins can be efficiently cross-presented. Also, the fact that $CD8a^+$ splenic DCs are more efficient than their $CD8a^-$ counterparts appears to be due to the relatively much less acidic pH environment in the phagosomes and endosomes dependent on the assembly of NOX2 driven by Rac2 (Savina et al. 2009), and the selectivity for MHC class I and MHC class II pathways in $CD8a^+$ and $CD8a^-$ subsets of DCs has been demonstrated (Iyoda et al. 2002; Dudziak et al. 2007).

Although the activation of iNKT cells as a cellular adjuvant can induce both $CD4^+$ and $CD8^+$ T cells, an important role for cross-presentation by in vivo matured DCs in a TAP dependent pathway has only been demonstrated for the activation of $CD8^+$ T cells (CTL) (Fujii et al. 2003, 2004; Hermans et al. 2003). As we previously demonstrated in an α-GalCer-loaded tumor vaccine model, the $CD8a^+$ subset of DCs that have acquired tumor derived debris produce more IL-12 than $CD8^-$ DCs, resulting in induction of adaptive immunity (Shimizu et al. 2007b; Fujii 2008). As discussed earlier, DC-iNKT coculture experiments showed a direct CD40L-dependent effect of iNKT cells on IL-12p70 production by $CD8a^+$DCs (Fig. 6.1).

Recently, many studies have been carefully focusing on the analysis of iNKT cell-induced DCs in situ and some remarkable findings have emerged. In the steady state, iNKT-deficient mice have a reduced number of $CD8a^+$ DCs with lower CD40 expression (Joyee et al. 2010). The homeostasis of DCs in vivo thus may be controlled by iNKT cells. More importantly, $CD8a^+$ DCs from $J\alpha18^{-/-}$ mice showed a significantly reduced ability to activate IFN-γ-producing T cells in vitro when compared to those from wild-type mice. For the generation of antigen-specific CTL mediated by the adjuvant activity of the invariant NKT cell ligand, both CTL and iNKT cells were severely impaired in mice selectively depleted of $langerin^+$ cells in vivo. Further analysis of subpopulation of $CD8a^+$DCs demonstrated that the langerin $(CD207)^+$ $CD8a^+$ DC population can produce IL-12p40 and also play a critical role in cross-priming (Farrand et al. 2009).

The adjuvant effects of α-GalCer induced by different injection routes and in different organs have also been compared. Lamina propria (LP)-DCs are responsible for antigen presentation after oral administration (Chang et al. 2008). After oral treatment with α-GalCer, the functional maturation of Ag-capturing LP-DC triggered by activation of iNKT cells in the small intestinal lamina propria (SI-LP) was found to be necessary for the abrogation of oral tolerance induction. There have been few studies of iNKT cell activity in the skin (Tripp et al. 2009). Although migratory skin DC and lymph node DC can present glycolipid to iNKT cells in vitro, intradermally applied glycolipids are presented predominantly by lymph node DC rather than by migratory skin DC to iNKT cells. On the other hand, it was reported in intraperitoneal injection model that $CD169^+$ macrophages localized in the subcapsular sinus in mediastinal lymph nodes can present antigen to iNKT cells at first before DCs can present antigen to iNKT cells (Barral et al. 2010). Intramuscular administration of α-GalCer was found to be much less potent than

the intravenous route in terms of maturation of DCs (Fujii et al. 2006). Therefore, the injection route affects the function of activated iNKT cells.

In addition, it has recently been reported that DCs that can be licensed for cross-priming by NKT cells have a different mechanism than that by helper T cells (Semmling et al. 2010). $CD8a^+$ DCs licensed by iNKT cells can produce CCL17, which attracts both $CD8^+$ T cells and iNKT cells expressing CCR4, whereas DCs licensed by $CD4^+$ helper T cells recruited $CD8^+$ T cells using CCR5 ligands.

Thus, design of any vaccine strategy will require consideration of DC subsets, the organs where they reside, as well as expressed chemokines and chemokine receptors, cytokine and cytokine receptors.

3.4 The Criteria for iNKT Cell-Induced Fully Functional Maturation of DCs

DC maturation by activated iNKT cells involves not only phenotypic changes, but also functional ones before the cell can be considered, a "fully mature" DC. We have summarized recent findings for determining the criteria for defining fully functional matured DCs in vivo in terms of their ability to link innate and adaptive immunity as follows (Fig. 6.1). (1) There is a marked increase in co-stimulatory molecules (CD40, CD80, and CD86) and MHC class II. (2) During maturation, DCs acquire the potential to produce inflammatory cytokines, such as TNF-α and IL-12. This is important since not only iNKT cells, but also other cells such as naïve T cells and NK cells express the mature form of the IL-12 receptor (IL-12R) and IL-12 has been shown to orchestrate immunity. (3) When DCs attain full maturation, they express CCL17 as another maturation marker to recruit both T cells and iNKT cells. (4) The DCs can acquire the capacity for strong stimulatory effect on T cell proliferation in the mixed leukocyte reaction. (5) The final criterion of in vivo DC maturation has to account for induction of antigen-specific T cell immunity.

4 Therapeutic Approaches Combining Innate and Adaptive Immunity by Harnessing the Adjuvant Effects of Activated iNKT Cells

4.1 Co-administrative Therapy of Tumor Antigen with α-GalCer

Based on the concept of iNKT cell-induced DC maturation in situ in linking innate and adaptive immunity (Fujii et al. 2007; Fujii 2008), several therapeutic strategies have been evaluated.

Induction of immunity was successfully accomplished in several therapeutic models by co-administration of antigen plus α-GalCer (Fig. 6.1). When mice were

immunized with the iNKT cell ligand and either an inactivated influenza A virus or HSV-2 glycoprotein D (gD), vaccine-induced $CD8^+$ T cell immunity as well as virus-specific IgG was generated systemically (Youn et al. 2007; Kamijuku et al. 2008; Guillonneau et al. 2009; Lindqvist et al. 2009). In addition, administration of antigen-specific DNA vaccine plus α-GalCer led to the successful protection from Leishmania and HIV-1 infection (Dondji et al. 2008; Huang et al. 2008).

As it has been reported that DCs preferentially uptake dying cells (Iyoda et al. 2002), co-administration of dying cell-associated antigen together with α-GalCer has been shown to induce Ag-specific T cell immunity (Fujii et al. 2003, 2007; Fujii 2008). Also, when mice were given irradiated tumor cells (J558 plasmacytoma cells) as dying cells and α-GalCer, the vaccinated mice were subsequently protected from the identical tumor (Liu et al. 2005). Both $CD4^+$ and $CD8^+$ T cells contribute to the tumor resistance induced through in vivo DCs capturing dying tumor cells.

4.2 Use of the Adjuvant Vector Cells to Harness In Vivo DC Targeting Therapy

The successful antitumor protection seen by co-administration of antigen and iNKT cell ligand has allowed extending this approach to several therapeutic models. Nonetheless, there is a limitation to this initial attempt to exploit the innate properties of DCs and iNKT cells at least against tumors in vivo. The problem is that the delivery of tumor antigen to DCs, while enhanced by the use of the i.v. route of injection, requires a relatively large dose of irradiated tumor cells in mice. Therefore, we changed the vaccine formulation from a combination of free α-GalCer and tumor cells to α-GalCer-loaded tumor cells.

To evaluate the problem of transferred cell number, the use of α-GalCer-loaded tumor cells has been assessed in several models, including B16 melanoma, J558 plasmacytoma, WEHI3B myelomonocytic leukemia, and EL4 thymoma.

Administration of tumor cells loaded with α-GalCer (tumor/Gal) has successfully induced iNKT and NK cell activation, as shown by protection against lung metastases (Fig. 6.2) (Shimizu et al. 2007a). The iNKT and NK-mediated lysis of tumor cells led to cross-presentation of the tumor antigens by host matured DCs elicited by iNKT cells. In addition, i.v. injection of mice with tumor/Gal induced protection against subcutaneous challenge with the same tumor (Fujii et al. 2007; Shimizu et al. 2007b). Moreover, after tumor regression, T cell-mediated antitumor responses to various epitopes were detected (Fig. 6.2) (Fujii 2008).

To elucidate the mechanism of these responses, when $CD11c^+$ DCs were isolated from tumor/Gal immunized mice and administered to naïve mice, they induced an antigen-specific T cell response. Also, when co-cultured with liver mononuclear cells (an enriched source of primary iNKT cells), $CD11c^+$ DCs were able to stimulate IFN-γ secretion in vitro. Therefore, DCs can cross-present both tumor antigens to $CD8^+$T cells and glycolipid to iNKT cells following i.v. injection of tumor/Gal (Fig. 6.2).

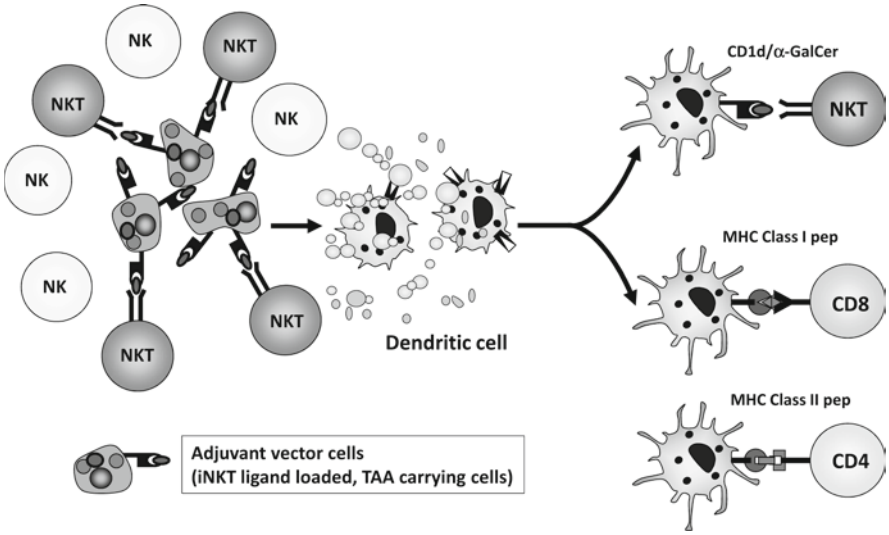

Fig. 6.2 iNKT cell-mediated adjuvant effects on antitumor protective responses are supported by two types of cross-presentation by DCs. Activated iNKT cells are responsible for DC maturation in mice immunized with tumor cells loaded with α-GalCer (Tumor/Gal) or tumor antigen mRNA-transfected fibroblasts loaded with α-GalCer (fibroblast-Ag mRNA/Gal). These α-GalCer-loaded, tumor associated antigen (TAA) carrying cells are killed by iNKT cells and NK cells, and then debris are captured by endogenous $CD11c^+$ DCs. These $CD11c^+$ DCs are then able to induce an antigen-specific T cell response. Also, the $CD11c^+$ DCs are able to cross-present glycolipid from phagocytosed tumor/Gal or fibroblast-TAA/Gal to iNKT cells in a CD1d-dependent manner

Based on these studies, we concluded that tumor/Gal can act as a powerful cellular adjuvant, since it can induce cross-presentation of tumor antigen as well as glycolipid to T cells and iNKT cells, respectively. Cells presenting both glycolipid and tumor antigens should also work well in vivo on the same DCs. Our findings suggest several possibilities for the development of future treatment strategies linking innate and adaptive immunity by designing "adjuvant vector cells" (Fig. 6.2).

We have attempted to develop such an approach based on the principles established with the α-GalCer-loaded tumor model but that would be more readily applicable. We established allogeneic fibroblast cells as adjuvant vector cells that could be loaded with α-GalCer as well as transfected with an appropriate tumor antigen-encoding mRNA (Fig. 6.2) (Fujii et al. 2009), thus combining the adjuvant effects of iNKT cell activation with delivery of antigen to DCs in vivo. As shown in the tumor/Gal model, these cells would express antigen protein and also potentiate the activation of NK and iNKT cells. When injected even into MHC mismatched mice, these vector cells elicited antigen-specific T cell responses and provided tumor protection, suggesting that these immune responses depend on endogenous DCs, but not the transferred cells (Fig. 6.2). The kinetics of activated iNKT cells made endogenous DCs to fit with the criteria of in vivo full maturation of DCs. Along with the iNKT cell activation, endogenous DCs were matured in terms of up-regulation of CD40, CD80/86,

and CD70. Also, T cell response induced by antigen-expressing fibroblasts loaded with α-GalCer was more potent than when the fibroblasts expressed NK cell ligands. Thus, glycolipid-loaded, mRNA-transfected allogeneic cells act as cellular vectors to promote iNKT cell activation, leading to DC maturation and T cell immunity. By harnessing the innate immune system and generating an adaptive immune response to a variety of antigens, this unique tool could prove clinically beneficial in the development of immunotherapies against malignant and infectious diseases.

5 Summary and Discussion

Initially, DC/Gal therapy informed us of the importance of IFN-γ producing iNKT cells and NK cells, and how these correlated with antitumor effects in murine models. These findings have been confirmed in clinical trials of solid tumor and hematological malignancies. The data thus far obtained with the iNKT cell targeting therapy are encouraging. In addition, the activation of iNKT cells has been accompanied by the activation of CD8 T and NK cells in some clinical studies, providing insight into possible strategies to harness the adaptive immune response. To develop a strategy bridging innate and adaptive immunity, the interactions between DCs and iNKT cells in situ have to be precisely understood. Particularly, as we have discussed here, on undergoing maturation, endogenous DCs can bridge iNKT cells to other innate cells as well as to adaptive immunity. Finally, to enhance the DC-iNKT cell interactions, we propose a new strategy and name it "adjuvant vector cell therapy," wherein fibroblasts express the tumor antigen and are loaded with α-GalCer. It is clear that DCs in situ are important potential therapeutic targets for iNKT cells. This novel adjuvant vector cell therapeutic strategy may prove clinically beneficial in the development of immunotherapies.

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Chapter 7
Therapeutic Approaches Utilising NKT Cells

Stephen R. Mattarollo and Mark J. Smyth

Abstract Natural killer T (NKT) cells are members of the immune armamentarium with profound immunoregulatory effects. They bridge the innate and adaptive immune systems, filling a niche in recognizing glycolipid antigens, and responding rapidly to prime subsequent immune responses. In cancer, type I NKT cells, defined by their semi-invariant T cell receptor (TCR) using Vα14Jα18 in mice and Vα24Jα18 in humans, are mostly host protective, by producing interferon-γ (IFN-γ) to activate and mature dendritic cells (DC) to make IL-12, which in turn activates NK and $CD8^+$ T cells. In contrast, type II NKT cells, characterized by more diverse TCRs recognizing lipids presented by CD1d, primarily inhibited anti-tumor immunity. This chapter will discuss the impact of CD1d-restricted NKT cells in tumor immune surveillance and immunotherapy and highlight recent therapeutic approaches in tumor mouse models with a focus on harnessing the anti-tumor activities of NKT cells.

1 Introduction

It is now widely accepted that the immune system is a crucial player in the outcome of cancer development. The term immunoediting has been used to describe the somewhat paradoxical capacity of the immune system to initiate both host-protective and tumor-sculpting functions in a dynamic process of cancer development that has been classified into three phases: elimination, equilibrium and escape (Dunn et al. 2002, 2004; Swann and Smyth 2007). The elimination phase defines the process of tumor immune surveillance, whereby immunity prevents against the development of tumors

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by identifying and eliminating cancerous or pre-cancerous cells prior to any resulting pathology. The adaptive immune system has gained much attention for its involvement in tumor immune surveillance, however, it has also been revealed that components of the innate immune system are equally, if not more important in this process (Smyth et al. 2007). CD1d-restricted natural killer T (NKT) cells are emerging as an important regulator of tumor immune surveillance (Smyth et al. 2000; Terabe et al. 2000, 2005, 2006; Crowe et al. 2002; Park et al. 2008; Swann et al. 2009; Bellone et al. 2010). These cells are often classified as an innate lymphocyte population based on their non-MHC-restricted recognition of lipid/glycolipid antigens in association with CD1d, and rapid production of cytokines and effector molecules upon activation.

At least two populations of CD1d-restricted NKT cells are associated with tumor immunity. The most extensively studied member, sometimes defined as type I NKT cells or iNKT cells, expresses a semi-invariant TCR, which in mice consists of a Vα14Jα18 TCR-alpha chain paired with beta chains Vβ8, Vβ7 or Vβ2. These cells preferentially recognize the glycolipid antigen α-galactosylceramide (α-GalCer) and can be divided into functionally distinct subsets based on NK1.1 and CD4 expression (Crowe et al. 2005; Godfrey et al. 2010). A homologous population of Type I NKT cells exists in humans, identified by an invariant Vα24/Vβ11 TCR. Other CD1d-restricted NKT cells have been identified in both humans and mice, and are collectively referred to as Type II NKT cells. These cells express a more diverse TCR repertoire and less is known as to their significance and role in regulating tumor immune surveillance, although accumulating evidence suggest that these cells have a regulatory, immune suppressive phenotype in some tumor models (Moodycliffe et al. 2000; Terabe et al. 2000; Ambrosino et al. 2007).

2 NKT Cell Responses in Anti-Tumor Immunity

2.1 Natural Anti-Tumor NKT Cell Activity in Immune Surveillance

2.1.1 Type I NKT Cells

NKT cells play a key role in modulating immune surveillance of certain tumors by promoting anti-tumor responses of innate and adaptive lymphocytes. The natural activity of NKT cells in tumor immune surveillance has been best characterized in a spontaneous tumor model initiated by a chemical carcinogen, methylcholanthrene (MCA). Mice deficient in NKT cells ($CD1d^{-/-}$ and $J\alpha18^{-/-}$) are more susceptible to MCA-induced tumors, exhibiting both higher tumor incidence and earlier onset of tumor development (Smyth et al. 2000). MCA-induced fibrosarcomas derived from $J\alpha18^{-/-}$ mice grow faster in these mice, however upon transfer into syngeneic, wild-type mice are rejected in a CD8 T cell-, NKT cell-, NK cell- and IFN-γ-mediated manner (Crowe et al. 2002). However, protection by NKT cells is unlikely requiring direct CD1d recognition on the tumor cells, since sarcomas that lack CD1d expression

are still susceptible to NKT cell-mediated rejection (Crowe et al. 2002). It is speculated that NKT cells contribute to immune surveillance of CD1d-negative tumors by promoting activation and activity of CD8 T cells and NK cells following recognition of tumor-associated glycolipids or altered self-glycolipids, presented on CD1d by host DC. Type I NKT cells were also implicated in host protection against pulmonary metastases produced by intravenous inoculation of the MCA-induced BALB/c sarcoma CMS5m (Nishikawa et al. 2003). In this model, Jα18$^{-/-}$ mice lacking type I NKT cells displayed more lung metastases and these mice were rescued by adoptive transfer of α-GalCer/CD1d tetramer-positive type I NKT cells. Moreover, the suppression of tumor immunity by CD4^{+}CD25^{+} T regulatory (Treg) cells induced by injection of SEREX (*Se*rological analysis of *R*ecombinant cDNA *EX*pression libraries)-defined tumor antigen was dependent on the reduction in the number of type I NKT cells in the lungs of these mice (Nishikawa et al. 2003). Thus, it appears not only that type I NKT cells can contribute to spontaneous tumor immunosurveillance, but may be cross-regulated by CD4^{+}CD25^{+} Treg.

In addition to MCA-induced fibrosarcomas, NKT cells have been implicated in protective tumor immune surveillance of more clinically relevant mouse models of cancer, such as tumors arising from mutations in the p53 tumor suppressor gene. NKT cells suppress the growth of osteosarcoma and hematopoietic tumors caused by loss of p53 in p53$^{+/-}$ mice (Swann et al. 2009). Both p53$^{+/-}$ CD1d$^{-/-}$ and p53$^{+/-}$ Jα18$^{-/-}$ mice displayed lower survival rates than their p53$^{+/-}$ wildtype counterparts, supporting a specific role for type 1 NKT cells in inhibiting tumorigenesis in the absence of functional p53 (Swann et al. 2009). More recently in a study using transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, investigators assessed whether NKT cells controlled the growth of spontaneous prostate cancer. In this model, the lack of NKT cells in TRAMP Jα18$^{-/-}$ mice correlated with more aggressive adenocarcinoma development in prostate tissue (Bellone et al. 2010). Despite the presence of a traceable tumor antigen-specific T cell response in these mice, no evidence was found to support a correlation between the presence of NKT cells and efficacy of the CTL response in this setting. Nevertheless, this study extends the list of spontaneously arising tumors in mice in which NKT cells are critical for natural immune surveillance. Consistent with these findings, reduced circulating NKT cell numbers have been observed in patients with a range of malignancies. The number of NKT cells appears to correlate with a more favourable prognosis in colorectal cancer, prostate cancer, head and neck cancer, and multiple myeloma (Dhodapkar et al. 2003; Tachibana et al. 2005; Molling et al. 2007).

2.1.2 Type II NKT Cells

The existence of CD1d-restricted T cells without expression of the invariant Vα14Jα18 TCR was first reported by Cardell et al. 1995; Cabaniols et al. 2001 and this NKT cell population is now called type II NKT cells (Godfrey et al. 2004). Distinct from type I NKT cells, type II NKT cells are defined as a heterogeneous population of CD1d-restricted NKT cells that lack Vα14Jα18. In fact although they express diverse TCRα chains, some subsets such as a Vα3.2Jα9 and Vα8 are

prominent (Park et al. 2001). Similar to type I NKT cells, type II NKT cells are a mixture of both $NK1.1^+$ and $NK1.1^-$ populations and produce both Th1 (interferon-γ) and Th2 (IL-4) cytokines upon the stimulation with CD1d antigen presentation (Chiu et al. 1999).

Since the antigens for type II NKT cells are not defined, what we know about these cells can only be inferred from studies comparing disease in $J\alpha18^{-/-}$ and $CD1d^{-/-}$ mice. At first it was seemingly paradoxical that NKT cells potentially suppressed tumor immunity (Moodycliffe et al. 2000; Terabe et al. 2000). The immunogenic 15–12RM fibrosarcoma grows-regresses-recurs in syngeneic BALB/c mice, however depletion of $CD4^+$ cells in vivo protected mice from tumor recurrence, indicating CD4 T cell-mediated suppression of CD8 T cell anti-tumor immunity (Matsui et al. 1997). Treg did not play a role (Terabe et al. 2005), but $CD1d^{-/-}$ mice were resistant to tumor recurrence, indicating that NKT cells might be the immune suppressors (Terabe et al. 2000). A complete study comparing the susceptibility of $J\alpha18^{-/-}$ and $CD1d^{-/-}$ in four different BALB/c tumor models indicated that $CD1d^{-/-}$ mice were resistant to tumor growth, whereas $J\alpha18^{-/-}$ mice behaved similarly to wild-type mice (Terabe et al. 2005). In the CT26 lung metastasis model, tumors seemed to grow faster at an early stage of tumor growth in $J\alpha18^{-/-}$ mice than in wild-type mice (Ambrosino et al. 2007). Collectively, these results suggested that type II NKT cells existing in wild-type and $J\alpha18^{-/-}$ mice were immune suppressive, whereas type I NKT cells lacking in $J\alpha18^{-/-}$ and $CD1d^{-/-}$ mice were tumor suppressive. Recently, a similar observation was reported in the BALB/c NS0 B cell lymphoma tumor (Renukaradhya et al. 2008). In addition, the anti-tumor effects of a combination of IL-12 and IL-18 against RENCA renal cell carcinoma (Subleski et al. 2006), and CpG oligodeoxynucleotide against B16 melanoma (Sfondrini et al. 2002), were increased in $CD1d^{-/-}$ mice compared with wild-type and $J\alpha18^{-/-}$ mice. These data are all consistent with an immunosuppressive role of type II NKT cells. Ambrosino et al. (2007) conducted a study in which they actively stimulated type I and type II NKT cells. Overall, it seemed that type II NKT cells suppressed tumor immunity, whereas type I NKT cells enhanced it (Terabe and Berzofsky 2007; Ambrosino et al. 2008; Berzofsky and Terabe 2008). Sulfatide was postulated as an activator of type II NKT cells in this work and although sulfatide is reportedly recognized by a fraction of type II NKT cells, this data remains controversial. The hypothesis that type II NKT cells are immune suppressive remains to be directly verified, since type II NKT cell-deficient mice do not yet exist and in the only studies comparing spontaneous tumors on both $J\alpha18^{-/-}$ and $CD1d^{-/-}$ backgrounds, no difference was observed in MCA-induced sarcomas and p53 mutant tumors (Swann et al. 2009).

2.2 Induction of Anti-Tumor NKT Cell Responses by Exogenous TCR Stimulus: A Focus on α-Galactosylceramide

NKT cells are activated in response to multiple stimuli – through their TCR, inhibitory and activation receptors shared with NK cells and some cytokine receptors. Activation of type I NKT cells results in TCR downregulation, proliferation and

prolonged cytokine secretion (Crowe et al. 2003; Wilson et al. 2003; Harada et al. 2004). In addition to the secretion of IL-4 and IFN-γ, activated iNKT cells have also been shown to produce IL-2, IL-3, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IL-21, TNF, TGFβ, GM-CSF, and as wide a range of chemokines (Coquet et al. 2008; Matsuda et al. 2008). It is becoming clear that the spectrum of T_H1- and T_H2-type cytokines that are produced by type I NKT cells is modulated by the strength of the type I NKT-cell TCR signaling events (Miyamoto et al. 2001; Schmieg et al. 2003; McCarthy et al. 2007) and by the type of APC presenting the type I NKT-cell agonists (Bezbradica et al. 2005; Schmieg et al. 2005; Yu et al. 2005).

The identity of endogenous glycolipids presented to CD1d-restricted T cells in the tumor setting remains controversial. However, the discovery of a synthetic glycolipid, α-GalCer, as a potent stimulator of Type I NKT cells has led to the realization that a variety of tumors can be controlled by appropriate manipulation of NKT cells. Since its discovery α-GalCer has proven to be an effective treatment against a broad spectrum of experimental and spontaneously-arising tumors (Nakagawa et al. 1998a, b; Hayakawa et al. 2003). The immunomodulatory activities of α-GalCer are vast and have been reviewed elsewhere (Hayakawa et al. 2004). Cytokines are central to the anti-tumor efficacy of α-GalCer. The initial IFN-γ release results in a cascade of events that begins with reciprocal activation and maturation of the presenting APC, most commonly a DC. The combination of DC maturation and IL-12 production and NKT cell-derived IFN-γ then leads to downstream induction of NK cell anti-tumor activity, as well as promoting the antigen-specific activities of CD8 and CD4 T cells. These interactions are bi-directional, since type I NKT cells receive signals from APC and vice versa. Signals are received through the TCR recognizing glycolipid–CD1d complexes, co-stimulatory receptors, such as CD40, CD27 and OX40 recognizing their ligands CD40L, CD70 and OX40L, respectively, as well as through cytokines. The anti-tumor activities of α-GalCer include, but are not exclusive to, an increase in cytotoxic potential via upregulation of effector molecules such as TRAIL (Smyth et al. 2001) and perforin (Smyth et al. 2002), which have been shown to be crucial to the anti-tumor effect of α-GalCer in some models. Production of IFN-γ itself can inhibit the growth of tumors by mechanisms which are not fully elucidated, but may involve inhibition of tumor angiogenesis (Hayakawa et al. 2002).

Although administration of soluble α-GalCer is effective, there are limitations with this approach. The window of opportunity, in which α-GalCer impacts on tumor growth, is narrow with single injection treatments. As a result, investigators have attempted multiple injections of α-GalCer. Despite the reported enhanced therapeutic efficacy with this approach, continual challenge with α-GalCer leads to deleterious effects including polarization of NKT cells towards Th2 cytokine production (Burdin et al. 1999) and long-term functional anergy (Fujii et al. 2002; Parekh et al. 2005; Uldrich et al. 2005).

It is reported that mice lacking DC fail to induce a normal α-GalCer-mediated NKT cell response (Bezbradica et al. 2005; Schmieg et al. 2005), indicating that DC are essential mediators in α-GalCer-induced anti-tumor immunity. In vitro studies have revealed that DC pulsed with α-GalCer are much more potent inducers of NKT cell activation than B cells, monocytes or macrophages (van der Vliet

et al. 2001b; Fujii et al. 2003c). Consistent with these observations, adoptive transfer of α-GalCer-pulsed DC is capable of inducing more potent anti-tumor effects than soluble α-GalCer injections, mainly by prolonging IFN-γ production by NKT cells and preventing the induction of NKT cell anergy (Toura et al. 1999; Fujii et al. 2002; Chang et al. 2005). These findings in mouse models have been largely supported by clinical trial data, which suggest that α-GalCer-pulsed autologous human DC are the most efficient mechanism of expanding and activating NKT cells in peripheral blood (Nieda et al. 2004; Chang et al. 2005; Ishikawa et al. 2005).

3 Harnessing NKT Cells for Enhanced Anti-Tumor Immunity: Recent Advances in Mouse Models (Summarized in Table 7.1)

3.1 α-GalCer as an Adjuvant in Tumor Vaccines

Since activation of NKT cells by α-GalCer aids DC maturation, immunization strategies to boost generation of tumor-specific T cells with concurrent administration of α-GalCer are currently under investigation. Initial studies showed that T cell priming following immunization with soluble or cell-associated ovalbumin (OVA) antigen was significantly enhanced with co-administration of α-GalCer (Fujii et al. 2003b; Hermans et al. 2003). This effect was demonstrated to be dependent on the ability of NKT cells to stimulate DC maturation and cross-presentation. Experiments with α-GalCer and peptide-pulsed DC showed that the effective adjuvant activity of α-GalCer was critically dependent on glycolipid and protein antigen being presented by the same APC (Hermans et al. 2003). A similar mechanism is speculated for soluble protein plus α-GalCer immunization, whereby simultaneous administration is required for effective enhancement of T cell responses (Fujii et al. 2003b; Hermans et al. 2003). Subsequent studies demonstrated that α-GalCer can also facilitate the induction of anti-tumor adaptive immune responses by vaccination with irradiated tumor cells. Vaccination with irradiated plasmacytoma or lymphoma cells failed to effectively prime anti-tumor immunity, but addition of α-GalCer to the vaccination resulted in potent tumor immunity mediated by both CD4 and CD8 T cells (Liu et al. 2005). In another study, adoptive transfer of bone-marrow derived DC co-pulsed with irradiated tumor and α-GalCer improved the priming of tumor-reactive CD8 T cells and conferred protection against tumor challenge. This activity was further enhanced by inactivation of Treg before DC transfer, using an anti-CD25 monoclonal antibody (Petersen et al. 2010). However, modulation of anti-tumor responses by NKT cells and Treg following antigen-loaded DC therapy appeared to work via independent mechanisms, as there was no evidence to suggest that NKT activation drove Treg proliferation, or that Treg suppressed NKT cell activation (Petersen et al. 2010).

Table 7.1 Summary of current strategies of harnessing NKT cell anti-tumor activity

Strategy	Benefits/advantages	Limitations/disadvantages
Soluble α-GalCer (single injection)	Simple Readily available	Not effective at low dose Toxic at high dose?
Soluble α-GalCer (multiple injections)	Enhanced therapeutic efficacy over single dose	Th2 cytokine polarization of NKT Functional anergy of NKT
α-GalCer-pulsed dendritic cells	Potent induction of NKT activation Prolongs NKT IFN-γ production Prevents NKT anergy	Requires inducing and expanding DC in vitro Time-consuming and resource heavy
α-GalCer-pulsed tumor cells	Tumor cells act as a source of tumor antigens in addition to vehicle for α-GalCer	Safety concern of administering tumor cells? – Cell irradiation dose critical
NKT cell adoptive transfer	Can administer large numbers of selected NKT cells Can expand and activate NKT away from suppressive influences of the host/tumor	Poor persistence in vivo Poor trafficking to lymphoid tissue or peripheral sites?
Agonistic CD1d-mAb based therapies	Can induce DC maturation in absence of functional NKT cells Can replace toxic mAbs (e.g. anti-CD40) in combination strategies	Might neutralize the activity of NKT cells May promote growth advantage of $CD1d^+$ neoplasms?

Interestingly, in addition to the capacity of NKT cells to promote the induction of an anti-tumor adaptive immune response, it has been reported recently that NKT cells can also enhance secondary $CD8^+$ and $CD4^+$ T cell responses in an experimental tumor setting. In a B16 melanoma re-challenge model in mice, it was observed that NKT cells could boost a recall CD8 T cell response and increase tumor rejection by enhancing both numbers and activity of tumor-specific $CD8^+$ T cells (Hong et al. 2009). This occurred under physiological conditions in the absence of exogenous NKT cell stimulus. In addition, administration of α-GalCer gave enhanced secondary responses in the setting of sub-optimal immunization and poor tumor rejection responses (Hong et al. 2009). In a follow-up study, NKT cells were shown also to enhance secondary antigen-specific $CD4^+$ T cell responses in an allergen-induced asthma model (Shin et al. 2010). It will be of interest to determine whether NKT cells can simultaneously enhance both memory $CD4^+$ and $CD8^+$ T cell activities in established tumor settings, potentially reducing the incidence of tumor metastases and patient relapse following surgical resection or other interventions.

NKT cell activation can also provide help to B cells, inducing B cell maturation, higher antibody titers and expansion of the B cell memory pool (Galli et al. 2003, 2007; Hermans et al. 2007; Silk et al. 2008). Targeting of NKT cell ligands to antigen-specific B cells can be achieved by using antigen and NKT cell agonists bound to particles, such as silica beads (Barral et al. 2008) or by synthesizing B cell antigens linked to α-GalCer (Leadbetter et al. 2008). NKT cells can facilitate B cell activation in response to particulate NKT-cell agonists, thus enhancing the

production of high titers of specific IgM and early class-switched antibodies. NKT cell-dependent induction of antigen-specific B- and T-cell responses is observed following the injection of α-GalCer through several routes, including intranasal (Ko et al. 2005; Kamijuku et al. 2008) and oral administration (Chung et al. 2004; Silk et al. 2004).

3.2 Tumor Cells as a Vehicle for α-GalCer Administration

The cumbersome nature of inducing and expanding DC from patients' peripheral blood monocytes for autologous α-GalCer-pulsed DC therapy has led to attempts of using irradiated tumor cells as a vehicle to deliver α-GalCer in vivo (Chung et al. 2007; Shimizu et al. 2007a, b; Petersen et al. 2010). This approach has the potential advantage that the tumor cells act as a source of tumor antigens in addition to delivering α-GalCer to DC. In a vaccination setting, administration of α-GalCer-pulsed B16 tumors conferred protection against the development of lung metastasis following intravenous injection of live B16 tumors (Shimizu et al. 2007a) and reduced the incidence of peripheral tumors after subcutaneous tumor inoculation (Shimizu et al. 2007b). In the lung metastasis model, innate immunity (NKT and NK cells) was sufficient for protection, however additional adaptive ($CD4^+$ and $CD8^+$ T cell) responses were required for protection against subcutaneous tumors. Although CD1d expression on the tumor cells increased the capacity for α-GalCer presentation and provided better protection, CD1d expression per se was not required for induction of anti-tumor activity. It is speculated that some initial tumor cell death, caused by irradiation of the cells or killing by NKT and NK cells, leads to release of α-GalCer and tumor antigens that are captured by DC. DC then process and present α-GalCer on CD1d to NKT cells and this cross-talk causes both IL-12 and IFN-γ production, which subsequently leads to further NK cell mobilization. Importantly, the maturing DC are then also able to induce the adaptive arm of the anti-tumor response by effectively priming $CD4^+$ and $CD8^+$ T cells (Shimizu et al. 2007b) (Fig. 7.1).

Administration of α-GalCer-pulsed tumor cells has not been efficacious in therapeutic settings when attempting to treat established tumors. The state of host immune responsiveness, especially that of NKT cells and DC, is critical for this approach and it is conceivable that immune modulation and immune suppression caused by factors released from the developing tumor may limit the effectiveness of this therapy in established tumors. An additional caution when developing this strategy for immunotherapy is the perceived safety concern of administering tumor cells into the patient. Cell irradiation techniques will need to be optimized for different tumor types to ensure that the cells are receiving sufficient dose to prevent tumor growth in the patient. However, preventing excessive irradiation is equally important as this will destroy the cells potentially along with their immunogenicity that may be induced by the immune sensitising effects of radiation (Demaria et al. 2005). Immune 'flagging' molecules induced by radiation may

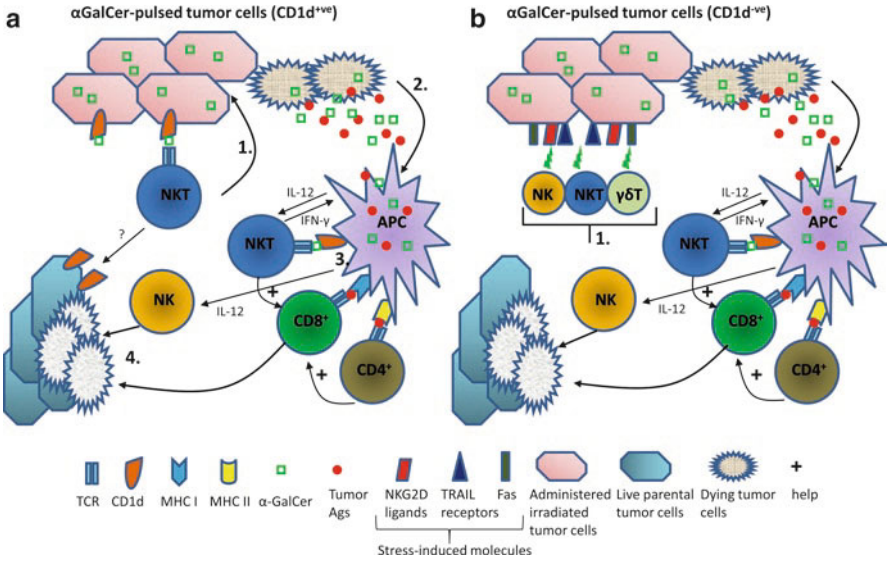

Fig. 7.1 Proposed mechanisms of innate and adaptive anti-tumor immunity elicited by administration of α-GalCer-pulsed tumor cells. (**a**) Pulsing of CD1d^{+ve} tumor cells allows direct tumor presentation of α-GalCer to type I NKT cells, resulting in CD1d-dependent, NKT cell-mediated cytotoxicity of the administered tumor cells (1). This initial tumor cell death causes release of both α-GalCer and tumor antigens which are captured by APC, such as DC (2). APC cross-present both α-GalCer on CD1d to NKT cells and tumor peptides on MHC class I to CD8 T cells. Peptides are also presented on MHC class II to CD4 T cells (3). The combination of NKT/APC cross-talk, DC maturation and help by co-activated CD4 T cells leads to potent generation and activation of tumor-specific CTL. IL-12 production from activated APC also leads to NK cell mobilization. The combination of innate (NKT cell, NK cell) and adaptive (CD8 T cell, CD4 T cell) anti-tumor activity causes destruction of the parental tumor (4). (**b**) CD1d^{-ve} tumors can be pulsed with α-GalCer, however elicitation of anti-tumor immunity upon administration is dependent on initial irradiation-induced cell death or up-regulation of stress molecules on the tumor cells. These stress-induced molecules "flag" to innate lymphocytes such as NK cells, NKT cells and γδ T cells, which can target and kill these tumor cells (1). Once this initial cell death occurs, α-GalCer and tumor antigens are released and the subsequent induction of adaptive immunity occurs in a similar fashion to CD1d^{+ve} tumors in *A*

prove critical for innate immune recognition of irradiated α-GalCer-pulsed tumor cells, particularly for CD1d-negative tumors where direct TCR recognition by NKT cells will not occur.

Another potential limitation is the availability of harvesting tumors cells from the patient, especially at early stages of disease. In this respect, the approach may be ideally suited for haematological malignancies, where a large number of tumor cells could be easily accessed from the blood or bone marrow. Patients with pre-malignant gammopathy or nonprogressive myeloma are obvious candidates for therapy with irradiated α-GalCer pulsed tumor cells based on evidence that NKT cell function is a negative prognostic factor in the development of multiple myeloma (Dhodapkar et al. 2003; Fujii et al. 2003a; Song et al. 2008).

3.3 Adoptive Transfer of NKT Cells

Vaccination strategies have an advantage over adoptive cell transfer methods in that they are generally more easily produced and made readily available for widespread administration. However, it is often difficult to elicit potent anti-tumor immune responses under suppressive influences of the tumor microenvironment or when patients are immunosuppressed as a result of their treatments. The challenge of inducing anti-tumor NKT cell activity in malignancies where it has been shown that NKT cell numbers or functions are impaired, may be overcome by expanding and activating these cells in vitro. Adoptive transfer of NKT cells into tumor-bearing mice, following expansion and functional elicitation in vitro by co-culture with DC plus α-GalCer, led to substantial anti-tumor effects (Molling et al. 2008). In addition, transfer of in vitro expanded human NKT cells in the presence of α-GalCer into mice harbouring CD1d-transfected neoplastic leukemic cells in a xenograft model, led to CD1d-dependent reduction in tumor mass (Bagnara et al. 2009). Two consecutive treatments of NKT cells and α-GalCer resulted in eradication of the tumor nodules in this model, implying that methods to prolong NKT cell persistence in vivo following adoptive transfer may be vital for effective anti-tumor therapy.

Adoptive transfer studies in NKT cell-deficient mice have also led to elucidation of NKT cell subsets that possess greatest anti-tumor activity. The ability of NKT cells to stimulate anti-tumor immunity upon transfer appears to be dependent on their organ of origin, since liver-derived NKT cells were capable of protecting $J\alpha18^{-/-}$ mice from the growth of MCA-1 sarcoma and B16-F10 melanoma (Crowe et al. 2002), whereas thymus and spleen-derived NKT cells were inefficient at stimulating tumor rejection (Crowe et al. 2005). Furthermore, $CD4^-$ liver-derived NKT cells were much more potent suppressors of tumor growth than their $CD4^+$ counterparts (Crowe et al. 2005), suggesting additional functional distinction of NKT cells within the same organ.

Although it may only be feasible to isolate and expand human NKT cells from peripheral blood, in vitro culture conditions have been optimized for maximal expansion, cytotoxic activity and IFN-γ production from peripheral blood human NKT cells, including from patients having reduced peripheral NKT cell numbers (Nishi et al. 2000; van der Vliet et al. 2001a; Lin et al. 2004; Mattarollo et al. 2006).

A clinical trial was performed using adoptively transferred type I NKT cells. Patients with non-small-cell lung cancer received two intravenous injections of up to 5×10^7 cells per m^2 in vitro-activated (with α-GalCer and IL-2) NKT cells (Motohashi et al. 2006). There were no severe adverse events in any of the patients, and in some cases increased frequencies of type I NKT cells were detected in blood samples post-immunization. Administration of activated type I NKT cells also induced IFN-γ production in vivo, mainly from NK cells. Nevertheless, none of the patients had any notable positive clinical response to this immunotherapy. In another study, eight patients received super-selective transcatheter intra-arterial infusion of activated Vα24 NKT cells into tumor-feeding arteries and nasal submucosal injections of α-GalCer-pulsed APC twice with a 1-week interval (Kunii et al. 2009).

The number of Vα24 NKT cells and interferon-gamma-producing cells in peripheral blood mononuclear cells increased in seven out of eight patients enrolled. Regarding the clinical responses, three cases exhibited a partial but significant response, four were classified as stable disease, and one patient continued to develop progressive disease.

The ability to administer large numbers of highly selected NKT cells as adoptive therapy has significant implications in combination therapies with conventional anti-cancer agents. This approach enables transfer of in vitro activated cells following administration of toxic or immune suppressive agents, such as chemotherapy and radiation, which would otherwise be detrimental to in vivo proliferating cells. Extensive evidence now exists for the tumor sensitising effects of chemotherapy and radiotherapy (Lake and Robinson 2005; van der Most et al. 2006; Haynes et al. 2008; Aymeric et al. 2010), to enable enhanced recognition and response from the immune system, including NKT cells (Mattarollo et al. 2006). Modification of the tumor microenvironment by these agents may also enhance trafficking of adoptively transferred NKT cells to the tumor site, an important characteristic of transferred effector cells, but lacking in many T cell adoptive therapy attempts. Therefore, combination treatment involving chemotherapy or radiotherapy followed by adoptive transfer of in vitro activated, autologous NKT cells may warrant further investigation.

3.4 CD1d Monoclonal Antibody-Based Therapy

The success of combination monoclonal antibody (mAb)-based anti-tumor therapy, including a "TriMab" combination of three mAbs (anti-DR5, anti-CD40 and anti-CD137) which induced rejection of various established experimental and carcinogen-induced tumors in mice (Uno et al. 2006), has led to development of combination mAb therapies which includes targeting of CD1d molecules and/or the activation of NKT cells (Teng et al. 2007, 2009a). Since agonists of CD40 are found to be toxic in patients (Vonderheide et al. 2001, 2007), it was replaced with glycolipid ligands for NKT cells as a substitute method to mature DC. These ligands were an effective replacement for anti-CD40 mAb and in combination with anti-DR5 and anti-CD137, termed NKTMab, rejected established mouse breast and renal tumors (Teng et al. 2007). NKTMab anti-tumor activity was dependent on CD4 and CD8 T cells, NKT cells and IFN-γ. Different α-GalCer glycolipid analogs given at high concentrations gave similar rates of tumor rejection, however toxicity was associated with high concentrations of α-GalCer, but not alpha-C-galactosylceramide (α-C-GalCer) (Teng et al. 2007).

Reduced numbers and defective function of NKT cells in tumor-bearing mice and advanced cancer patients suggest that poor host NKT cell responsiveness might limit the success of the NKTMab approach. Therefore, bypassing NKT cell activation, by specifically targeting CD1d molecules on DC using an agonistic anti-CD1d mAb, was attempted for immunotherapy of established subcutaneous tumors. Anti-mouse

CD1d mAb demonstrated modest anti-tumor activity by means of inducing specific release of IL-12 from CD1d-positive APC and an increase in serum levels of IL-12, IFN-γ and IFN-α (Teng et al. 2009b). To determine whether these properties of anti-CD1d mAb were beneficial in combination therapy, a subsequent study determined the anti-tumor efficacy of anti-CD1d mAb in combination with anti-DR5 and anti-CD137 (termed 1DMab) in three different established subcutaneous tumor models; R331 renal carcinoma, 4T1 mammary carcinoma and CT26L5 colon adenocarcinoma (Teng et al. 2009a). Similar to glycolipid ligand substitution of anti-CD40 mAb in NKTMab (Teng et al. 2007), anti-CD1d mAb effectively induced rejection of established tumors in 1DMab combination therapy. In addition, 1DMab therapy was specifically more efficacious than TriMab therapy in the eradication of 4T1 and CT26L5 tumors (Teng et al. 2009a). 1DMab-induced tumor rejection was completely dependent on CD8 T cells, IFN-γ and CD1d, and partially dependent on NK cells and IL-12. Although type I NKT cells did not appear critical in the anti-tumor activity of 1DMab therapy, it is not clear whether anti-CD1d mAbs might also neutralize the activity of type I NKT cells. It will be interesting to assess whether the anti-CD1d mAb clone (1B1) used in these studies can inhibit or potentiate glycolipid ligand-mediated anti-tumor activity in the same tumor models. The experimental tumor models used – R331, CT26 and 4T1 were by design CD1d negative. However, a large proportion of hematopoietic tumors in mice and humans do express CD1d. It is currently unknown, but conceivable, that the ability of anti-CD1d mAb to stimulate CD1d itself may be undesirable if it promotes a growth advantage for CD1d-positive neoplasms. Therefore, further studies to investigate the efficacy of 1DMab against hematopoietic tumors that express CD1d are required to determine the feasibility of anti-CD1d mAb-based therapies in different tumor settings.

4 Conclusion

In the absence of more than just correlative studies of NKT cells and patient outcome or disease progression in humans, there is clearly a need for more studies determining the role of NKT cells in spontaneous and carcinogen-induced tumors in mice. Thus far, in just the few models examined, it would appear the type I NKT cell is a key host protective mechanism from tumor development. Whether type II NKT cells have any effects in these same mouse models of cancer is yet to be demonstrated. Indeed, the definition of type II NKT cells requires far more progress, before the role of these cells can be satisfactorily addressed. At present, only a simple comparison between tumor growth on Jα18-deficient and CD1d-deficient backgrounds has been possible.

Since the discovery of type I NKT cells over 20 years ago, it has become clear that type I NKT cells have extremely versatile responses to activation. It is clear that α-GalCer and similar agonist CD1d ligands are powerful adjuvants for driving both cell-mediated and antibody responses in mice. Activated NKT cells can positively modulate DC and B cells, and their pharmacological activation in the presence of

antigenic proteins can enhance antigen-specific B- and T-cell responses. Current vaccination strategies are generally failing to elicit T-cell responses that are similar to those generated by natural infections. The challenge remains to understand how to activate and manipulate the different subpopulations of type I NKT cells and how to translate these results into the clinical setting to control the presentation of NKT-cell agonists by different APCs in different microenvironments both quantitatively and qualitatively.

Although numerous synthetic CD1d-binding ligands have been described, it is important to ensure that the type I NKT-cell agonists used in the clinic can be repeatedly injected into patients without inducing type I NKT-cell over-activation, resulting in a type I NKT-cell anergy or cytokine storm. This may be possible by identifying type I NKT-cell agonists that have a reduced affinity for the type I NKT-cell TCR and at the same time ensure optimal DC maturation. The next generation of type I NKT-cell agonists should be capable of targeting specific APC populations to ensure preferential binding to DCs and/or antigen-specific B cells. To date, no clinical trials have been carried out in which type I NKT-cell agonists are co-injected with subunit vaccines and only one clinical trial has looked indirectly at the ability of α-GalCer to enhance the response of the adaptive immune cells in the clinic. Clinical trials should be carried out to assess whether results that have been obtained in mouse models, which show that type I NKT-cell activation can enhance antigen-specific B- and T-cell responses, can be extended to humans, that have a lower and far more heterogeneous composition of type I NKT cells.

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Chapter 8
NKT Cells of Cancer Patients and How Models Can Inform Therapeutic Plans

Mark A. Exley, Lydia Lynch, and Michael Nowak

Abstract Multiple immune cells contribute to antitumor activity. Adaptive immune cells have specificity and amplified responses to repeated antigen, but require targeting to tumor to avoid excessive autoimmune effects. Innate elements can directly kill tumor cells, have specificity and even a form of memory, and can recruit adaptive responses, but have a finite spectrum of targeting ligands. Invariant natural killer T cells (iNKT) are a subset of innate-like CD1d-restricted T cells with roles in regulating immunity, including immune surveillance against certain pathogens and tumors. iNKT recognize lipid antigens presented by monomorphic MHC-like CD1d expressed by dendritic cells (DC) and other antigen presenting cells (APC) as well as many hemopoietic, prostate, and some other solid tissues and corresponding tumors. Studies of human cancer have revealed frequent and selective numerical and functional defects in iNKT, which correlate with progression, but are reversible in vitro (at least). iNKT can contribute to physiological antitumor responses in animal models. iNKT activation with αGalCer (as originally identified) and newer analogs promotes model tumor rejection and protection from metastasis. However, CD1d-restricted either (either "noninvariant" or iNKT) NKT can also suppress antitumor responses through regulatory cytokine(s). Defects of iNKT appear to be mediated by both intrinsic causes and by impaired stimulatory capacity of DC present in tumor microenvironment (TME). iNKT have great therapeutic potential.

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Although this potential has been limited to date, apparently by such iNKT defects, combinations such as αGalCer with IL-12 can reverse model defects. This review summarizes progress, pitfalls, and new opportunities to exploitation of NKT cells in cancer therapy.

1 CD1d-Restricted T Cell Subsets: Prototypic (Type I) iNKT and "Noninvariant" Type II NKT Cells

iNKT cells express invariant TCR Vα14-Jα18 in mice (originally called Vα14-Jα281; human Vα24-Jα18, originally called Vα24-JαQ) and have preferred Vβ usage (Vβ2, −7, and −8 in mice and rats, Vβ11 in humans and nonhuman primates). iNKT are restricted by the nonpolymorphic MHC class 1-like CD1d glycoprotein (Bendelac et al. 2007; Matsuda et al. 2008). CD1d presents exogenous CD1d ligands, including analogs of the prototypic high-affinity lipid alpha-galactosylceramide (αGalCer) from pathogenic as well as nonpathogenic microorganisms (Parekh et al. 2005; Kronenberg and Kinjo 2009; Salio et al. 2010; Venkataswamy and Porcelli 2010). The identity of physiological endogenous ligands that can also mediate CD1d-dependent iNKT cell activation remains supposed, but ambiguous (Kronenberg 2005; Godfrey et al. 2006). iNKT can also be activated by cytokine combinations (Reilly et al. 2010). iNKT in turn rapidly secrete large amounts of cytokines upon activation, thereby impacting antitumor and other immune responses (Bendelac et al. 2007; Kronenberg and Kinjo 2009). These products include both regulatory factors (e.g., IL-4, IL-10, IL-13) as well as pro-inflammatory agents such as IL-2, IL-17, TNFα, CCL3 (MIP1α), and IFNγ, reflecting their capacity to suppress or stimulate Th1, Th2, Th17, and other immune responses. iNKT are often referred to as "Type I" CD1d-restricted T cells (as distinct from Th1-biased NKT) (Godfrey and Kronenberg 2004). Identification of iNKT is described in detail in other chapters, but use of CD1d multimers loaded with higher affinity ligands such as α-GalCer (Gumperz et al. 2002; Lee et al. 2002) or anti-iNKT TCR mAb 6B11 (Exley et al. 2008) is widely accepted standard, whereas even combinations of other mAbs such as Vα24 and Vβ11 both miss some iNKT (i.e., Vβ11-negative iNKT; Exley et al. 1997) and can detect non-Jα18$^+$ T cells also using Vα24 (Berzins et al. 2011). While these differences may appear subtle, the variable and often extremely low levels of true iNKT make even small errors in values of high significance, as well as making data uncomparable cross studies.

Many human T cells express NK cell markers such as CD56 and especially CD161. Most of these, however, are not CD1d restricted and are typically CD8$^+$ MHC class 1-restricted recently activated or memory-type T cells. Other polyclonal "noninvariant" functionally CD1d-restricted T cells predominate in some organs (Godfrey and Kronenberg 2004; Arrenberg et al. 2009), and indeed need not express NK markers (Durante-Mangoni et al. 2004). These diverse polyclonal CD1d-reactive T cells are often referred to as "Type II" NKT cells (Godfrey and Kronenberg 2004). Depending on organ and health or disease, noninvariant CD1d-restricted T cells can be Th1, Th2, or Th0 in cytokine bias (Exley and Koziel 2004; Arrenberg et al. 2009; see below).

2 Contribution of CD1d-Restricted T Cells to Prototypic Th1 Immune Responses

Considerable data has shown that despite the initial interest in iNKT for rapid IL-4 production in Th2 responses, a major role of iNKT is to amplify Th1 responses in concert with dendritic cells (DC) and other CD1d$^+$ myeloid antigen presenting cells (APC) (Hegde et al. 2010). iNKT IFNγ helps to stimulate APC maturation and IL-12 production, which in turn augment iNKT IFNγ (Tomura et al. 1999; Kitamura et al. 1999; Godfrey and Kronenberg 2004; Fujii et al. 2007). This iNKT:APC Th1-biased amplification loop helps to explain the contribution of CD1d-restricted T cells, in general, and iNKT, in particular, to immunity against a wide range of mostly (but not exclusively) intracellular pathogens (reviewed in Behar and Porcelli 2007; Cohen et al. 2009; Balato et al. 2009). Indeed, iNKT-induced Th1 responses are critical in immunity against a number of viruses (Exley and Koziel 2004; Behar and Porcelli 2007; Cohen et al. 2009; Tessmer et al. 2009), although they can also have regulatory roles in infections (Diana and Lehuen 2009). The contribution of iNKT cells to immune surveillance is at least partly based on their capacity to mature DC and subsequently activate NK cells via IL-12/IFNγ to become potent cytotoxic cells and to enhance overall IFNγ and both B and T cell immunity (Fujii et al. 2007; Galli et al. 2007; Lang 2009). Upon recognition of CD1d:lipid complexes and costimulatory molecules such as CD80/86 on the surface of DCs, iNKT cells up-regulate IL-12R and CD40 molecules. Subsequently, and mediated by CD40L, iNKT then induce maturation and production of IL-12 in DCs. This IL-12 release in turn potently increases IFNγ production by iNKT (Godfrey and Kronenberg 2004; Fujii et al. 2007; Cohen et al. 2009). As detailed later and in other chapters, analogous results demonstrate that iNKT stimulation of similar Th1-type immunity also contributes to protective antitumor responses.

3 CD1d-Restricted T Cell Populations in Cancer Models

As described in detail in other chapters in this volume, numerous studies have shown that CD1d-restricted T cell subsets can contribute to model physiological antitumor responses and iNKT ligand α-GalCer and analogs have been shown to inhibit tumor growth and metastases in experimental tumors (Swann et al. 2007; Zaini et al. 2007; Berzofsky and Terabe 2009; Taniguchi et al. 2010; Exley et al. 2011; O'Konek et al. 2011). iNKT can exert their antitumoral effects indirectly through IFNγ and by induction of APC IL-12 leading to activation of NK cells as well as other T cells. Human as well as rodent CD1d-restricted T cell subsets also possess potent direct CD1d-dependent and possibly LAK-like cytotoxicity via classic granule-mediated mechanisms (Exley et al. 1998; Nicol et al. 2000; Metelitsa et al. 2001; Konishi et al. 2004) as well as TNF family members (e.g., TNFα, FasLigand, and TRAIL; Kawano et al. 1998).

Importantly, however tumors can suppress iNKT and antitumor activity can be mediated through regulatory activity such as production of IL-13 and/or TGF-beta by some iNKT and Type II CD1d-restricted T cells (Terabe et al. 2003; op cit 2005; op cit 2006; Yanagisawa et al. 2006; Park et al. 2008; Renukaradhya et al. 2008; ang et al. 2011). Therefore, as with other immune elements, the presence of iNKT or other CD1d-restricted T cells should not be taken as evidence of antitumor response in the absence of functional data. Furthermore, results from mice given tumor cell lines can be misleading compared to tumor-bearing models such as transgenic-based cancers.

4 CD1d-Restricted T Cell Populations of Cancer Patients

Because they have the invariant TCR and can therefore be directly identified, iNKT are by far the best-studied CD1d-restricted T cell population in human health and disease, including in cancer. Available results for Type II CD1d-restricted T cells are noted at the end of this and the clinical trials sections.

4.1 Specific Quantitative Defects in Cancer Patient iNKT

Numerous studies have described iNKT from cancer patients, chiefly although not exclusively, from peripheral blood. Statistically significant population-wide decreases in circulating iNKT cell numbers in multiple types and stages of cancer have been described in many but not all cases, including advanced prostate cancer, melanoma, colon, lung, and breast cancers, head and neck squamous cell carcinoma (HNSCC), myelodysplastic syndromes (MDS), and progressive multiple myeloma (MM) (Tahir et al. 2001; Dhodapkar et al. 2003; Fujii et al. 2003a; Crough et al. 2004; Molling et al. 2005; op cit 2007). This mirrors the situation in a wide range of inflammatory conditions, in which iNKT are also generally reduced (van der Vliet et al. 2001). However, there is considerable variation in healthy person circulating iNKT fraction "set points" as well as overlap between proportions of healthy and diseased group iNKT levels, so these might appear to not therefore be of direct diagnostic value. Interestingly, however, healthy individuals seem to maintain their set points over some time (Lee et al. 2002) before eventually declining with age (Crough et al. 2004; Molling et al. 2005), so once established in a given individual, there is the potential for observation of consistent changes to be informative, as indeed found for cancer patients (Molling et al. 2007). Of course, reports giving only % iNKT may also miss changes or find them when no change in absolute numbers of iNKT has actually occurred, so care should be used in cases where only fractions are presented.

4.2 Selective Qualitative Defects in Cancer Patient iNKT

Of greater interest than modest quantitative defects, decreased iNKT cell numbers are frequently accompanied by functional defects. In particular, selectively decreased IFNγ production (Tahir et al. 2001; Dhodapkar et al. 2003; Fujii et al. 2003a; Molling et al. 2005; van der Vliet et al. 2008) of iNKT cells have been found in advanced prostate cancer, progressive MM, MDS, and other cancer patients, resulting in a net detrimental type 2 *cytokine* profile of residual iNKT cells from such advanced cancer patients. Proliferative potential of iNKT can also be impaired (Tahir et al. 2001; Yanagisawa et al. 2002; Konishi et al. 2004). Importantly, these defects are relatively iNKT specific, since net conventional T cell responses from the same patients were normal (Tahir et al. 2001; Dhodapkar et al. 2003).

Interestingly, both IFNγ production and proliferative defects are reversible in vitro (Tahir et al. 2001; Yanagisawa et al. 2002; van der Vliet et al. 2003), and therefore potentially also clinically. For example, IL-2 reverses the iNKT proliferative defect (Tahir et al. 2001; Yanagisawa et al. 2002), similarly to how IL-2 can overcome some forms of T cell anergy. Furthermore, IL-12 can reverse the block in IFNγ production in response to αGalCer in vitro (Tahir et al. 2001), reflecting the in vivo block and also reversibility that we subsequently identified in a tumor model of the same type of cancer, prostate cancer (Nowak et al. 2010b; see below).

Not all tumors or stages are associated with defects in iNKT. For example, no such defect was observed in glioma subjects by the same group who identified iNKT-specific IFNγ production defects in MM and MDS (Dhodapkar et al. 2004). Similarly, melanoma and lung cancer patient iNKT did not appear to be defective in IFNγ production (Kawano et al. 1999; Motohashi et al. 2002). Interestingly, like on their normal counterparts, CD1d can be expressed not only by a range of many hemopoietic and some other tumors (Zheng et al. 2002; Metelitsa et al. 2003; Fais et al. 2005; Nebozhyn et al. 2006; Kenna et al. 2007; Metelitsa 2011), but also murine and human prostate tumors and primary epithelium (Nowak et al. 2010b). Therefore, functional iNKT cancer defects may partially reflect expression of CD1d on tumor, although also on infiltrating immune cells and potentially stroma in various tumors and stages (Metelitsa 2011), as well as in response to some therapies.

A hypothesis consistent with a central role for interactions with $CD1d^+$ APCs in these iNKT-specific defects is that iNKT traffic to tumors and encounter tumor-induced tolerogenic DCs (or other tumor infiltrating APC-like cells, such as myeloid suppressor cells or macrophages), which fail to stimulate or more actively suppress/deviate iNKT (e.g., Gabrilovich 2004). Alternatively as above, and not mutually exclusively, myeloma cells (Chang et al. 2005; op cit 2008) or other especially hematological tumor cells, which can express CD1d like their normal counterparts, could suppress iNKT cell function as nonprofessional APC (Nowak et al. 2010a). For example, we have found prostate tumors and epithelium can express CD1d (Nowak et al. 2010a) and other nonhematological tumors can also do so (Metelitsa 2011), although this is unlikely to be the case for many solid

tumor types. Therefore, although some tumors express CD1d and thus might directly be recognized by iNKT, the majority of human and model solid tumors have been assumed to be CD1d-negative. Significantly, while tumor CD1d effects on iNKT could be via tumor tolerogenic APC cross-presentation of tumor lipids (Wu et al. 2003; Chang et al. 2008a, b), iNKT:tumor interactions can also directly induce Th2 bias in vitro (Nowak et al. 2010a). Functional consequences of CD1d on model tumor cells and impact on local and therefore eventually systemic iNKT are further described later. Finally, iNKT cells may also be directly suppressed by soluble or matrix-bound factors such as TGF-beta produced in the tumor microenvironment (TME). Indeed, it maybe that biasing may turn iNKT from friends into foes of antitumor immunity in these and other ways not yet described. Sampling iNKT at various stages within the tumor itself and draining lymph nodes, where clinically justifiable (e.g., resections), is clearly important to fully understand the roles of iNKT in human cancers.

4.3 Cancer Patient Organ iNKT Cells

For practical reasons, most studies utilize patient blood iNKT and therefore address systemic effects. However, human iNKT associated with tumors have been studied in some cases directly from tumor resected tissue ex vivo. Results to date show that tissue and tumor-infiltrating lymphocyte (TIL) iNKT and other CD1d-reactive "NKT"-type cells are distinct and can be enriched relative to matched blood (Kenna et al. 2003; Bricard et al. 2009; Lynch et al. 2009). Hepatic iNKT cytokine responses were Th1 biased in hepatic primary and metastatic tumor tissue (Kenna et al. 2003; Lynch et al. 2009), unlike the equivalent cells in at least normal mouse liver, which produce Th2 as well as Th1 cytokines (Kronenberg 2005; Bendelac et al. 2007). This human hepatic NKT bias is consistent with studies of iNKT and total human hepatic CD1d-restricted T cells in hepatitis and healthy liver, which are apparently mostly Type II NKT cells and show a notable Th1 bias (Exley et al. 2002; Durante-Mangoni et al. 2004; Kenna et al. 2007), except in advanced fibrosis/cirrhosis (de Lalla et al. 2004; Durante-Mangoni et al. 2004). Interestingly, however, hepatic tumor TIL can be enriched for $CD4^+$ iNKT (Bricard et al. 2009), which at least from healthy donors in vitro produce higher levels of regulatory/Th2 cytokines (Lee et al. 2002; Gumperz et al. 2002; Bricard et al. 2009). Tumor iNKT can also kill autologous tumor cells ex vivo even in the absence of exogenous ligand such as αGalCer (Lynch et al. 2009). This activity appears predominantly CD1d-dependent (Lynch et al. 2009), like healthy person and patient iNKT with model targets (Exley et al. 1998; Nicol et al. 2000; Metelitsa 2011), and could contribute to antitumor activity in individuals with $CD1d^+$ tumors and who do not progress. iNKT can be depleted from some tissues in cancer patients, in some cases apparently being replaced by Type II "noninvariant" NKT (Kenna et al. 2007). Where a Th1-bias is maintained by Type II NKT, this may still enhance antitumor responses.

4.4 Summary: Cancer Patient CD1d-Restricted T Cells

In vivo results from tumor models, in vitro and limited ex vivo patient results implicate CD1d-restricted T cell populations in antitumor activity. Finally, there is also the remarkable finding that iNKT and other peripheral blood CD1d-reactive NKT-type cell populations specifically capable of producing IFNγ in vitro are strongly and selectively associated with improved prognosis in patients with glioma (Metelitsa 2011), colon cancer, HNSCC, and hematological malignancies (Tahir et al. 2001; Molling et al. 2007; Shaulov et al. 2008; de Lalla et al. 2011). Therefore, even systemic CD1d-restricted T cell subsets can reflect responses relevant to the tumor, although whether this reflects a causative rather than effect relationship remains to be determined. Association of CD1d-restricted T cell Th1 responses with good prognosis combined with the other results earlier has justified and helped in the immunomonitoring of a number of clinical trials exploiting the NKT system. Further details of these trials are provided later, as well as in other chapters in this volume.

5 iNKT and Other CD1d-Restricted T Cell-Related Clinical Trials

The observations of frequent functional but reversible iNKT defects in human cancers, in conjunction with the potent antitumor effects of αGalCer in mice, have triggered several phase I clinical studies in advanced-stage cancer patients. Other chapters describe several of these in detail. Of note, although αGalCer is highly specific and apparently produces no effect in mice lacking just iNKT and other Jα18^{+} T cells, there is a report that it can induce an innate-type cell response, inducing type-1 IFN (Mehta et al. 2004), which could conceivably enhance (or complicate) human therapeutic use.

Briefly, in the first clinical phase I study, the iNKT cell ligand αGalCer was intravenously administered to advanced cancer patients (Giaccone et al. 2002; Schneiders et al. 2010). In this study, circulating iNKT cells were decreased pretreatment in most of the cancer patients, and immunological responses to αGalCer administration (increases in GM-CSF and TNFα levels) were only observed in patients with normal or above-normal iNKT cell numbers. No signs of dose limiting toxicity were reported in this trial and the maximum tolerated dose could not be established with the available formulation. In the second reported trial, Nieda et al. studied the effects of adoptive transfer of purified αGalCer-pulsed immature monocyte-derived DC. There were only minor systemic side effects in 9 of 12 patients, including fever, malaise, lethargy, and headache, which were temporally related to immunologic responses (Nieda et al. 2004). The majority of patients experienced temporary exacerbation of tumor symptoms, many of which were clearly inflammatory (e.g., tender enlargement of palpable tumor deposits or involved lymph nodes, bone pain, respiratory

symptoms in subjects with pulmonary metastases) and interpreted as inflammatory responses to the tumor because they also had a strong temporal relationship to study therapy, were reproducible in terms of timing and nature with subsequent treatment episodes, were transient (generally lasting only 1–3 days), and did not occur outside the study period. In four patients, sustained decreases in tumor markers were observed, and in one patient extensive tumor necrosis was noted. This study showed reproducible immunological responses with activation of iNKT cells, subsequent activation of T- and NK-cells, enhanced NK cell cytotoxicity, and increased serum levels of IL-12 and IFN-γ, as also seen in many murine model therapeutic studies.

Ishikawa et al. investigated adoptive transfer of autologous cell preparations of αGalCer-pulsed PBMC in patients with recurrent lung cancer or advanced nonsmall cell lung cancer (Ishikawa et al. 2005). No serious adverse events were reported, and several patients had an increase in the circulating number of iNKT cells. Notably again, immunological responses were restricted to patients having "normal" pre-treatment iNKT cell numbers. Although no partial or complete clinical responses were noted, 2 patients out of 11 had stable disease for over 1 year. Chang et al. similarly evaluated the effects of intravenous administration of purified αGalCer-pulsed mature monocyte-derived DC in five patients with hematological malignancies and observed more than 100-fold expansion of circulating iNKT cell numbers in all original five patients (Chang et al. 2005; Dhodapkar and Richter 2011). This expansion was sustained for up to 6 months postvaccination and an increase in adaptive T cell immunity was suggested by an increase in viral antigen-specific memory $CD8^+$ T cells. In this study, no more than grade 1 toxicity was observed, although one patient developed rheumatoid factor and transient positive antinuclear antibody at follow-up. More recently, Motohashi et al. reported on clinical trials of cell preparations enriched for αGalCer-activated iNKT cells and/or αGalcer-loaded adherent matured PBMC in patients with advanced nonsmall cell lung cancer or HNSCC (Motohashi et al. 2006; op cit 2009; op cit 2011). iNKT cells were activated and expanded in vitro among PBMC using αGalCer, and patients were treated with up to tens of millions of iNKT equivalents/M^2, with no reported toxicity. Transient increases in iNKT in peripheral blood were found at the higher doses, and there were increases in the number of IFNγ-producing cells in vitro after αGalCer stimulation, although systemic immune activation was not noted.

Overall, the studies in mice and these small phase I clinical trials using αGalCer are promising and support continued efforts to further enhance iNKT cell function in cancer patients. The therapeutic approach we are involved with is similarly adoptive transfer of ex vivo expanded autologous iNKT cells to restore iNKT cell numbers and function. Highly enriched iNKT cells (median ~50% pure) are isolated after leukapheresis using a monoclonal antibody that we developed, which specifically recognizes the invariant TCRα of iNKT cells, and in vitro expansion with anti-CD3 (Tahir et al. 2001; Exley et al. 2008). This permits infusion of large numbers (up to $>10^8$ iNKT equivalents/infusion) of relatively pure in vitro-expanded and well-characterized iNKT cells, and after initial safety testing ($n=3$), subsequent patients also receive GM-CSF on second and third cycles of iNKT infusions to potentially mobilize and enhance DC activation. This ongoing study will determine

the feasibility and safety profile of this treatment, with intensive phenotypic and functional immune monitoring to gain further insight into the therapeutic potential of this approach in humans. Our working hypothesis is that a central function of iNKT cells is to traffic into peripheral tissues and regulate the activation and maturation of immature DCs, which can then migrate to lymph nodes and initiate immune responses as mature DCs. This is being directly tested through manipulations in mouse models, with implications for immune monitoring of the ongoing iNKT clinical trial and for the design of subsequent combination clinical trials.

Further NKT-related clinical trials have been reported. Not all directly target iNKT cells or use αGalCer. NKT, in general, are resistant cells, which can also recover faster than other T cells from potent stimulations and/or insults, such as pharmacological doses of IL-12, anti-CD3 mAb, steroids, hemopoietic stem cell transplantation (HSCT), etc. (Kohrt et al. 2010; Beziat et al. 2010). Noninvariant NKT, in particular, can have suppressive effects on graft-versus-host disease ("GvHD") and yet maintain Graft-versus-Leukemia (GvL) activity (Kohrt et al. 2010). Nonmyeloablative HSCT regimens with protective effects dependent upon NKT populations and which minimize acute GvHD have been developed in animal models. Interestingly, some of the related human trials concomitantly increase "NKT"-type T cells (Lowsky et al. 2005; Kohrt et al. 2009) and in some models have been shown to augment and indeed depend upon iNKT cells themselves (Lan et al. 2003; Morris et al. 2005; Pillai et al. 2007; Kohrt et al. 2010; Saito et al. 2010). Conversely, however, iNKT DC activation and IL-12 production downstream of αGalCer can exacerbate GvHD (Kuns et al. 2009; Morris et al. 2009).

6 Future iNKT Adjuvant and Other Clinical Trials

As described in other chapters, numerous distinct ligands for iNKT have been described. While the first generation of these were either inactive on human iNKT or had major solubility or stability issues, second and third generation analogs show more promise for specific types of response and application in appropriate diseases. Most seem to induce a Th2 iNKT bias likely not ideal in cancer. However, a recent publication describes a beta-mannosylceramide which is antitumor in models, like αGalCer, but via an apparently IFN-γ-independent mechanism more reminiscent of other nitric oxide-related iNKT effects in tumor-bearing mice, active with human iNKT, and synergistic with αGalCer (O'Konek et al. 2011).

Finally, a compelling case has been made for adjuvant activities of iNKT cells for both cellular and antibody-dependent immunity (Fujii et al. 2003b, 2007; Hermans et al. 2003; Cerundolo et al. 2009; Padte et al. 2010). These results lead to the conclusion that iNKT simulation could be synergistic with many types of vaccines, including those directed against cancer. Indeed, iNKT are essential for GM-CSF-based vaccine efficacy in mice (Gillessen et al. 2003). Interestingly, similar results have been published for CpG vaccine iNKT dependency and iNKT:DC interactions enhanced by CpGs (Hafner et al. 2001; Montoya et al. 2006; Paget et al. 2009),

although this can be complicated by Th2-promoting activity attributed to Type II noninvariant CD1d-reactive T cells (Sfondrini et al. 2002). Furthermore, iNKT interact with TLR ligand signals and are likely to be synergistic with these and certain other classes of vaccine adjuvants. iNKT contribute to Th1-biased responses to several TLR ligands through interactions with DC (Paget et al. 2007). Several groups are now moving toward trials testing iNKT stimulation along with various types of vaccines against cancer or infections.

7 Impaired iNKT Cells and Their Cytokine Responses in Tumor-Bearing Individuals: Learning from Models

Clinical cancer and mouse models can display similar quantitative and/or qualitative defects of iNKT cells in advanced cancer and the believed basis for their roles in antitumor immune responses, as well as in the activity of cancer vaccines (reviewed Swann et al. 2007; Berzofsky and Terabe 2009; Taniguchi et al. 2010; Dhodapkar and Richter 2011; Exley et al. 2011). Figure 8.1 shows a model for progressive defects in iNKT Th1 responses of tumor-bearing individuals. In particular, the Tramp mice model has similar functional iNKT defects (Nowak et al. 2010a) to advanced prostate cancer patients (Tahir et al. 2001).

Cancer-bearing mice can exhibit numerical and/or functional iNKT cell defects comparable to those reported in cancer patients (see above). For example, tumor bearing mice are relatively refractory to αGalCer therapy compared to recently transplanted individuals. This has been traced to $CD11b^{+}Gr\text{-}1^{+}$ "myeloid suppressor"-like cells, which did so in a nitric oxide-dependent fashion, but which could be reversed by retinoic acid (Yanagisawa et al. 2002). We also evaluated iNKT in the MDR2 KO model (Popov et al. 2005) of fibrosis/cirrhosis leading to liver cancer (Fig. 8.2). iNKT were largely and selectively depleted in advanced fibrotic/early cirrhotic livers when microscopic liver cancer is beginning (8 months old). Despite the tendency toward larger livers of cirrhotic mice, total lymphocyte and T cell fractions were very similar. Since iNKT levels do not rebound in other cancer models, this suggests that endogenous iNKT loose the "battle" with cirrhosis/early liver cancer in this model.

In contrast to humans, where relatively large cohorts of cancer patients have been compared to controls (see above), TRAMP mice exhibit iNKT frequencies comparable to age-matched WT mice. iNKT cell frequencies from spleens and livers of TRAMP mice showed age-dependent increases in liver and splenic iNKT cell frequencies (Nowak et al. 2010a), as in normal mice (cf. Faunce et al. 2005).

TRAMP mice injected with exogenous CD1d ligand αGalCer to activate iNKT cells systemically, however, produce relatively reduced levels of IFNγ upon αGalCer administration and IL-12 is undetectable compared to normal mice (cf. Parekh et al. 2005; Taniguchi et al. 2010; Reilly et al. 2010; Nowak et al. 2010a). Collectively, these results show variable but pronounced defects in composition of iNKT and/or the cytokine production of both DC and iNKT cells in tumor-bearing mice. The cytokine defect predominantly involved Th1 cytokines.

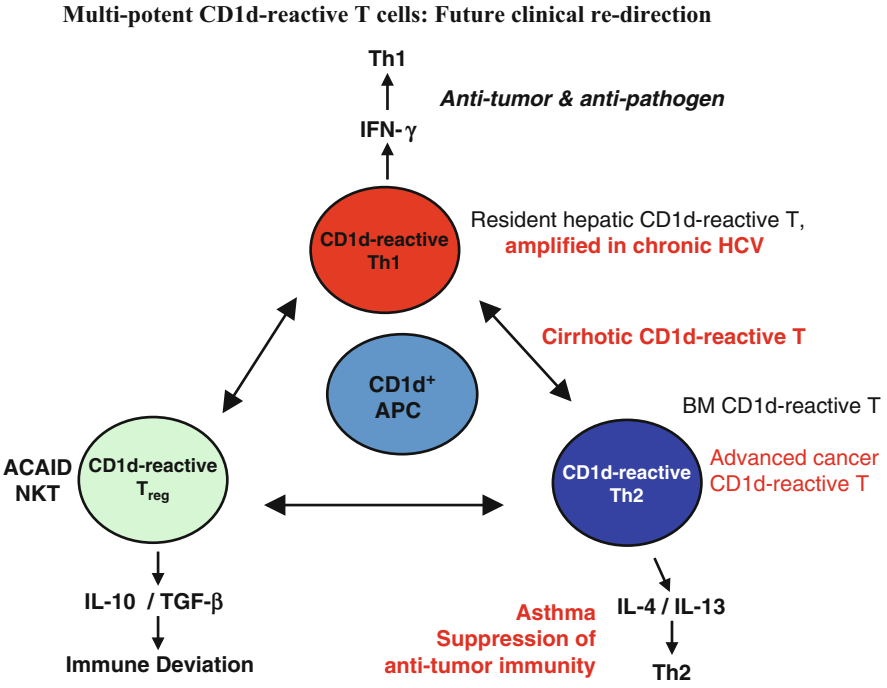

Fig. 8.1 Model for suppression of iNKT in tumors. iNKT cells can produce antitumor IFN-γ. However, iNKT subsets can be re-polarized by suppressive activity to tumor and or tumor-associated APC, as shown. Therapeutic potential of NKT subsets lies in reversing their polarization

CD1d is expressed primarily by hematopoietic cells (Exley et al. 2000; Roark et al. 1998; Brossay et al. 1998, Cohen et al. 2009), but there is low-level expression by hepatocytes and intestinal epithelial cells (Bleicher et al. 1990; Mandal et al. 1998). With the exception of tumors arising from naturally CD1d$^+$ tissues and lymphoid and myeloid neoplasms (reviewed in Metelitsa 2010), expression of CD1d on tumor cells was thought to be rare. However, TRAMP tumor cell lines show clear CD1d surface expression, confirmed by western blot and fluorescence microscopy (Nowak et al. 2010a). Prostate tumor cells from TRAMP mice also express CD1d (Nowak et al. 2010a). Finally, human prostate cancer cell lines express CD1d, as do even untransformed human prostate epithelial cells (Nowak et al. 2010a). Therefore, normal prostate epithelium and prostate tumors CD1d expression can directly interact with iNKT cells.

7.1 Prostate Tumors Defectively Activate iNKT Cells

IL-12 mediates its effects by binding to a heterodimeric receptor comprised of two chains, of which the β1 chain is up-regulated during activation of conventional T cells and expressed at low levels on resting iNKT cells (Reddy et al. 2005).

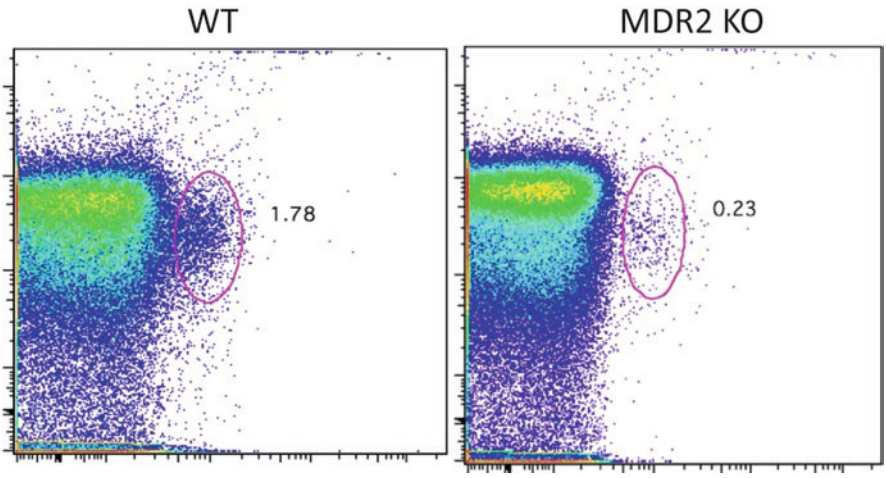

Fig. 8.2 iNKT levels in age-matched WT control and MDR2 KO mice liver preparations. Whole liver cell extracts (without perfusion) from 8-month-old males (cirrhotic/microscopic HCC) were overlaid on percoll to isolate lymphocytes. Cells were stained with CD3 (*Y*-axis), CD4 and αGalcer analog PBS-57-CD1d tetramer (*X*-axis; NIH Tetramer Facility). Note ~10 times more iNKT in control WT liver. Total CD3+% and iNKT CD4/DN ratios were similar between WT and KO (not shown). (thanks to Y. Popov and D. Schuppan for MDR2 KO mice)

In light of the results showing that IL-12 stimulates IFNγ production by iNKT cells cultured with αGalCer pulsed TRAMP-C2 cells, we examined expression of the IL-12Rβ1 chain (Nowak et al. 2010a). The IL-12Rβ1 chain is up-regulated after iNKT contact with TRAMP-C2 cells without addition of αGalCer. As expected based on responses to IL-12, IL-12Rβ1 was strongly induced by αGalCer-pulsed TRAMP-C2. In contrast, DC led only to a small increase in IL-12Rβ1 expression on iNKT, whether or not in the presence of αGalCer. Concomitantly, exposure to TRAMP-C2 (± αGalCer) increased expression of PD-1 on iNKT, whereas BM-DC failed to induce this effect (Nowak et al. 2010a). When iNKT cells express PD-1 upon activation in vivo, they can subsequently enter an anergic-like state (Chang et al. 2008a, b; Parekh et al. 2009). How iNKT PD-1 is regulated remains unclear.

In light of these unexpected results, we further characterized TIL-iNKT present in TRAMP tumors. Although PD-1 and CD25 are not up-regulated on iNKT in tumor-bearing TRAMP mice, there is strong up-regulation of IL-12Rβ1 on TIL-iNKT in tumor but not from spleens (Nowak et al. 2010a). Up-regulation of IL-12Rβ1 is specific to iNKT cells, as it is not detectable on conventional T cells (Nowak et al. 2010a), further corroborating this CD1d-mediated iNKT partial activation. Additionally, iNKT present in TRAMP tumors express higher levels of early activation marker CD69 compared to iNKT in spleens (Nowak et al. 2010a). Finally, the presence of Tramp tumor cells results in reduced phospho-STAT4 in response to either mitogen or IL-12 stimulation (Nowak et al. 2010a), suggesting a defect in this pathway.

These in vitro findings are corroborated by ex vivo data showing elevated expression of some activation markers on iNKT in model prostate tumors relative to the periphery.

However, TIL-iNKT cells do not express CD25. Whether this is due to a higher turnover of iNKT cells in the tumor through apoptosis remains to be clarified. In this context, like some human tumors and cell lines (Metelitsa et al. 2004), murine prostate tumor cells can secrete chemokine CCL2 and attract iNKT into the TME without detectable depletion of iNKT elsewhere, which argues for a higher turnover. Specifically, only iNKT in prostate tumors but not elsewhere show a defective hyperactivated phenotype. This suggests that presence of $CD1d^+$ tumor cells is required for this effect. Collectively, these data indicate that murine prostate tumor cells induce a partial activation state in iNKT cells. Although iNKT cells in this state up-regulate the IL-12R, they are unable to respond to IL-12.

7.2 IL-12 Restores iNKT Cell Activation in Response to Prostate Tumor Cells

iNKT produce high levels of IL-2, IL-4, and IFNγ in response to αGalCer-pulsed DC (see above). However, iNKT produce some IL-2, comparable IL-4 to DC as APC, but little if any IFNγ in response to αGalCer-pulsed TRAMP-C2 cell line (Nowak et al. 2010a). IL-12 is an adjuvant for IFNγ production by iNKT (Tomura et al. 1999; Kitamura et al. 1999; Cerundolo et al. 2009). Addition of IL-12 enhances TRAMP-C2-induced IFNγ production of iNKT (Nowak et al. 2010a), as previously described with other APC (see above). IL-12 stimulating iNKT with TRAMP-C2 alone induces significant but minimal levels of IFNγ produced by iNKT (Nowak et al. 2010a). Addition of IL-12 to αGalCer-pulsed TRAMP-C2 as stimulators is sufficient to induce comparable levels of IFNγ as DC do from iNKT (Nowak et al. 2010a). These data show that TRAMP-C2 stimulates iNKT to produce IL-4 but not IFNγ, which could not be induced by addition of αGalCer alone. The addition of both high-affinity CD1d agonist αGalCer and the adjuvant IL-12 are both required to induce IFNγ production in iNKT cells in response to tumor cell CD1d, restoring the Th1 response of iNKT.

8 Endogenous iNKT Ligands in Cancer

The data earlier suggests that iNKT respond to ligands expressed by various $CD1d^+$ cells, including tumor cells, although these result in iNKT polarization away from optimal antitumor activity. We have also found that ligand-like antagonists of iNKT can inhibit CD1d reactivity (Nieuwenhuis et al. 2005; not shown), providing further indirect evidence for ligands of iNKT. Others have identified ligand-like activity in tumor cells (Wu et al. 2003; Chang et al. 2008a, b). Indeed, tumor iNKT can kill autologous tumors in the absence of exogenous ligands such as αGalCer (Lynch et al. 2009). Thus, it seems likely that physiological as well as pathological ligands for iNKT will be defined in the next few years.

9 The Role of Adenosine Receptors in iNKT Cell Defects

The purine nucleoside adenosine is an important anti-inflammatory molecule, inhibiting a variety of immune cells by adenosine receptor-mediated mechanisms. NKT and regulatory T cells (Treg) share expression of the ecto-nucleotidases CD39 and CD73, which generate adenosine from ATP via ADP/AMP. Expression of both enzymes is required for Treg suppression (Deaglio et al. 2007). Similarly, CD39-deficient iNKT fail to produce IL-4 upon CD1d-mediated activation (Beldi et al. 2008), suggesting that endogenous adenosine modulates their cytokine production.

Mechanisms polarizing iNKT cytokine patterns in vivo are elusive, but adenosine can suppress iNKT IFNγ production in a mouse model of liver ischemia–reperfusion injury (Lappas et al. 2006). It was uncertain whether iNKT were generally inhibited or their cytokine profile was skewed. Mouse iNKT express all four known adenosine receptors A1R, A2aR, A2bR, and A3R, and IL-4 production in primary mouse iNKT and a primary human iNKT line is efficiently inhibited by A2aR blockade (Nowak et al. 2010b). A2AR-deficient mice exhibit highly decreased levels of IL-4, IL-10, and TGFβ concomitantly with an increase of IFNγ in response to αGalCer in vivo (Nowak et al. 2010b). These data suggest adenosine:A2aR-mediated mechanisms can control the cytokine secretion pattern of iNKT and suggest a way to control iNKT. A2BR KO mice have similar defects and A2A/BR DKO are most profoundly defective (collaboration with Drs. A. Ohto and M. Sitkovsky, NEU). Based on the above results, it is possible that treating cancer with longer half-life adenosine receptor antagonists might provide benefit through controlling iNKT as well as effects on Treg, i.e., "suppressing the suppressors."

10 CD1d mAb Based-Therapy Bypasses iNKT in Cancer Models

As described in Teng et al. (2009a, b) for tumor cell line models, CD1d mAb therapy induces a potent Th1 and type 1 interferon response both in vitro but also in vivo (Yue et al. 2005; op cit 2010). This approach is potentially synergistic with other approaches, both conventional and immunotherapeutic (Teng et al. 2009b). Therefore, this may be a future way to bypass defects in NKT cells of cancer patients.

11 Conclusions

Cancer immunotherapy has yet to reach its full potential. Selective but reversible systemic numerical and functional iNKT cell defects have been observed in cancer patients. Figure 8.1 illustrates the dynamic interactions between tumor, APC populations, and iNKT cells which can lead to suppression of this arm of immunity in progressive cancers. Figure 8.3 shows an overview of NKT cell activities in health

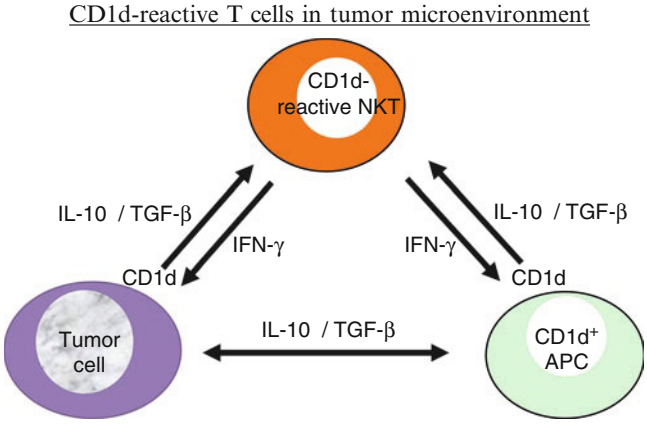

Fig. 8.3 Model for polarization of NKT in physiological responses and potential therapeutic use. In *Blue-Black*: physiological roles of NKT populations in various tissues. In *Red*: example pathogenic roles of NKT cell types/Unlike classic T cells, NKT subsets can be re-polarized as shown. Therapeutic potential of NKT subsets lies in controlling their reversible polarization

and disease. iNKT cells of advanced cancer patients show a significant reduction of IFNγ and proliferation in response to TCR-mediated activation in vitro. Importantly, however, those who retain Th1-biased NKT have better prognosis, suggesting a direct protective role for NKT populations. A large amount of model cancer data also suggests that iNKT defects can be causative. The mechanism(s) underlying iNKT cell defects in cancer remain to be completely understood. In one class of tumors, including many hemopoietic malignancies and prostate and some other cancers, CD1d expression by the actual tumor is a common denominator. iNKT from prostate tumor-bearing mice exhibit functional defects similar to those observed in humans, specifically, diminished ratio of antitumor Th1 cytokines IL-12 and IFNγ relative to Th2/regulatory responses upon activation with iNKT ligand αGalCer.

The interaction of tumor cells with iNKT cells reveals that $CD1d^+$ tumor cells reversibly inhibit iNKT, but this can be overcome by provision of strong TCR signals, such as provided by the high affinity ligand αGalCer in combination with Th1-promoting IL-12. We conclude from these findings that tumor cells can directly block production of IFNγ cytokines in iNKT cells. iNKT may store cytokine mRNA, allowing them to respond rapidly upon TCR-mediated activation (Matsuda et al. 2003). Thus, it is conceivable that tumor cells, in addition to CD1d-mediated signals activating iNKT, deliver additional signals that block translation or export of iNKT IFNγ. Whether IFNγ blockade is mediated by tumor-derived soluble factors is not yet known. In conclusion, model prostate tumor cells induce a novel and partial activation state in iNKT reminiscent of defects in patients. Concomitantly, prostate tumor cells directly inhibit secretion of IFNγ as an effector function in iNKT. This effect is resistant to IL-12. However, correction of these iNKT cell defects by CD1d ligand αGalCer in combination with IL-12 might support future development of

IL-12R and iNKT-targeted therapeutic approaches. The next few years will bring this and various other potentially improved NKT-related approaches to clinical trials in cancer and other diseases.

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Chapter 9
Understanding the Role of Natural Killer T Cells in Hematologic Malignancies: Progress and Challenges

Natalia Neparidze and Madhav V. Dhodapkar

Abstract Natural Killer T (NKT) cells have emerged as important effector cells in tumor immunity. Hematologic malignancies are particularly attractive targets of NKT mediated anti-tumor effects as most hematologic tumors express CD1d. Patients with several hematologic malignancies have been reported to carry quantitative or qualitative defects in NKT cells. Preliminary clinical studies suggest the capacity of alpha-galactosylceramide (α-GalCer) loaded dendritic cells to stimulate NKT cells in vivo. However harnessing the effects of NKT cells in the clinic will also require attention to reversing the defects in NKT function in vivo. Improved understanding of the cross-talk between type I and II NKT cells, as well as the nature of naturally occurring CD1d binding ligands in these patients is needed to optimally target NKT cells in patients with hematologic malignancies.

1 Diversity of Natural Killer T cells

Natural killer T cells (NKT) cells are distinct innate lymphocytes that recognize lipid antigens in the context of CD1d (Berzofsky and Terabe 2009; Bendelac et al. 2007). The recognition of lipids by these cells fills a distinct niche in the immune system in contrast to conventional CD4 or CD8+ T cells that recognize peptide antigens. Importantly, these cells have a distinct developmental pathway and follow distinct modes for antigen recognition which perhaps help explain their functional properties (Godfrey et al. 2011). At least one subset of these cells rapidly secrete cytokines in response to ligand-dependent stimulation, and can lead to rapid downstream activation of several other immune cells, including NK, dendritic and T cells (Munz et al. 2005). Thus NKT cells may serve as an important bridge between

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innate and adaptive immunity in the context of signals derived from self or pathogen-derived lipid antigens. NKT cells may serve as a link between innate and adaptive immunity, and therefore have been implicated in immune regulation of diverse disease states, including regulation of responses to pathogens and cancer, as well as allergy, inflammation and autoimmunity (Wu and Van Kaer 2010; Dhodapkar 2009; Godfrey and Kronenberg 2004). In this review, we will primarily focus on the current state of studies dissecting the role of NKT cells in the context of hematologic malignancies.

While the original criterion for definition of NKT cells was simply as a T-cell subset expressing both T and NK markers, the term is now used to represent a broader repertoire of lipid-reactive T cells (Godfrey et al. 2004). This approach has led to the recognition of broadly three distinct subsets of NKT cells. The best studied of the subsets are those expressing the invariant T-cell receptor (Vα14Jα18 TCR in the mouse or Vα24Jα18 in the human), called type I NKT or invariant NKT (iNKT) cells. A major breakthrough in this regard was the identification of α-galactosylceramide (α-GalCer; KRN-7000) as a potent agonist for these cells. This spurred considerable research on the properties of both human and murine type I NKT cells. In humans, type I NKT cells can be further divided into CD4+ and CD4–CD8– DN subsets, and a small subset of human NKT cells may even express CD8αα (Gumperz et al. 2002; Lee et al. 2002). NKT cells have also been distinguished by their tissue localization. For example in the mouse, up to 30% of cells in the liver are type I NKT cells (Bendelac 1995). The liver-resident cells also show different functional characteristics. For example, it has been suggested that liver NKT cells are more protective against tumors than NKT cells from the spleen or thymus (Crowe et al. 2005). Type I NKT cells have a phenotype of activated T cells, and rapidly secrete cytokines in response to antigen stimulation, which is a property in part due to the presence of preformed mRNA in these cells. These cells can be rapidly activated by a synthetic glycolipid ligand, α-galactosylceramide (α-GalCer), which allows facile detection of these cells using α-GalCer-loaded CD1d multimers. In mice, NKT activation by α-GalCer leads to rapid downstream activation of NK cells and induction of adaptive immunity via activation of dendritic and T cells (Fujii et al. 2007). This property of NKT cells is being targeted to help improve the efficacy of adjuvants. Invariant NKT cells are evolutionarily conserved, thus suggesting an important role in the immune system. Another subset of NKT cells that are also CD1d-restricted but thought to express more diverse TCRs were termed type II NKT cells (Terabe and Berzofsky 2007). Compared to type I NKT cells, the current understanding of the biology of type II NKT cells in humans is very limited. However, it is likely that these cells have broader repertoire of antigenic specificities and diverse roles in the context of disease states. Finally, there is another heterogeneous subset of T cells with diverse TCRs that express NK markers, but are not CD1d-restricted or glycolipid-reactive, and have been termed type III NKT cells. This distinction between different subsets of NKT cells is critical in the context of tumor immunity as they appear to have distinct functional roles in this regard.

2 Dual Roles of CD1d-Restricted T Cells in Antitumor Immunity

Much of the early work regarding the potential roles of NKT cells in tumor immunity suggested primarily a protective role, which was linked to their capacity to secrete interferon γ (Smyth et al. 2002). Several studies described the antitumor activity of iNKT cells stimulated by α-GalCer in vivo (Crowe et al. 2002, 2003). Studies using mice deficient in iNKT cells illustrated an important role for NKT cells in immune surveillance against tumors in several models. These mice have increased propensity for cancer in both spontaneous as well as carcinogen and oncogene-induced models, and this may be particularly relevant in the setting of p53 deficiency (Crowe et al. 2002, 2003; Hayakawa et al. 2002, 2003). The anti-tumor effects of NKT cells are thought to be mediated by several distinct pathways, including enhancement of immune effectors, particularly IFN-γ-mediated activation of NK cells and antiangiogenesis (Hayakawa et al. 2002, 2003). Syngeneic DCs pulsed with α-GalCer were found to have antitumor effects and could treat established liver metastases of the B16 melanoma (Fujii et al. 2002). Alteration of biochemical properties of the α-GalCer backbone has been explored to skew NKT cells more toward an interferon-γ response, and this led to greater antitumor activity (Fujii et al. 2006). In addition to the role of IFN-γ in recruiting NK cells, NKT cells were shown to activate DCs to make IL-12, and this mechanism may also play an important role in antitumor activity (Fujii et al. 2003a; Kitamura et al. 1999). Another new target of INKT-mediated antitumor effects may involve inhibition of myeloid-derived suppressor cells (Song et al. 2009; De Santo et al. 2008). An important concept was proposed by Terabe et al., who described differential effects of type I versus II NKT cells in mice in several cancer models (Terabe and Berzofsky 2007; Terabe et al. 2000, 2003). In this concept, the balance of type I versus type II NKT cells regulates antitumor immunity (Terabe and Berzofsky 2007). Therefore, improved understanding of the regulation of this axis and how it is altered during tumor progression will be critical to be able to effectively harness the properties of NKT cells in the clinic.

3 Hematologic Malignancies as a Model to Study NKT/CD1d Axis

Hematologic malignancies represent a diverse group of tumors that share hematopoietic system as the cell of origin. Several aspects of hematologic tumors make them attractive potential candidates for immune regulation via NKT cells (Table 9.1). First, most of these tumors are derived from cells of lymphoid or myeloid lineage, and commonly express CD1d, as is the case with the cells or origin (Dhodapkar et al. 2003; Neparidze and Dhodapkar 2009; Metelitsa et al. 2003; Takahashi et al. 2003; Fais et al. 2004; Stoiber et al. 2004; Yoneda et al. 2005; Chan et al. 2010;

Table 9.1 Rationale for targeting CD1d/NKT system in hematologic malignancies

- Common expression of CD1d by primary tumor cells.
- Susceptible to lysis by iNKT cells as well as NK cells.
- Correlation of CD1d expression with outcome (e.g., in myeloma).
- Correlation of iNKT function and clinical course (e.g., in myeloma).

Xu et al. 2010; Imataki et al. 2008). This is an important distinction from several epithelial cancers, wherein the tumor cells often do not express CD1d. There is also a growing body of evidence that the capacity of NKT cells to regulate tumor growth may be linked to the expression of CD1d by tumor cells (Haraguchi et al. 2006). Due to the expression of CD1d, tumor cells in hematologic malignancies are often sensitive to direct lysis by iNKT cells (Dhodapkar et al. 2003; Song et al. 2008). In some tumors such as myeloma, the expression of CD1d may also be linked to survival pathways within tumor cells. Indeed, the development of progressive extramedullary disease in myeloma is often associated with loss of CD1d expression by tumor cells, which in turn correlates with disease progression (Spanoudakis et al. 2009). Therefore, there may be a more direct impact of CD1d expression or CD1d-targeted therapies on the biology of tumor cells. Third, many of the hematologic tumors such as leukemia and myeloma typically grow in the bone marrow, which is known to be a site enriched in CD1d-restricted T cells, albeit mostly type II cells in humans (Exley et al. 2001). Finally, as with other tumors, these malignancies are also linked to inflammation characterized by alteration of bioactive lipids, which are now emerging as important ligands for CD1d-restricted T cells (Chang et al. 2008; Salio and Cerundolo 2009). Below, we discuss the current data about changes in CD1d/iNKT system in hematologic tumors, with a particular emphasis on myeloma, which has been the primary focus of our own efforts in this area.

4 Multiple Myeloma

4.1 Role of CD1d and Alteration in NKT Function in Multiple Myeloma

Multiple myeloma (MM) is a common hematologic malignancy characterized by the growth of malignant plasma cells in the bone marrow. The precursor lesion to MM is termed as monoclonal gammopathy of undetermined significance (MGUS). Clinically, MGUS is a common entity, seen in 3–4% of the population older than 50 years of age (Kyle et al. 2002). It is now reasonably well established that most if not all of MM lesions are preceded by an asymptomatic precursor state. Interestingly, tumor cells in MGUS carry many of the known cytogenetic changes seen in MM,

suggesting that interactions between tumor cells and the microenvironment may regulate the progression of MGUS to clinical malignancy (Dhodapkar 2004). In prior studies, we have shown that primary tumor cells in MM bone marrow commonly express CD1d (Dhodapkar et al. 2003). Surprisingly, MM cell lines, which are typically derived from patients with advanced disease lack CD1d (Dhodapkar et al. 2003). These data were confirmed in another study which also found a loss of CD1d expression with disease progression, and that this loss of CD1d expression by tumor cells was predictive of adverse survival (Spanoudakis et al. 2009). Together, these data suggest that CD1d-related signaling pathways may have important implications for homing and/or survival of tumor cells. Consistent with this, engagement of CD1d on the surface of tumor cells by anti-CD1d mAbs induces cell death of myeloma cell lines with restored CD1d expression and primary myeloma cells (Spanoudakis et al. 2009). Nonetheless, the possibility that CD1d expression has important effects on the biology of human MM suggests that immunologic manipulation of CD1d-restricted T cells may also have important implications for disease biology. Few other lines of evidence also point to a potential link between lipids and myeloma. Patients with Gaucher's disease, a lipid storage disorder have been shown to carry an increased risk for developing MM, which has now been particularly manifest, as these patients are living longer (Rosenbloom et al. 2005). It would certainly be of interest to find out which lipid antigens, if any, drive this disease. Finally, one of the best known models of murine plasmacytoma involves intraperitoneal injection of an oil (pristane) in Balb mice. While this model does differ from human MM in several respects, it illustrates how lipid-driven inflammation may lead to a clonal plasma cell tumor.

As discussed earlier, the antitumor properties of iNKT cells are strongly linked to their capacity to secrete interferon-γ. Therefore, we analyzed the functional properties of natural killer T cells from the blood and tumor bed in patients with MGUS or MM. NKT cells from patients with progressive MM, but not nonprogressive MM or MGUS, had a marked deficiency of ligand-dependent interferon-γ production (Dhodapkar et al. 2003). This functional defect could be overcome in vitro using dendritic cells pulsed with the NKT ligand, α-GalCer. Importantly, primary MM cells were found to be sensitive to lysis by NKT cells in a CD1d-dependent manner. As discussed above, NKT activation may in principle also mediate antitumor effects by anti-angiogenesis, as well by activation of NK cells, and tumor-specific cytotoxic T cells (Neparidze and Dhodapkar 2009). These data suggest that clinical progression in patients with monoclonal gammopathies is associated with an acquired but potentially reversible defect in NKT cell function and support the possibility that these innate lymphocytes play a role in controlling the malignant growth of this incurable tumor. Thus, measurement of NKT cell function may be a useful predictor of clinical outcome in MM patients. However, this needs to be tested in larger cohorts of patients, preferably in the context of prospective studies (Berzins et al. 2011). Strategies to harness the functional properties of NKT cells or restoring their function might be of therapeutic benefit in MM.

4.2 Probing Antigenic Targets of CD1d-Restricted T Cells in Human MM

The cause of loss of ligand-dependent IFN-γ-secreting function in NKT cells from MM patients is not known. However, the finding that the effector function of conventional T cells was preserved in at least some of the patients suggested that the effects on NKT function were not due to a global alteration of T-cell function (Dhodapkar et al. 2003). Therefore, we posited that understanding the nature of ligands recognized by NKT cells may provide novel insights into the mechanism of observed NKT dysfunction. Over the past few years, there has been considerable interest in trying to understand the nature of endogenous ligands recognized by NKT cells. Autoreactivity has long been recognized as a property of NKT cells (Hegde et al. 2010). Studies have shown that type I NKT cells can recognize some microbial glycolipids (such as those from sphingomonas and Borrelia) and self-antigens, such as isoglobotrihexosylceramide (iGb3) (Brutkiewicz 2006). However, whether iGb3 is the critical self ligand for iNKT cells is now somewhat controversial. The nature of antigens specifically recognized by type II NKT cells is even less clear. Some studies have shown sulfatide and nonlipidic small molecules as capable of binding these T cells (Jahng et al. 2004; Van Rhijn et al. 2004). In some settings, GD3 and tumor-derived glycosphingolipids was implicated as a ligand for mouse iNKT cells (Wu et al. 2003). However, the nature of the specific endogenous ligands recognized by either type I or II NKT cells in inflammation or cancer in humans remains obscure. In order to identify the nature of CD1d binding ligands in the context of human myeloma, we isolated bulk lipids from plasma of myeloma patients, and used it to load CD1d multimers and stain human T cells. Interestingly, these CD1d multimers stained a subpopulation of both Vα24+ and Vα24 – T cells. Elution of CD1d-bound lipid led to the discovery of lyso-phosphatidylcholine (LPC) as a CD1d-binding ligand recognized by human NKT cells. Interestingly, the great majority of T cells that stained with CD1d multimers loaded with this lipid lacked the expression of Va24 and therefore were type II NKT cells (Chang et al. 2008). The presence of increased proportion of LPC-specific T cells could also be demonstrated in MM patients. Interestingly, these T cells were skewed toward a Th2 phenotype, particularly expressing IL13 upon TCR-based stimulation. The concept that PC and LPC can bind CD1d has now been confirmed by several groups. Using a mass spectrometry-based approach, the Cresswell lab recently also demonstrated the binding of phospholipids such as PC and LPC to CD1d (Yuan et al. 2009). Their hypothesis is that PC may in particular play an important role in loading of endogenous ligands on CD1d. The Th2 bias of T cells in response to such ligands may relate to weaker affinity of binding to CD1d (Im et al. 2009). Gumperz et al. (2000) had initially identified phospholipids as CD1d-binding ligands in mice. More recently, this group has also analyzed human NKT cell recognition of CD1d-associated cellular ligands (Cox et al. 2009; Fox et al. 2009; Wang et al. 2008). In these studies, the most antigenic species again was LPC. In addition, lysosphingomyelin, which shares the phosphocholine head group of LPC, also activated

human NKT cells. Antigen-presenting cells pulsed with LPC were capable of stimulating increased cytokine responses by NKT cell clones and by freshly isolated peripheral blood lymphocytes. Taken together these results demonstrate that human NKT cells recognize cholinated lyso-phospholipids as antigens presented by CD1d. We are currently trying to clone out these cells in order to better understand their properties. Since these lyso-phospholipids serve as lipid messengers in normal physiological processes and are present at elevated levels during most inflammatory responses including cancer, these emerging insights point to a novel link between NKT cells and cellular signaling pathways that are associated with human cancer and inflammation (Salio and Cerundolo 2009). It is notable that while the detection of these cells adds another dimension to inflammation-associated signaling in MM, the nature of tumor-derived CD1d-binding lipid ligands still remains obscure. In particular, it will be important to identify which lipids derived directly from tumor cells are recognized by CD1d-restricted T cells, and whether CD1d expressed by MM cells is capable of presenting endogenous ligands. Whether or how inflammation-associated ligands might alter the properties of iNKT cells is an area of active research, and may provide novel insights into how inflammation may alter or disable this innate arm of the immune system.

4.3 Harnessing iNKT Cells in Human MM

The body of data discussed above provides the rationale for harnessing the properties of iNKT cells in human MM. Two broad types of strategies are currently moving to the clinic. One involves adoptive transfer of iNKT cells expanded and activated ex vivo (Song et al. 2008). Another approach involves adoptive transfer of antigen-loaded dendritic cells to expand iNKT cells in vivo (Chang et al. 2005). The discovery and availability of α-GalCer as a clinical grade reagent has allowed studies employing this ligand to enhance NKT cells in vivo. Despite encouraging initial preclinical data in mice using α-GalCer, injection of the ligand alone in humans with cancer led to only modest and transient effects without substantial NKT expansion (Giaccone et al. 2002). In contrast, in a phase I trial, injection of glycolipid-loaded DCs led to >100-fold expansion of iNKT cells in all patients tested (Chang et al. 2005). Therefore, the response to NKT ligands may differ depending on the nature of antigen-presenting cell (Fujii et al. 2002, 2003b). However, the functional properties of even the NKT cells expanded in vivo using DCs were impaired in the patients with advanced cancer. Therefore, strategies to improve the effector function of iNKT cells are of interest. One strategy might be the combination with immune-modulatory drugs such as Revlimid (Song et al. 2008; Chang et al. 2006). We and others have shown that this combination can lead to enhanced activation and costimulation of NKT cells and greater secretion of interferon-γ. We are currently testing this combination approach in the setting of patients with early-stage MM. In this trial, patients receive a low dose of Revlimid (10 mg/day), along with three monthly injections of α-GalCer-loaded DCs.

These studies should provide important insights into whether costimulation is an essential feature for optimal activation of iNKT cells in the clinic (Singh et al. 2010; van den Heuvel et al. 2010).

5 B-Cell Lymphoma

Evidence in the literature suggests that NKT cells might also play an important role in other lymphoproliferative disorders (Imataki et al. 2008; Renukaradhya et al. 2008; Kawano et al. 1999). This is not surprising in view of CD1d expression in several lymphoid tumors. Preclinical studies show that type I NKT cells may exert cytotoxic effect toward lymphoma cells, or confer protection in CD1d-dependent manner. In one study, invariant Va24/Vb11 NKT cells displayed a potent cytotoxic activity against Daudi lymphoma (Kawano et al. 1999). Here again, type I versus II NKT cells may have opposing functional effects. Renukaradhya et al. (2008) have analyzed the roles for various NKT-cell subsets in the host's innate anti-tumor response against a murine B-cell lymphoma model in vivo. In tumor-bearing mice, they found that type I NKT cells conferred protection in a CD1d-dependent manner, whereas type II NKT cells exhibited inhibitory activity.

The potential for NKT-based approaches in lymphoma is beginning to inspire preclinical studies targeting NKT cells. One simple approach is to pulse the lymphoma cells with NKT ligand, α-GalCer. This strategy takes advantage of the capacity of CD1d-expressing tumor cells to directly present α-GalCer and activate iNKT cells in the proximity of the tumor, which may mediate direct antitumor cytotoxicity. The hypothesis is that NKT- mediated activation of DCs that take up dying tumor cells in vivo can then lead to the induction of adaptive immunity (Moreno et al. 2008). Single vaccination with α-GalCer-loaded A20 lymphoma cells elicited effective anti-tumor immunity against tumor challenge. This vaccination strategy also induced significant tumor regression in A20-bearing mice. Importantly, the survivors from primary tumor inoculation were all resistant to tumor rechallenge, indicative of established adaptive memory immunity (Chung et al. 2007). Collectively, these data suggest that there are distinct roles for NKT-cell subsets in response to lymphoid neoplasms, pointing to potential novel targets to be exploited in immunotherapeutic approaches against lymphoproliferative neoplasms.

6 Myelodysplastic Syndromes

Myelodysplastic syndrome (MDS) comprises a group of clonal bone marrow disorders characterized by ineffective hematopoiesis, cytopenias, and increased predisposition to acute myeloid leukemia. The causes of MDS remain poorly defined, but several studies have reported that NKT-cell compartment of patients with MDS is

deficient in number and functional effectiveness. Patients with myelodysplastic syndromes were found to have a severe deficiency of glycolipid-reactive Vα24+/Vβ11+ natural killer T (NKT) cells, but not NK cells or CD4+ or CD8+ T cells. Neither the blood nor marrow of MDS patients had detectable interferon-γ-producing NKT cells in response to the NKT ligand, α-GalCer, although influenza-virus-specific effector T-cell function was preserved (Fujii et al. 2003c). Another study using flow cytometry and TCR spectratyping demonstrated that iNKT or iTCR+ cells, unlike other T-lymphocyte subpopulations, were disproportionally decreased in the bone marrow of patients with aplastic anemia and MDS (Zeng et al. 2002). Changes in iNKT cells or their function have also been evaluated in the context of Revlimid therapy, which is particularly effective in the subset of MDS patients with 5q- cytogenetic abnormality. Chang et al. (2006) observed that in MDS patients responding to Revlimid, the previously observed decline in iNKT cells and ligand-dependent interferon-γ secretion was restored in some patients. However, another recent study found no consistent changes in frequency of iNKT cells in MDS patients responding to Revlimid (Chan et al. 2010). Evaluation of NKT function in the latter study was not based on ligand-dependent stimulation, as with prior studies. The reasons behind the differences in these studies are not clear, but may relate to the heterogeneity of MDS, relatively small numbers of patients studied thus far, as well as technical differences in the methodologic approach as noted above. Further studies are needed to better address this issue.

The finding of apparent decline in circulating iNKT numbers is not unique to MDS, but has been reported in other hematologic malignancies as well. Yoneda et al. (2005) assessed the Vα24+ NKT cell numbers in peripheral blood from 30 healthy donors and 70 patients with hematopoietic malignancy including chronic myelogenous leukemia (CML), malignant lymphoma (ML), acute myelogenous leukemia (AML), and MDS. It was demonstrated that circulating Vα24+ NKT cell numbers were significantly decreased in all the patients with hematopoietic malignancy in comparison with that in healthy donors. In particular, the decline was particularly notable for the CD4– CD8– (double negative) subset (Yoneda et al. 2005). Taken together, while changes in iNKT cells in MDS have been reported in some studies, this finding has not been consistent in at least one study and therefore needs further clarification.

7 Acute Myeloid Leukemia

Evidence for antitumor properties of NKT cells prompted the investigators to also evaluate these cells in the setting of acute leukemia. Previous in vitro studies have shown susceptibility of several leukemia cell lines to cytotoxic activity of Vα24+ NKT cells (Nicol et al. 2000). The same group subsequently reported that myeloid leukemia cells from patients with the monocytic subtype of AML (M4), but not from patients with less differentiated AML (M0 or M1), undergo apoptosis following culture with TRAIL-expressing autologous or allogeneic healthy donor Vα24NKT cells (Nieda et al. 2001). Several leukemia cell lines such as Jurkat and U937 express

CD1d and are susceptible to lysis by NKT cells. Killing of the latter was greatly augmented in the presence of α-GalCer (Metelitsa et al. 2001, 2003).

Metelitsa et al. evaluated NKT cells with myeloid leukemia cells from 14 patients and purified NKT cells that were α-GalCer/CD1d reactive. CD1d was expressed by 80–100% of cells in three of seven acute myeloid leukemias (AMLs) and by 28–77% of cells in five of six juvenile myelomonocytic leukemias (JMML). CD1d+ AML cells were myelomonocytic or monoblastic types, and CD1d+ JMML cells were differentiated and lacked the expression of CD34. NKT-mediated cytotoxicity of human leukemia required CD1d expression on tumor cells, was increased by α-GalCer and inhibited by anti-CD1d mAb (Metelitsa et al. 2003). Together these findings suggest that iNKT cells may be of therapeutic benefit in the setting of some subtypes of AML, particularly those with monocytic differentiation.

8 Bone Marrow Transplant and Graft-Versus-Host Disease

Allogeneic bone marrow transplantation (BMT) is a potentially curative therapy for leukemia and lymphoma, but graft-versus-host disease (GVHD) remains a major complication (Kohrt et al. 2010). In preclinical models, bone marrow-derived NKT cells have been shown to potently inhibit GVHD in a mouse model where NKT cells were cotransferred with allogeneic bone marrow into lethally irradiated recipient (BALB/c) mice (Kohrt et al. 2010; Pillai et al. 2007; Zeng et al. 1999).

Studies indicate a critical contribution of NKT cells to the induction of allograft tolerance in murine transplantation models. In one report the authors found that the CD8+ NKT cells produced very high levels of IFN-γ, were highly cytotoxic against a wide range of tumor cell targets in vitro and BCL1 leukemia cells in vivo, and could prevent GVHD (Baker et al. 2001). Seino et al. reported that long-term acceptance of the grafts was observed only in WT but not Vα14 NKT cell-deficient mice. Adoptive transfer with Vα14 NKT cells restored long-term acceptance of allografts in Vα14 NKT cell-deficient mice (Seino et al. 2001).

Human Vα24+ NKT cells have also been implicated in allograft tolerance (Haraguchi et al. 2004, 2005). In one report NKT cells were reconstituted within 1 month after transplantation in peripheral blood stem cell transplantation recipients, while their numbers remained low for more than 1 year in BMT recipients. The number of Vα24+ NKT cells in BMT recipients with acute GVHD was lower than that in patients without acute GVHD, and both the CD4+ and CD4- Vα24+ NKT subsets were significantly reduced. With regard to chronic GVHD, BMT recipients with extensive GVHD had significantly fewer Vα24+ NKT cells than other patients. Furthermore, the number of CD4+ Vα24+ NKT cells was also significantly reduced in patients with chronic extensive GVHD. Together these studies raise the possibility that the number of Vα24+ NKT cells could be related to the development of GVHD (Haraguchi et al. 2004, 2005). In another study, injection of the synthetic NKT cell ligand α-GalCer to recipient mice on day 0 following allogeneic BMT

promoted Th2 polarization of donor T cells and a dramatic reduction of serum tumor necrosis factor, a critical mediator of GVHD. A single injection of α-GalCer to recipient mice significantly reduced morbidity and mortality of GVHD. However, the same treatment was unable to confer protection against GVHD in NKT cell-deficient CD1d knockout indicating the critical role of host NKT cells (Hashimoto et al. 2005).

9 EBV-Related Malignancies

There is also evidence that frequencies of CD8(+) NKT cells in patients with EBV-associated Hodgkin's lymphoma or nasopharyngeal carcinoma are significantly lower than those in healthy EBV carriers. These CD8(+) NKT cells in tumor patients were also noted to be functionally impaired. Adoptive transfer with EBV-induced CD8(+) NKT cells significantly suppressed tumorigenesis by EBV-associated malignancies. Thus, immune reconstitution with EBV-induced CD8(+) NKT cells could be a useful strategy in management of EBV-associated malignancies (Xiao et al. 2009; Yuling et al. 2009).

10 Challenges to Harnessing NKT Cells in Humans

In spite of the large body of preclinical data suggesting a regulatory role for NKT cells in diverse medical conditions including cancer, approaches to specifically harness these cells toward clinical benefit remain in their infancy (Dhodapkar 2009). One reason behind this may be the relative frequencies of NKT cells in mice versus humans. In particular, NKT cells are much more frequent in mice than in humans (Bendelac et al. 2007). Therefore, the effects of NKT activation may be more amplified in mice versus humans. As a result, approaches targeting NKT cells in humans are now considering a combination approach to both increase their frequency and their functional properties. Improved understanding of naturally occurring ligands for these cells, and understanding the balance between type I and II NKT cells may facilitate therapeutic targeting of these cells in humans. In particular, the property of these cells to serve as a link between innate and adaptive immunity provides the potential for NKT-targeted approaches to recruit long-term immune memory and mediate immune regulation (Bendelac and Medzhitov 2002). The ability to reliably alter the function of these cells may be of clinical benefit in diverse medical conditions including cancer, autoimmunity, inflammation, and allergy.

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Chapter 10
Clinical Trials with α-Galactosylceramide (KRN7000) in Advanced Cancer

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Abstract For over a century, research has sought ways to boost the immune system of cancer patients in order to eradicate tumors that arose after escaping presumptive immunosurveillance. With increasing knowledge of the immune system, immunotherapeutic strategies to break tolerance to the tumor evolved from largely nonspecific to more specific and increasingly potent forms of cancer vaccination. Overall however, immunotherapeutic strategies for the treatment of cancer have had limited clinical success and an urgent need exists, therefore, to introduce more effective, knowledge-based therapeutic approaches. Invariant natural killer T (iNKT) cells constitute an evolutionary conserved T lymphocyte lineage with dominant immunoregulatory and antitumor effector cell properties. iNKT specifically recognize the glycolipid α-galactosylceramide (α-GalCer/KRN7000) in the context of the CD1d antigen-presenting molecule resulting in their activation. Activated iNKT have been shown to promote the development of a long-lasting Th1-biased proinflammatory antitumor immune response in a variety of murine tumor-metastasis models of liver, lung and lymph nodes, including colon and lung carcinoma, lymphoma, sarcoma, and melanoma, suggesting broad clinical applicability. Here, we will provide an overview of the preclinical data of α-GalCer that formed the basis for subsequent clinical trials in advanced cancer patients, review these clinical trials, focusing on our own experience with α-GalCer, and discuss future perspectives.

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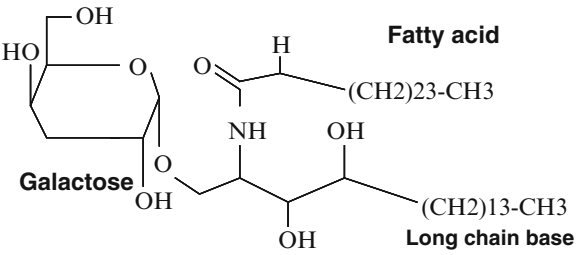

Fig. 10.1 Chemical formula of KRN7000, or (2S, 3S, 4R)-1-O-(α-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1, 3, 4-octadecanetriol. Molecular formula: $C_{50}H_{99}NO_9$. Reproduced from Giaccone et al. (2002) with permission from AACR

1 Introduction

Invariant natural killer T (iNKT) cells were first described in 1987 as murine thymocytes expressing a restricted T-cell antigen receptor (TCR) repertoire in combination with the NK cell marker NK1.1 (Fowlkes et al. 1987). The restricted TCR repertoire consists of a Vα14.Jα18 chain paired with Vβ2, Vβ7 or Vβ8.2 in mice and a Vα24.Jα18 chain preferentially paired with Vβ11 in man (Koseki et al. 1991; Dellabona et al. 1994; Exley et al. 1997). Research sparked off when the agelasphin derivative α-galactosylceramide (α-GalCer/KRN7000) from the Okinawan marine sponge *Agelas mauritianus* was identified as a ligand for iNKT cells in a screen for novel antitumor agents by the KIRIN Pharmaceutical Research Laboratory in Japan (Fig. 10.1) (Kobayashi et al. 1995). KRN7000, which consists of a galactose and a ceramide molecule linked in an α-configuration, was found to be presented to iNKT cells by the monomorphic HLA class I-related molecule CD1d and selected as the most potent agent from a series of related compounds based on structure–activity relationship studies (Kobayashi et al. 1995; Kawano et al. 1997; Spada et al. 1998). The crystal structure of CD1d revealed that its Ag binding groove was deeper, narrower, and much more hydrophobic than that of related MHC class 1 and 2 molecules and therefore well-suited to present hydrophobic Ags, including α-GalCer (Zeng et al. 1997). Upon presentation of α-GalCer by CD1d-expressing Ag presenting cells (APC), iNKT are activated and rapidly produce large amounts of predominantly IFN-γ but also IL-4 that cumulatively result in the activation of NK cells, $CD4^+$ and $CD8^+$ T cells, B cells, neutrophils, macrophages and the DCs with which they interact. It is this cascade of events that is believed to result in the development of a Th1-biased proinflammatory antitumor immune response that is pivotal for the antimetastatic activity of α-GalCer and the generation of a long-lasting antitumor effector cell population (reviewed by van der Vliet et al. 2004). Here, we will (a) summarize the preclinical data of α-GalCer that formed the basis for subsequent clinical trials in advanced cancer patients, (b) review our clinical experience with α-GalCer and provide a summary of phase 1 clinical trials performed by other groups, and (c) discuss the relevance and limitations of these studies and propose alternative iNKT cell based immunotherapeutic approaches that merit further investigation.

2 Pre-Clinical Background

Several years before the elucidation of its mechanism of action, KRN7000 was found to prolong the survival period of mice inoculated with B16 melanoma or EL-4 lymphoma. In these models, KRN7000 was more potent than, e.g., mitomycin C, a typical chemotherapeutic agent. In liver metastases models of EL-4 lymphoma and colon26 adenocarcinoma KRN7000 was found to dose-dependently induce a cytokine storm with detectable levels of IL-2, IL-4, IL-12, and IFN-γ and to augment the NK cell lytic activity of hepatic mononuclear cells resulting in tumor growth inhibition. Again, effects were more marked than several chemotherapeutic agents as well as IL-12, could be observed in the setting of established tumors and resulted in a relatively high rate of cured mice. These mice were resistant to a subsequent tumor challenge suggesting the emergence of tumor-specific immune memory (Kobayashi et al. 1995; Nakagawa et al. 1998a, b). α-GalCer-activated iNKT cells have now been shown to bridge innate and adaptive immune responses by stimulating the generation of classical cytotoxic T lymphocytes, by activating NK cells and memory $CD4^+$ and $CD8^+$ T cells in an IFN-γ and IL-12 dependent manner, and by activating dendritic cells. The sequential production of IFN-γ, sparked off by iNKT cells and subsequently by NK cells, is pivotal for the antimetastatic activity of α-GalCer and stimulates $CD8^+$ T -cell effector functions in vivo (reviewed by van der Vliet et al. 2004). An immunohistological study by Nakagawa et al. further illustrated this cascade of events by demonstrating that α-GalCer induced an increase in NK cells, $CD8^+$ T cells, and macrophages among tumor infiltrating leukocytes (Nakagawa et al. 1998a).

In these early preclinical models, i.v. and i.p. administration of KRN7000 was found to induce antitumor activity against multiple tumor types (e.g., melanoma, sarcoma, colon and lung carcinoma, and lymphoma) in multiple models including spontaneous tumor models (Kobayashi et al. 1995; Kawano et al. 1997; Nakagawa et al. 1998a, b, 2001; Toura et al. 1999; Hayakawa et al. 2001; Crowe et al. 2002). Antitumor effects of KRN7000 were observed using doses ranging from 0.01 to 100 μg/kg and apart from microgranuloma formation in liver and spleen (when very high doses were used), no side effects were reported in mice, rats, and monkeys with doses of KRN7000 up to 220, 440, and 660 μg/kg, respectively, given for 28 consecutive days. The administration of α-GalCer (100 μg/kg) in mice resulted in a transient increase in alanine aminotransferase (ALAT) levels. Based on data indicating that repeated administration of KRN7000 with intervals was superior to single injections, 1-week intervals were selected for the first-in-man clinical phase 1 study of i.v. KRN7000 given in patients with refractory solid tumors (Kobayashi et al. 1995; Giaccone et al. 2002).

Clinical benefit for cancer patients was anticipated, because both human and mouse CD1d can present KRN7000 to iNKT from either species, illustrating a strongly evolutionarily conserved immune recognition system (Spada et al. 1998).

3 Clinical Trials

3.1 Intravenous Administration of KRN7000

First clinical experience with KRN7000 was obtained in a phase 1 study in patients with refractory solid tumors. This study, performed in the VU University Medical Center in Amsterdam, and the Radboud University Medical Center in Nijmegen, was an open-label nonrandomized dose-escalation study with the identification of the maximum tolerated dose (MTD) and the optimum biologically active dose (OBAD) of KRN7000 as major endpoints (Giaccone et al. 2002). In this study, a total of 24 patients were included between October 1998 and February 2000 and, based on preclinical data (Kobayashi et al. 1995), treated by slow i.v. administration of KRN7000 on days 1, 8, and 15 of 4-weekly cycles. KRN7000 was supplied as vials containing lyophilized KRN7000 (0.2 mg), sucrose (56 mg), L-histidine (7.5 mg), and polysorbate 20 (5 mg). For pharmacokinetics of KRN7000, serial serum sampling was performed. Urines were collected in 24-h aliquots both before and after drug administration. KRN7000 concentrations in urine and serum were determined with ^{13}C-labeled KRN7000 as an internal standard using validated high-performance liquid chromatography tandem mass spectrometry. To assess biological activities of KRN7000, immunomonitoring was performed, including phenotyping of PBMCs and serum cytokine levels. Radiological assessment of tumor response was performed for every two cycles. The 24 patients (14 male, 10 female; median age 54 year (range 31–69 year), see Table 10.1 for patient characteristics) were evaluated in seven dose levels (with doses of KRN7000 varying from 50 to 4,800 μg/m^2). In each dose level three patients were entered, with the exception of the lowest dose level, in which six patients were entered because distinct biological changes were seen in this cohort. A total of 69 cycles was given (median 2 (range 1–6) per patient). Toxicity, assessed using the National Cancer Institute Common Toxicity Criteria (NCI-CTC), was minimal and included a case of grade 3 fever with headache, vomiting, chills and malaise 2–3 h after the first injection, a case of transient flush, and a case of sneezing. Although KRN7000 induces liver toxicity in mice, we found no signs of KRN7000-induced liver toxicity (Osman et al. 2000; Nakagawa et al. 2001; Giaccone et al. 2002). No serious drug-related adverse event occurred. The MTD, defined as the dose associated with dose-limiting toxicity in at least two of six patients, was not reached because further dose escalation could not be performed using the available drug formulation. Pharmacokinetic analyses revealed no evidence for drug accumulation or serum saturation of KRN7000 with weekly administrations. Moreover, KRN7000 was not detectable in the urine at any dose level.

The number of circulating iNKT cells among the cancer patients that were included in our trial was significantly lower than that of healthy volunteers (Fig. 10.2, $P = 0.0001$, Wilcoxon two-sample test). As iNKT cells constitute the primary cellular target of KRN7000, an iNKT-high group and an iNKT-low group were defined using the median number of iNKT in patients (333 cells/ml peripheral blood) as a cut-off value (Giaccone et al. 2002). Strikingly, the number of peripheral blood

Table 10.1 Patient characteristics of phase 1 study with i.v. KRN7000 in patients with solid tumors

Total number entered	24
Median age (range)	54 (31–69)
Gender (male/female)	14/10
Performance status (0/1/2)	5/16/3
Prior chemotherapy	22
Prior immunotherapy	6
Tumor types	
Melanoma, esophagus	Three each
Breast, thymus, head & neck, stomach, lung, kidney	Two each
Bladder, pancreas, prostate, biliary tract, chondrosarcoma, liver	One each

Main inclusion criteria: patients were required to have a WHO performance score ≤2, a life expectancy of >3 months, a histological or cytological diagnosis of progressing solid tumor for which no standard therapy was available. Prior immunotherapy was not allowed in a period of 12 weeks prior to inclusion into this trial, and chemo- or radiation therapy was not allowed in the 4 weeks prior to inclusion into this trial. Patients were required to have a normal bone marrow reserve and normal organ functions. Patients with a history of autoimmune disease were excluded

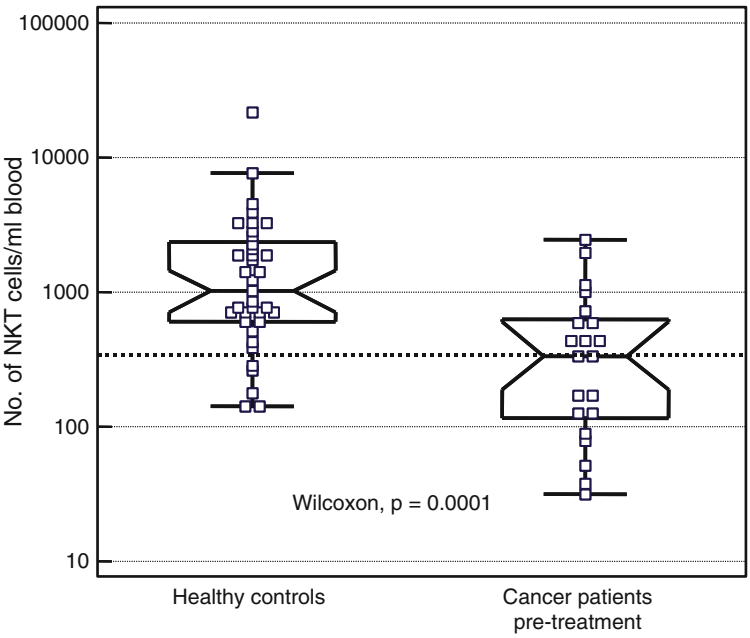

Fig. 10.2 iNKT cell numbers in peripheral blood of patients ($n=21$) and healthy volunteers ($n=37$; 17 males, 20 females, age range: 21–52 year). The number of iNKT cells in cancer patients (median = 333 cells/ml) was significantly lower than that of healthy volunteers (median = 1,013 cells/ml) ($P=0.0001$). Reproduced from Giaccone et al. (2002) with permission from AACR

iNKT cells decreased to undetectable levels within 24 h after the first administration of KRN7000 at all of the dose levels, and recovery to the pre-administration levels was not observed within a week (Fig. 10.3a). Although KRN7000 did not induce consistent changes in the numbers of circulating granulocytes, monocytes, B cells, and $CD4^+$ or $CD8^+$ T cells, both the number and lytic activity of NK cells, reported to play an essential role in the inhibition of tumor metastases in mice treated with KRN7000 (Nakagawa et al. 1998a; Hayakawa et al. 2001), transiently decreased in seven out of ten patients with high pretreatment iNKT numbers. No changes in NK cell numbers or lytic activity were observed in the iNKT cell-low group. Importantly, immunological effects induced by the administration of KRN7000 appeared to be restricted to the subgroup of patients with relatively high pretreatment iNKT cell numbers. For example, while only slight fluctuations of GM-CSF and TNF-α serum levels were seen after KRN7000 administration in the iNKT-low group, five of ten patients in the iNKT-high group showed an increase in both GM-CSF and/or TNF-α levels, peaking 4–6 h after KRN7000 administration (Fig. 10.3b). In one patient, who had a relatively high pretreatment iNKT cell count, a faint but detectable increase in serum IFN-γ was observed at 6 h after the first KRN7000 administration, followed by a peak in serum IL-12 at 8 h after KRN7000 administration. Detectable levels of IFN-γ or IL-12 (>15 or 0.8 pg/ml, respectively) were never observed in the iNKT-low group. Intriguingly, KRN7000-induced immune responses (iNKT cell depletion, changes in serum cytokine levels) could be observed even at the lowest dose level (50 $\mu g/m^2$) and therefore the effects of KRN7000 in our study appeared to be mostly determined by the size of the circulating iNKT cell pool. As none of the immunological parameters tested showed clear KRN7000 dose-dependent effects, an OBAD could not be identified.

No partial or complete tumor responses were observed in this trial, but disease stabilizations were observed in seven patients [median time of stable disease 123 days (range 83–216 days)]. The absence of clinical responses in combination with the low frequency of patients in which signs of immunological activation were observed, precluded further evaluation of this schedule of administration in a phase 2 study.

Emerging preclinical data subsequently indicated that i.v. administration of α-GalCer-pulsed DC exerted greater antitumor activity compared to i.v. α-GalCer alone. Furthermore, as treatment with α-GalCer-pulsed DC resulted in an expansion of iNKT cells in the lungs of treated animals and a more prolonged IFN-γ-producing iNKT cell response, this approach, with variations in APC platform, was subsequently evaluated in clinical trials (Toura et al. 1999; Nieda et al. 2004; Chang et al. 2005; Ishikawa et al. 2005; Uchida et al. 2008) as is summarized below.

3.2 Intravenous Administration of α-GalCer Pulsed Immature Monocyte Derived DC

The first of these trials was an open-label phase 1 clinical study involving 12 patients with metastatic malignancy (Nieda et al. 2004). Patients were i.v. treated

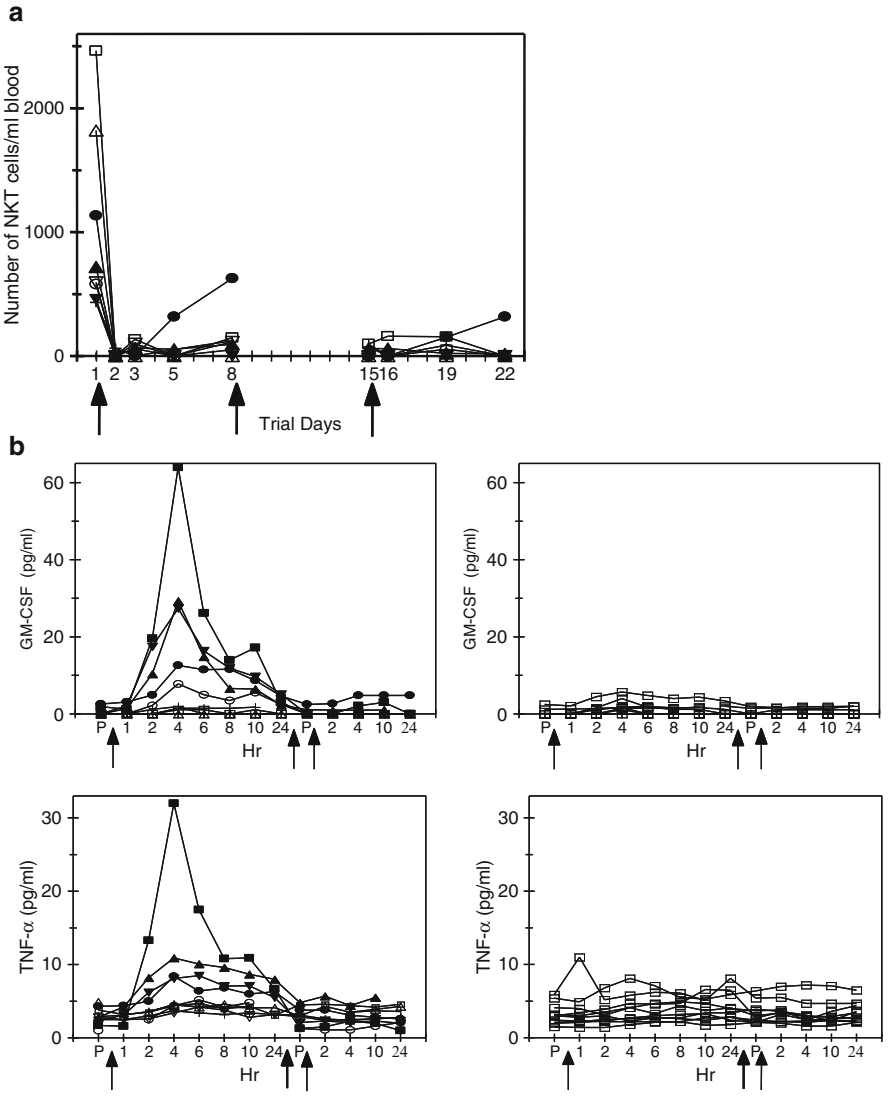

Fig. 10.3 (**a**) Reduction of peripheral blood iNKT cell numbers upon KRN7000 injection in patients with high (>333/ml) pretreatment iNKT cell numbers. Data of nine out of ten patients are shown, since in one patient only the pretreatment iNKT number was known. PBMC were collected on day 1 (before the first administration), on days 2, 3, 5, 8 (before the second administration), 15 (before the third administration), 16, 19, and 22. *Arrows* represent the three first weekly injections. (**b**) Serum GM-CSF and TNF-α levels in patients with high (>333/ml, $n=10$, left panel) and low (<333/ml, $n=11$, right panel) pretreatment iNKT cell numbers. Serum samples were collected before the first administration, 1, 2, 4, 8, 10, and 24 h after the first administration, and on days 15 (before the third administration), 16, 19, and 22. *Arrows* represent the three first weekly injections. Reproduced from Giaccone et al. (2002) with permission from AACR

with a median of 5×10^6 CD1d-expressing immature monocyte-derived (mo)DC which resulted in strikingly reproducible immune responses. Peripheral blood iNKT, NK, T, and B-cell levels reproducibly but transiently fell to a nadir around 1–2 days after each treatment. This decrease was most notable for iNKT cells (up to 18-fold decrease; mean decrease, 3-fold), and less so for NK cells (up to 9-fold decrease; mean decrease, 1.9-fold) and T cells (up to 3-fold decrease; mean decrease, 1.8-fold). The degree to which NK cells decreased correlated with the relative decrease in iNKT cells, supporting published data that the specific interaction between α-GalCer-pulsed DC and iNKT cells was responsible for the observed changes in the NK cell population. Serum levels of IFN-γ transiently but significantly increased in all evaluable cases ($n = 10$) and serum IL-12 levels increased in six of nine evaluable cases. Of interest, a substantial priming effect in the degree to which iNKT levels fell below baseline levels was observed as the second i.v. administration of α-GalCer-pulsed immature moDC resulted in a greater drop in circulating iNKT cell numbers in more than 90% of cases. This priming effect translated into secondary immune effects that were faster and greater in magnitude. For example, upon the second administration of α-GalCer-pulsed immature moDC larger numbers of NK and T cells were activated, and when the initial (more rapid and deep) reduction in T and NK cell numbers had passed, subsequent peak levels of NKT and NK cells were higher and more sustained. Furthermore, serum IFN-γ levels were higher and systemic symptoms suggestive of immune activation were more frequent. Notably, the majority of patients experienced a temporary exacerbation of tumor symptoms following the injection of α-GalCer-pulsed immature moDC, many of which were clearly inflammatory. Furthermore, in several patients sustained decreases in tumor markers, extensive tumor necrosis, and improvements in hepatocellular enzyme levels in patients with hepatic tumor infiltration were observed (Nieda et al. 2004).

3.3 Intravenous Administration of α-GalCer Pulsed Mature Monocyte Derived DC

Mature moDC have been shown to provide an immunologically more potent stimulus to responding T cells compared to immature moDC (Ridge et al. 1998) and can therefore be expected to induce more pronounced iNKT cell responses when loaded with α-GalCer. This approach was evaluated in a study in which mature α-GalCer-pulsed moDC were i.v. administered to five patients with advanced cancer (Chang et al. 2005). In all patients injection resulted in the substantial expansion of several iNKT cell subsets. These increased levels were sustained for up to 6 months after vaccination, which is in contrast with the reported transient iNKT cell activation that was observed with either i.v. α-GalCer alone, or i.v. α-GalCer-pulsed immature moDC. iNKT cell activation was associated with an increase in serum levels of IL-12p40 and IFN-γ inducible protein-10. In addition, an in vivo increase in cytomegalovirus specific memory $CD8^+$ T cells was observed in response to α-GalCer-loaded mature

moDC. This study demonstrated that administration of mature α-GalCer-loaded moDC resulted in a sustained expansion of iNKT cells in patients with advanced cancer, and suggested that this activation of iNKT cells boosts adaptive T cell immune responses in vivo (Chang et al. 2005).

3.4 Intravenous Administration of α-GalCer-Pulsed APC Fractions Generated from PBMC Using GM-CSF and IL-2

Ishikawa et al. performed a phase 1 study to assess the feasibility and toxicity of i.v. administration of α-GalCer-pulsed APC fractions in 11 patients with advanced or recurrent non-small cell lung cancer (Ishikawa et al. 2005). In this study APC were generated from peripheral blood mononuclear cells (PBMC) using GM-CSF and IL-2. No major (above NCI-CTC grade 2) toxicity or severe side effects were observed. In addition no clear relationship was found between the number of circulating iNKT cells and the administration of α-GalCer-pulsed APC. No patients had a complete or partial response; in five patients stable disease was noted. The authors concluded that i.v. administration of α-GalCer-pulsed APC had no major side effects and was well tolerated in patients with advanced stages of lung cancer.

3.5 Intranasal Administration of α-GalCer-Pulsed APC Fractions Generated from PBMC Using GM-CSF and IL-2

Similar APC fractions were used in a phase 1 study in which α-GalCer-pulsed APC were administered in the nasal submucosa of nine patients with unresectable or recurrent head and neck squamous cell carcinoma (HNSCC) (Uchida et al. 2008). Patients received two injections of 1×10^8 α-GalCer-pulsed autologous APC in the nasal submucosa with an interval of 1 week. During the clinical study period no serious adverse events (NCI-CTC version 3.0 > grade 3) were observed. After the first and the second administration of α-GalCer-pulsed APC, an increased number of circulating iNKT cells was observed in four patients, while an enhancement of NK cell lytic activity was detected in the peripheral blood of nine patients. The administration of α-GalCer-pulsed APC into the nasal submucosa was found to be safe and, importantly, induced anti-tumor activity in several patients (Uchida et al. 2008).

3.6 Intravenous Administration of Autologous In Vitro Expanded iNKT Cell Fractions

As mentioned, numerical defects have been observed in the peripheral blood of many cancer patients and correlate with poor clinical outcome in e.g., HNSCC,

colorectal cancer, and neuroblastoma (Metelitsa et al. 2004; Molling et al. 2005, 2007; Tachibana et al. 2005). Motohashi et al. (2006) evaluated the effects of reconstitution of the iNKT cell pool in a phase 1 study using i.v. transfer of autologous in vitro expanded cell fractions that were enriched for iNKT. A total of six patients with advanced or recurrent non-small cell lung cancer received i.v. injections of activated iNKT cells (level 1: $1 \times 10^7/m^2$ and level 2: $5 \times 10^7/m^2$). After the first and second injection of activated iNKT cell fractions, an increased number of peripheral blood iNKT cells was observed in two of three cases in dose level 2. Furthermore, in all three patients included in dose level 2, an increase in the number of IFN-γ-producing cells in PBMC could be observed. Adoptive transfer of these activated iNKT-cell-enriched fractions was well tolerated and carried out safely with minor adverse events in patients with advanced diseases. However, no patient was found to meet the criteria for either a partial or a complete response.

Common observations from these trials indicate that administration of α-GalCer results in a rapid decrease in circulating iNKT cell numbers. Though not definitively clarified in human, this apparent decrease could be caused by, e.g., activation-induced TCR downregulation or apoptosis (Osman et al. 2000; Wilson et al. 2003). In our phase 1 trial with i.v. administered KRN7000 we found no consistent KRN7000-induced changes in circulating numbers of other lymphocyte subsets, but trials with α-GalCer-pulsed DC resulted in a transient decrease in peripheral blood NK, T, and B cells. Of interest, this transient drop in circulating NK-, T-, and B cell numbers was also observed in a phase 1–2 trial with i.v. administered KRN7000 in patients with chronic HCV infection (Veldt et al. 2007), revealing either schedule- and/or disease-dependent differences in response to KRN7000. Administration of α-GalCer-pulsed moDC resulted in the secondary activation of NK cells and T cells and, following the initial drop in cell numbers, subsequent peak levels of NKT and NK cells were higher and more sustained (Nieda et al. 2004; Chang et al. 2005). Striking was the observation that immunological effects induced by the administration of α-GalCer appeared to be most pronounced in the subgroup of patients with relatively high pretreatment iNKT cell numbers. Importantly, clinical responses were only observed when KRN7000 was presented by professional APC (Giaccone et al. 2002; Nieda et al. 2004; Chang et al. 2005; Ishikawa et al. 2005; Motohashi et al. 2006; Uchida et al. 2008).

In conclusion, KRN7000 based immunotherapeutic approaches in cancer patients have proven to be well tolerated. Clinical responses have been observed using KRN7000-pulsed APC, but these are presently not sufficiently consistent and predictable to allow widespread clinical application.

4 Discussion and Future Perspectives

An important finding of the phase 1 study performed by Giaccone et al. (2002) was the observation that the initial size of the circulating iNKT-cell pool in cancer patients was significantly lower compared with healthy controls. Quantitative and

qualitative defects in the iNKT-cell pool have subsequently been observed in many tumor types, including colon cancer, lung cancer, breast cancer, melanoma, head and neck squamous cell cancer, prostate cancer, myelodysplastic syndromes, and progressive malignant myeloma (reviewed by van der Vliet et al. 2004). Reduced iNKT cell numbers were found to be independent of tumor type or load, and did not recover after effective removal of tumor by surgery or radiotherapy (Molling et al. 2005). Although it cannot be excluded that iNKT cell numbers would eventually recover after tumor removal, the present data suggest that a low number of circulating iNKT cells precedes the development of cancer and thus might represent a risk factor for the development of malignancies. The latter hypothesis would be in accordance with the reported role of iNKT cells in tumor immunosurveillance (Crowe et al. 2002). Regardless of the cause of the reduced size of the iNKT cell population, the low number of iNKT cells in cancer patients likely contributed to the lack of immunoreactivity observed after i.v. administrations of KRN7000 as signs of immune activation (e.g., increases in serum cytokine levels of GM-CSF and TNF-α and changes in circulating NK cell numbers) were only observed in those patients that had relatively high pre-treatment iNKT cell numbers (Giaccone et al. 2002). Importantly, the generally observed reduced size of the iNKT cell population in cancer patients is also by itself clinically relevant as it was associated with a strikingly poor clinical outcome in patients with HNSCC treated with curative-intent radiotherapy (Molling et al. 2007). Similarly, reduced intratumoral iNKT cell numbers have been linked to poor outcome in colon cancer and neuroblastoma (Metelitsa et al. 2004; Tachibana et al. 2005). Novel clinical approaches could be based on increasing the size of the iNKT cell population e.g., by adoptive transfer of ex vivo expanded autologous iNKT cells. In order to optimize the effects of adoptive transfer it might be important to select Th1 cytokine-producing iNKT cells (such as $CD4^{-}CD8^{-}$ iNKT cells; Gumperz et al. 2002; Lee et al. 2002) for transfer or alternatively use type 1 polarized α-GalCer-pulsed DC for ex vivo expansion of iNKT cells as this simultaneously supports their expansion and Th1 polarization (van der Vliet et al. 2003). The immunomodulatory drug lenalidomide, a derivative of thalidomide, has been shown to enhance ligand-dependent activation and Th1 polarization of iNKT cells and can be considered for both in vitro and in vivo use in order to optimize iNKT-cell-based cancer immunotherapy (Song et al. 2008).

In several of the clinical trials a rapid reduction in (residual) circulating iNKT cells from the circulation was observed. In our phase 1 trial with repeated i.v. administration of KRN7000 the exact duration of the iNKT-cell depletion was not investigated, but iNKT-cell repopulation was not observed in the week following the injection of KRN7000 (Giaccone et al. 2002). Whether this decreased circulating iNKT cell frequency is caused by tissue redistribution, apoptosis, or temporary TCR downregulation has not been elucidated in man. However, murine studies have shown that the response of murine iNKT to α-GalCer is characterized by surface receptor down-modulation and subsequent expansion providing an explanation for the apparent loss of iNKT cells after in vivo TCR engagement (Wilson et al. 2003). As TCR down-regulation after antigenic stimulation is a well established phenomenon for conventional T cells and may be a means for protecting T cells against

overstimulation and activation induced cell death (AICD), TCR down-regulation by iNKT cells may protect these cells from overstimulation and prevent extensive tissue damage (Wilson et al. 2003). Though an early depletion of iNKT cells was also observed in the trials using α-GalCer-pulsed APC this was frequently followed by an in vivo expansion. The iNKT cell depletion was accompanied by an increase in the expression of activation markers on T and NK cells and a temporary drop in their frequency. These phenomena were also observed in our follow-up clinical trial of KRN7000 in patients with chronic HCV (Veldt et al. 2007), though repeated administrations in this case resulted in increasingly blunted responses as also observed in our phase 1 clinical trial in cancer where KRN7000-induced signs of immune activation only occurred after the first administration (Giaccone et al. 2002). Of interest, although the decrease in e.g., circulating NK cell numbers at first glance contrasts with murine studies, it should be noted that a temporary loss of NK cells in the spleen, accompanied the KRN7000-induced increase in liver NK cells in mice (Osman et al. 2000). Furthermore, repeated administrations of α-GalCer similarly resulted in blunted responses in mice by inducing iNKT cell anergy (Sullivan and Kronenberg 2005).

Importantly, in the trials in which iNKT cells were activated using α-GalCer-loaded moDC the initial decrease in iNKT cell numbers was frequently followed by an expansion of the iNKT cell pool. There are however some practical restraints that hamper further clinical exploration of treatment with α-GalCer-pulsed DC. In general, DC used for clinical trials are derived from monocytes that are differentiated and matured to mature DC during an in vitro culture period of up to 10 days using combinations of IL-4, GM-CSF and TNF-α. These DC can acquire different states of function, including tolerogenic (immature DCs), proinflammatory (mature DC), and inhibitory (mature exhausted DC). Clearly, the combination of a relatively long in vitro culture period and functional heterogeneity hampers broad clinical application and could cause detrimental effects (Moser and Brandes 2006). A distinct subset of T cells termed Vγ9Vδ2-T cells has recently been shown to be able to acquire professional Ag-presenting capacities, including the uptake, processing, and presentation of peptide-Ag to $CD4^+$ (via MHC class II) and $CD8^+$ T cells (via MHC class I). When compared to moDC, the use of Vγ9Vδ2-T-APC could provide major advantages with respect to clinical (tumor immunotherapeutic) application. For example, Vγ9Vδ2-T cells are substantially more numerous compared to DC-precursors and, more importantly, they can be matured into professional APC within 24 h using phosphoantigens. Furthermore, Vγ9Vδ2-T APC have better lymph node homing characteristics and a more uniform and consistent proinflammatory functional status (Brandes et al. 2005; Moser and Brandes 2006). Vγ9Vδ2-T APC might therefore constitute an attractive alternative APC platform for α-GalCer. It is tempting to consider co-loading of APC with both α-GalCer and tumor associated antigen in order to further promote the induction of tumor-specific immune responses.

Interesting clinical responses have also been observed after intranasal administration of α-GalCer-loaded APC in patients with HNSCC suggesting that the activation of iNKT cells within the tumor microenvironment could be quite effective (Uchida et al. 2008). Indeed, other clinical trials with KRN7000 relied on systemic activation of iNKT cells, providing no specific trigger for iNKT cells to accumulate

in the tumor where they can be expected to most effectively propagate the induction of antitumor immunity. Stirnemann et al. explored an indirect local targeting approach using constructs consisting of α-GalCer/CD1d molecules fused to tumor Ag specific scFv fragments in a murine melanoma model, and reported that specific tumor targeting of iNKT indeed induced substantially more potent antitumor responses compared to α-GalCer alone. Furthermore, while repeated i.v. administration of α-GalCer induced the expected iNKT cell anergy, repeated administration of CD1d-targeted constructs resulted in sustained iNKT cell activation (Stirnemann et al. 2008). Tumor targeting of iNKT cells or α-GalCer pulsed APC might therefore constitute a powerful yet unexplored clinical approach.

Other approaches that need to be explored with respect to their capacity to enhance the efficacy of iNKT-cell-based cancer immunotherapy include (a) alternative routes of administration of KRN7000, including the subcutaneous route of administration as this prevents the iNKT cell anergy observed with i.v. administration and results in superior tumor protection (Bontkes et al. in press), (b) combining iNKT cell activation with depletion of immunosuppressive $CD4^+CD25^+Foxp3^+$ (natural) regulatory T cells as the latter population is expanded in cancer patients and has been shown to inhibit the development of conventional T as well as iNKT cell responses (La Cava et al. 2006; van der Vliet et al. 2007), and (c) the use of alternative glycolipid ligands such as the nonglycosidic compound threitolceramide which was shown to efficiently activate iNKT cells, resulting in DC maturation and the priming of Ag-specific T and B cells, while preventing ligand-induced iNKT cell lysis of pulsed DCs as well as the activation-induced anergy of iNKT (Silk et al. 2008).

In conclusion, though immunological, biochemical, and even clinical responses have been observed in patients treated with α-GalCer/KRN7000 results are not consistent. Several factors might contribute to the lack of consistency in the induction of antitumor immune responses in the clinical trials that have been performed to date. The fact that iNKT cells have been shown to be clinically relevant in multiple tumor types and to be able to induce clinical responses even in aggressive tumor types without causing substantial toxicity provides sufficient fuel to justify the design and evaluation of novel iNKT-cell- based approaches. The question is no longer whether iNKT cells can induce clinical responses, but how to make them more consistent and predictable.

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Chapter 11
Clinical Trials of Invariant Natural Killer T Cell-Based Immunotherapy for Cancer

Shinichiro Motohashi, Yoshitaka Okamoto, and Toshinori Nakayama

Abstract Human Vα24$^+$Vβ11$^+$ invariant NKT (iNKT) cells are distinct lymphocytes that play an important role in tumor immunity. iNKT cells have the capability to positively modulate the function of a wide variety of immune cells in both direct and indirect manner, thereby bridging innate and acquired immunity. Abnormalities in the number and function of iNKT cells have been observed in patients with malignant diseases, and their presence is associated with a poor clinical outcome. Therefore, therapeutic strategies focused on the reconstitution of the impaired iNKT cell pool and amelioration of their function would be a reasonable rationale for the treatment of cancer. In this chapter, the progress made in the clinical trials of iNKT cell-based immunotherapy is briefly reviewed, and the role of iNKT cells in tumor immunotherapy is highlighted.

Abbreviations

α-GalCer	α-Galactosylceramide
APCs	Antigen presenting cells
NKT	Natural killer T
DCs	Dendritic cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IL	Interleukin
PBMCs	Peripheral blood mononuclear cells

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1 Introduction

Invariant natural killer T (iNKT) cells are innate immune cells that are critical for the immune regulation that links the innate and adaptive immune responses (Taniguchi et al. 2003a, b). Human iNKT cells co-express some NK markers and an invariant TCR α chain encoded by the Vα24Jα18 gene segment paired with Vβ11. In contrast to conventional $CD8^+$ and $CD4^+$ T cells, the iNKT cell TCR does not recognize peptide antigens which are presented by the polymorphic major histocompatibility complex (MHC) class I or class II. Instead, this invariant TCR recognizes glycolipid antigen presented by CD1d, a monomorphic nonclassical antigen-presenting molecule (Brigl and Brenner 2004). The α-galactosylceramide (α-GalCer; KRN7000), originally extracted from the marine sponge *Agelas mauritianus,* was discovered to be a potent synthetic ligand for iNKT cells during studies initiated by Kirin Pharmaceuticals to identify natural anticancer agents (Kawano et al. 1997; Kobayashi et al. 1995; Morita et al. 1995). α-GalCer is the most potent stimulator of iNKT cells and has proven to have direct cytotoxic activity against a wide range of tumors (Kawano et al. 1998, 1999). Various ligands for iNKT cells have been synthesized, and some ligands (such as α-C-GalCer) showed a more potent Th1-type response than α-GalCer (Fuhshuku et al. 2008; Fujii et al. 2006). At present, however, α-GalCer is the only agent available for clinical trials.

iNKT cells directly and indirectly interact with many other immune cells such as NK cells, T cells, B cells, and dendritic cells (Barral et al. 2008; Carnaud et al. 1999; Fujii et al. 2003; Smyth et al. 2002). Upon stimulation with α-GalCer, iNKT cells rapidly produce IFN-γ and IL-4, and up-regulate co-stimulatory molecules, such as CD40 ligand (Fujii et al. 2004). In addition to these molecules which augment the antitumor immune responses, iNKT cells also regulate the function of immune suppressive cells (De Santo et al. 2008; Song et al. 2009). This networking between iNKT cells and other immune cells may be an important factor in the promotion of each of these populations to exert a potent antitumor immune response (Cerundolo et al. 2009) (Fig. 11.1).

A decreased number and diminished function of iNKT cells have been observed in patients with various malignant diseases, and it has been shown that these are related to a poor clinical outcome (Molling et al. 2007; Motohashi et al. 2002). On the other hand, an increased number of tumor infiltrating iNKT cells has been shown to correlate with better survival (Metelitsa et al. 2004; Tachibana et al. 2005). Therefore, the reconstitution of a functionally efficient iNKT cell population could represent an appropriate and promising strategy for immunotherapy in patients with malignant diseases (Motohashi and Nakayama 2008, 2009).

In this chapter, we summarize and discuss the results of translational research aimed to utilize the powerful antitumor activity of α-GalCer-activated iNKT cells. One of the most important points of these studies is to elucidate the iNKT cell specific immune response, in short, to achieve quantitative detection of the activation of iNKT cells in vivo, and the subsequent induction of adjuvant effects, such as the enhancement of NK cell cytotoxicity and Th1 responses.

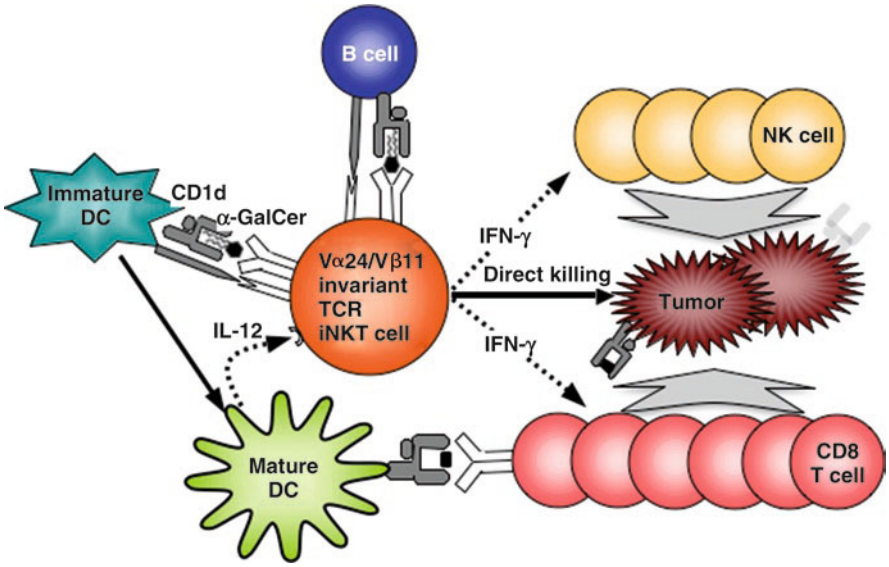

Fig. 11.1 The role of iNKT cells as a bridge between innate and adaptive immunity. Immature DCs present α-GalCer on CD1d towards the Vα24/Vβ11 invariant T cell receptor on iNKT cells. After the recognition of α-GalCer, iNKT cells are activated and exert a direct tumoricidal activity through molecules such as perforin/granzyme, FasL and/or TRAIL. The activated iNKT cells induce IFN-γ production, which subsequently activates other immune cells, such as NK cells in innate immunity as well as $CD8^+$ T cells in the adaptive immune system. NK cells can kill MHC^- tumor cells and $CD8^+$ T cells recognize tumor antigen in conjunction with MHC class I and attack these MHC^+ tumor cells. iNKT cells also stimulate immature DCs or B cells via the CD40/40 L pathway, which in turn induces the production of IL-12 from the fully matured DCs leading to the activation of NK and iNKT cells

2 Administration of Soluble α-GalCer for Various Solid Tumors

A phase I clinical trial of soluble α-GalCer (KRN7000) injection was performed as a nonrandomized dose-escalation study (Giaccone et al. 2002). Twenty-four patients with advanced solid tumor were enrolled in the study, and severe adverse events related to KRN7000 administration were observed in one patient at dose level 1 (50 $\mu g/m^2$), characterized by fever and shivering. In this patient, increased serum levels of IFN-γ, IL-12, GM-CSF, and TNF-α were detected after the first administration of α-GalCer. No other severe adverse event was reported. The immunological findings showed that peripheral blood iNKT cells rapidly disappeared from the circulation after KRN7000 administration. Increased serum GM-CSF and TNF-α levels were only observed in patients with relatively high levels of iNKT cells (Table 11.1). No objective tumor reduction was observed, but seven patients had stable disease (SD) for a median duration of 123 days (Table 11.1). These results suggest that other therapeutic strategies, such as α-GalCer-pulsed APCs or adoptive

Table 11.1 Immunological responses and antitumor effects of α-GalCer-related treatments

	Treatment	Number of patients	Tumor types	Immunological responses	Antitumor effects (number of patients)
Giaccone et al.	α-GalCer	24	Melanoma, kidney sarcoma, various carcinoma	Increase in serum GM-CSF and TNF-α iNKT cell depletion	SD (7)
Ishikawa et al.	α-GalCer-APC	11	Lung ca.	iNKT cell expansion augmentation of IFN-γγ mRNA level	SD (3)
Motohashi et al.	α-GalCer-APC	17	Lung ca.	iNKT cell expansion augmentation of IFN-γ spot forming cells	SD (5)
Uchida et al.	α-GalCer-APC	9	HNSCC	iNKT cell expansion augmentation of IFN-γ spot forming cells	PR (1), SD (7)
Nieda et al.	α-GalCer-immature DC	12	Melanoma, RCC, various carcinoma	iNKT cell expansion up-regulation of CD69 expression on T and NK cells, increased NK cell cytotoxicity, increase in serum IL-12 and IFN-γ	Reduction of tumor markers (2), extensive necrosis of tumor (1)
Chang et al.	α-GalCer-mature DC	5	Myeloma, anal ca., RCC	iNKT cell expansion increase in memory CMV-specific $CD8^+$ T cells, increase in serum IL-12 and IP-10	Reduction of serum or urine M protein (3), SD (1)
Motohashi et al.	α-GalCer activated iNKT cell	6	Lung ca.	iNKT cell expansion augmentation of IFN-γ spot forming cells	SD (2)
Kunii et al.	α-GalCer-APC/ α-GalCer activated iNKT	8	HNSCC	iNKT cell expansion augmentation of IFN-γ spot forming cells	PR (3), SD (4)

Abbreviations: *ca* cancer, *HNSCC* head and neck squamous cell carcinoma, *RCC* renal cell carcinoma, *SD* stable disease, *PR* partial response, *CMV* cytomegalovirus, *IP-10* IFN-γ-inducible protein-10

transfer of α-GalCer-activated NKT cells, aimed at reconstitution of the deficient iNKT cell population in cancer patients might be warranted.

3 α-GalCer-Pulsed Antigen-Presenting Cell Immunotherapy

We and others have previously observed that α-GalCer-pulsed DCs produce a greater antitumor effect in mice than α-GalCer (Fujii et al. 2002; Toura et al. 1999). α-GalCer-pulsed DCs effectively activated NKT cells in vivo, resulting in the inhibition of tumor metastasis. In addition, α-GalCer induced the expansion of iNKT cells in the lungs and resulted in the efficient inhibition of tumor growth in vivo in murine lung cancer models (Akutsu et al. 2002; Motohashi et al. 2002). Based on the in vivo effects of α-GalCer-pulsed DC in the therapeutic setting, we started to consider a clinical trial using α-GalCer pulsed DCs in patients with lung cancer.

3.1 The Use of Antigen-Presenting Cells to Produce Active Immunotherapy

Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system and ex vivo-generated DCs pulsed with tumor antigens are capable of activating tumor-specific cytotoxic T cells, which induce protective antitumor immunity (Banchereau and Steinman 1998; Figdor et al. 2004). Most clinical studies to date have used monocyte-derived DCs. When cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for several days, the monocytes develop into immature DCs, and can be further matured by a subsequent 1- to 2-day culture period using proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and prostaglandin E2. Since it is difficult to obtain large numbers of monocyte-derived DCs (moDCs) under good manufacturing practice (GMP) procedure, we established an alternative method for preparing APCs for efficient stimulation of iNKT cells via α-GalCer-loaded CD1d expressing APCs. In our clinical trial, whole PBMCs were cultured with GM-CSF and IL-2 for 1 or 2 weeks, then all cultured cells were pulsed with α-GalCer and used as antigen-presenting cells (IL-2/GM-CSF-cultured PBMCs). Ishikawa et al. demonstrated that α-GalCer-pulsed IL-2/GM-CSF-cultured PBMCs induce efficient expansion of autologous Vα24Vβ11 iNKT cells, which were superior to moDCs (Ishikawa et al. 2005b). $CD11c^+$ cells in α-GalCer-pulsed IL-2/GM-CSF-cultured PBMCs develop a mature phenotype through the production of TNF-α produced by $CD11c^-CD3^+$ T cells in the α-GalCer-pulsed IL-2/GM-CSF-cultured PBMCs. Due to the enhanced maturation of immature DCs in the culture, the α-GalCer-pulsed IL-2/GM-CSF-cultured PBMCs provided efficient expansion and activation of iNKT cells and subsequent

iNKT cell-mediated Th1 responses. Moreover, the preparation of α-GalCer-pulsed IL-2/GM-CSF-cultured PBMCs is simpler than preparation of moDCs since the process for separating nonadherent cells which is essential for generating moDC is no longer required. Therefore, α-GalCer-pulsed IL-2/GM-CSF-cultured PBMCs appear to represent a potent alternative material for active immunotherapy aimed at the in vivo activation of iNKT cells. For purposes of simplicity, in the rest of this report, we refer to these whole PBMCs cultured with GM-CSF and IL-2 as "α-GalCer-pulsed APCs."

3.2 α-GalCer-Pulsed APCs for Lung Cancer

From 2001 to 2002, we performed a phase I dose-escalation study of α-GalCer-pulsed APCs in patients with advanced or recurrent non-small cell lung cancer (NSCLC) at Chiba University Hospital, Japan (Fig. 11.2a) (Ishikawa et al. 2005a). In this first clinical study, 11 patients refractory to the standard treatments were enrolled in the study, and the safety profile, immunological and clinical responses were investigated. On day 0, apheresis was performed and α-GalCer-nonpulsed APCs were administered intravenously on day 7, and α-GalCer-pulsed APCs were administered on day 14, 21, 63, and 70. No severe adverse events were observed in any of the patients, and only minor adverse events such as general fatigue, cystitis, or headache occurred. A striking increase in the peripheral blood iNKT cell number was observed in one case, and a modest increase was noted in two cases at the level three doses ($1 \times 10^9/m^2$) of α-GalCer-pulsed APCs. Simultaneously, IFN-γ mRNA from purified iNKT cells from patient peripheral blood was increased after the first and second injections of α-GalCer-pulsed APCs. Although objective tumor regression was not observed in any of the 11 patients, the patient who showed a remarkable increase in peripheral blood iNKT cells had long-term stable condition of intrathoracic lesion for more than two and half years with a good quality of life, and survived for 59 months without additional chemotherapy (Table 11.1). In summary, this study indicates that α-GalCer-pulsed APCs were well tolerated, and it could be done safely even in patients with advanced diseases.

Based on these findings, we extended our study and performed a phase I–II study of α-GalCer-pulsed APC administration in patients with NSCLC to evaluate the safety, immunological responses, and clinical outcomes (Motohashi et al. 2009). In the 17 cases enrolled, 4 injections of α-GalCer-pulsed APCs were well tolerated and no severe adverse events related to the treatment were observed. One patient experienced the recurrence of a deep vein thrombosis (grade 3). The Chiba University Quality Assurance Committee on Cell Therapy concluded that no clear relationship between the cell therapy and DVT was established. Regarding the iNKT cell-specific immune response, the absolute number of peripheral blood iNKT cells increased in 6 patients after α-GalCer-pulsed APC administration. The number of IFN-γ producing cells in PBMCs after restimulation with α-GalCer in vitro was assessed by an enzyme-linked immunospot (ELISPOT) assay. In 10 patients, the number of IFN-γ producing cells was obviously

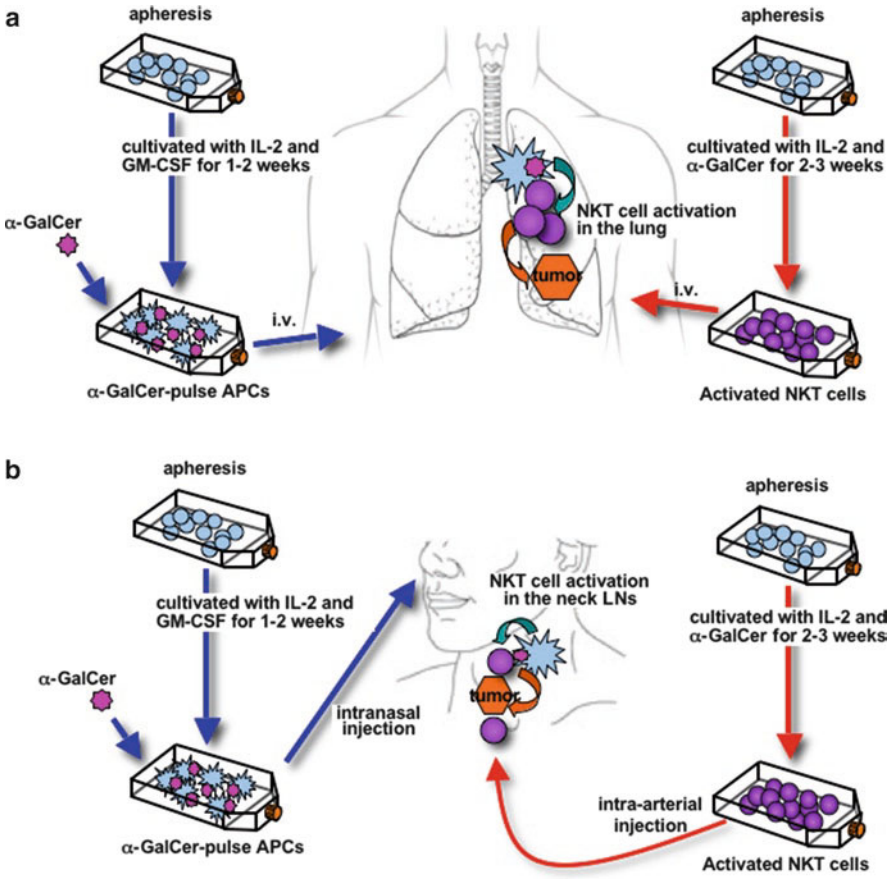

Fig. 11.2 Overview of the cell processing and stimulation procedures. (**a**) Treatment of lung cancer with α-GalCer-pulsed APCs or activated iNKT cells: PBMCs were collected by apheresis and cultured with GM-CSF and IL-2 for 1 or 2 weeks. One day before administration, the cultured cells were pulsed with α-GalCer, then cells were administered intravenously. To induce in vitro activation of iNKT cells, PBMCs were cultured with α-GalCer and IL-2 for 2 or 3 weeks, and then the activated iNKT cells were administered intravenously. (**b**) Treatment for head and neck cancer with α-GalCer-pulsed APCs or activated iNKT cells: PBMCs were cultured with GM-CSF and IL-2 for 1 week, then the cultured cells (1×10^8 cells/200 μl) were injected into the nasal submucosa, ipsilateral to the tumor location. To induce in vitro-activated iNKT cells, PBMCs were cultured with α-GalCer and IL-2 for 2 or 3 weeks, and then the activated iNKT cells were administered intra-arterially

increased (good responder group) while the remaining 7 patients did not show any increase in the number of IFN-γ producing cells (poor responder group). We followed up the prognosis of all 17 patients, and the median survival time (MST) was 18.6 months. The MST of the good responder group was 29.3 months, which was significantly better than that of the poor responder group (MST, 9.7 months) (Fig. 11.3). From these results, the increased IFN-γ producing capacities upon α-GalCer stimulation could be concluded to correlate with the prolonged overall survival after α-GalCer-pulsed APCs.

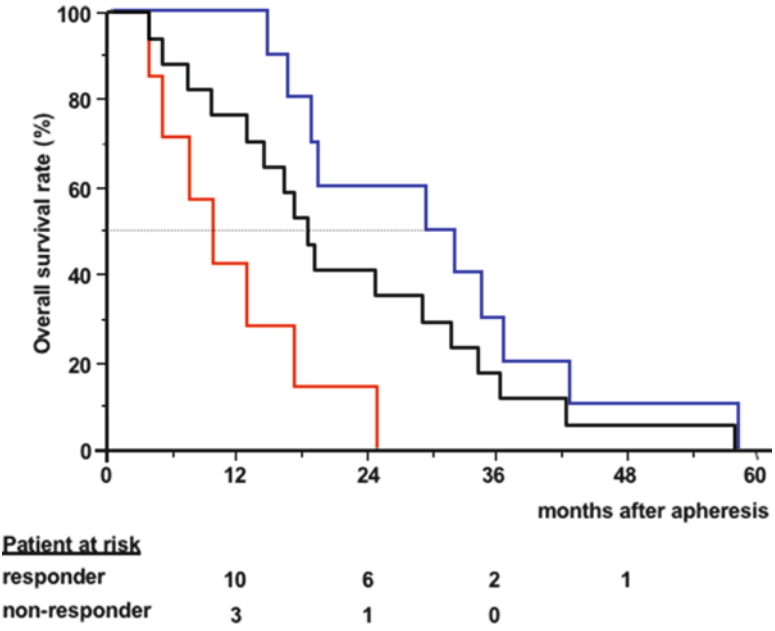

Fig. 11.3 Overall survival curve after administration of α-GalCer-pulsed APCs. Overall survival curve of all patients i.v. administered four times with α-GalCer-pulsed APCs. *Blue line*, overall survival of good responders ($n = 10$) with increased IFN-γ-producing cells. *Red line*, poor responders ($n = 7$) without increased IFN-γ production

3.3 α-GalCer-Pulsed APCs for Head and Neck Cancer

In 2004, we designed a new protocol using α-GalCer-pulsed APCs in patients with head and neck cancer at Chiba University Hospital. Uchida et al. (2008) reported the results of nasal submucosal injections of α-GalCer-pulsed APCs in patients with head and neck squamous cell carcinoma (HNSCC). In this trial, we harnessed the nasal submucosa as a novel route for administration of APCs. The antigen-presenting cells that were injected into the nasal submucosa quickly migrated to the ipsilateral regional neck lymph nodes, and remained there for more than 1 week (Horiguchi et al. 2007). Therefore, injection of α-GalCer-pulsed APCs into the nasal submucosa appears to be sufficient for the induction of regional antitumor immune responses. Based on these observations, α-GalCer-pulsed APCs were administered into the inferior turbinate submucosa in patients with unresectable or recurrent head and neck squamous cell carcinoma (Fig. 11.2b). Between 2005 and 2006, 9 patients were administered α-GalCer-pulsed APCs twice, with a 1-week interval between injections, into the nasal submucosa ipsilateral to the tumor. This treatment procedure was well tolerated without pain and no severe adverse events were observed. According to the immunological results, relatively smaller numbers of APCs were able to evoke conspicuous immune responses compared to those produced following

systemic administration, as evidenced by the results in the peripheral blood iNKT cell number and ELISPOT assay (Table 11.1). In addition to the immune responses, apparent tumor regression was observed in one patient (Table 11.1). Therefore, the study design was refined, and this injection method was included for use in the combination iNKT cell therapy (refer Sect. 5).

3.4 Other α-GalCer-Pulsed DCs for Malignant Tumors

The results of a phase I study of α-GalCer-pulsed monocyte-derived immature DCs in patients with various metastatic malignancies were reported (Nieda et al. 2004; Okai et al. 2002). Twelve patients in this study received α-GalCer-pulsed immature DCs induced from the plastic adherent monocytes cultured with GM-CSF and IL-4. The majority of the patients experienced tumor flares and minor adverse events. A mild and transient increase in peripheral blood iNKT cells was observed after α-GalCer-pulsed DC injection and then the number of activated T cells and NK cells increased after an augmentation of serum IFN-γ and IL-12 (Table 11.1). Tumor markers significantly decreased in two patients for 4 and 12 months, respectively, and one patient with renal cell carcinoma showed extensive tumor necrosis (Table 11.1).

Another phase I clinical study of α-GalCer-loaded mature DCs was reported (Chang et al. 2005). In this study, five patients received α-GalCer-pulsed monocytes-derived mature DCs matured with IL-1β, IL-6, TNF-α, and PGE_2. No serious adverse events related to the treatments were noted. The α-GalCer-pulsed mature DCs led to a more than 100-fold increase in circulating iNKT cells, and the expansion continued for 3 months in all patients. iNKT cell activation was associated with an increase in the serum levels of IFN-γ-inducible protein (IP)-10, IL-12 p40 and macrophage inflammatory protein (MIP)-1β (Table 11.1). Regarding the clinical response, three patients had a clinically relevant decrease in the level of urine or serum M protein and one patient showed stable metastatic lung tumors (Table 11.1).

The administration of both immature DCs and mature DCs loaded with α-GalCer was safe and well tolerated. The peripheral blood iNKT cell activation and expansion may be correlated to the maturation levels of DCs loaded with α-GalCer because the fully matured DCs appeared to induce considerably better responses in the magnitude and the duration of elevated numbers of iNKT cells in PBMCs in comparison to the injection of the immature DCs. However, this will need to be addressed more carefully in future trials with patients with more similar backgrounds, and not only the proportion of iNKT cells but also the functional changes of iNKT cells will need to be monitored.

4 Adoptive Immunotherapy with Activated iNKT Cell

Abnormalities in the number and function of iNKT cells have been observed in patients with various malignant diseases. Therefore, the therapeutic strategies focused on the direct regeneration of a functional iNKT cell population could be a

reasonable approach for the immunotherapy of cancer patients if sufficient numbers of autologous functional iNKT cells could be prepared in vitro. Since both the population and expansion capacity of peripheral blood iNKT cells from cancer patients is lower than healthy volunteers, it has not been possible to collect a large number of iNKT cells. However, it was reported that iNKT cells were able to expand following the repeated stimulation of peripheral blood mononuclear cells with α-GalCer in vitro (Rogers et al. 2004).

Based on these results, a phase I study of in vitro expanded iNKT cell therapy was conducted (Motohashi et al. 2006). The primary endpoint was to test the safety profile, and the secondary endpoints were to measure the iNKT cell-specific immune response and clinical response to the treatment (Fig. 11.2a). Six patients with recurrent lung cancer after standard treatment were enrolled in the study. To induce the activated iNKT cells in vitro, whole PBMCs were stimulated with α-GalCer and IL-2 for 2 or 3 weeks. Total cultured cells containing activated iNKT cells were intravenously injected twice with a 1-week interval between injections. The administered iNKT cell number was 1×10^7 per injection (level 1) and 5×10^7 per injection (level 2). The transferred iNKT cells predominantly expressed either $CD8^+$ (mean 48.7%) or were double negative (mean 29.0%). The in vitro expanded iNKT cells appear to be suitable for use in the treatment of cancer patients, because the expanded iNKT cells showed a potent antitumor activity in human tumor cell lines both in vivo and in vitro.

Regarding the primary endpoint, a few minor adverse events occurred but no severe adverse events were developed. As for the secondary endpoints, the absolute number of circulating $V\alpha24^+V\beta11^+$ iNKT cells increased in 2 of 3 cases that received a level 2 dose, and IFN-γ production (mainly from iNKT cells and NK cells) was augmented (Table 11.1). Although no objective tumor regression was observed, two patients who received the level 2 dose and showed up-regulation of IFN-γ production had SD for 9 and 12 months and survived for 33.3 and 76.6 months, respectively (Table 11.1).

5 Combination Immunotherapy with In Vitro Activated iNKT Cells and α-GalCer-Pulsed APCs

A phase I clinical study of the intra-arterial administration of in vitro-expanded iNKT cells in patients with recurrent refractory head and neck squamous cell carcinoma in combination with nasal submucosal injection of α-GalCer-loaded APCs was performed (Kunii et al. 2009). Because the blood supply of most head and neck cancers is provided by a terminal artery, such as a branch of the external carotid artery, it is possible to selectively infuse the anticancer drugs or effector cells to tumor feeding artery. Eight patients, who had locally recurrent malignant neoplasms refractory to standard therapy were enrolled, and underwent leukapheresis on day 0. The patients received an injection of 1×10^8 α-GalCer-loaded APCs into the nasal submucosa on day 7 and 13, and then received superselective transcatheter arterial

infusion of 5×10^7 iNKT cells into a tumor-feeding artery on day 14. After these therapies, 3 of 8 patients showed moderate increases in the number of circulating iNKT cells and 4 patients showed greater increases. Moreover, 4 patients showed moderate increases in IFN-γ producing cells and 3 patients showed greater elevations in PBMCs. The induction of an immunological response in the peripheral blood was not observed in one case that had received bilateral neck lymphatic dissection in the previous treatment, which might have prevented the migration of nasal submucosally injected APCs. According to a CT scan examination, 3 patients showed PR, 4 had SD, and 1 patient had PD. Grade 3 toxicity with a pharyngocutaneous fistula related to local tumor reduction was observed in one patient and mild adverse events with grade 1–2 symptoms occurred in seven patients. Therefore, this combination therapy with intra-arterial infusion of activated iNKT cells and submucosal injection of α-GalCer-loaded APCs was mostly well tolerated. The use of the intra-arterial infusion of activated NKT cells and the submucosal injection of α-GalCer-pulsed APC has been shown to induce significant antitumor immunity and had beneficial clinical effects in the management of advanced HNSCC. The use of such therapeutic modalities may therefore be helpful in the management of tumors and therefore needs to be explored in further detail.

6 Perspective

We have summarized the clinical trials of iNKT cell-based immunotherapy, and have demonstrated the novel immunological results accumulated during these studies. In these clinical trials, we and others assessed the immune responses using PBMCs, since PBMCs are the only sample type that can be easily and repeatedly accessed. The next step will be to investigate whether the immunological responses in the peripheral blood are comparable to those at the tumor site. Therefore, in the next stage of translational research, we would like to focus on the immunological analyses of the tumor site. In the surgically resected specimens of lung cancer, the frequencies of intratumor iNKT cells increased significantly following treatment (Motohashi et al. 2002). The accumulation of iNKT cells in the region of colorectal cancer was related to a better survival. Therefore, a study focusing on the immunological analyses of the tumor region, including the assessment of functional properties of tumor infiltrating iNKT cells will be crucial. In addition, histological and functional studies of tumor infiltrating lymphocytes may provide conclusive results regarding the antitumor mechanisms of the activated iNKT cells. Further investigation may provide a more sophisticated protocol that can generate meaningful immunological results.

Although the number of enrolled patients was small, some HNSCC patients (3 out of 8 cases) experienced objective antitumor responses after combination therapy with α-GalCer-pulsed APCs and activated iNKT cells (Kunii et al. 2009). In the case of lung cancer, prolonged SD conditions and prolonged overall survival after iNKT cell-based immunotherapy was observed. To improve the efficacy of

the iNKT cell therapy, the identification of adequate biomarkers which can predict the responses to iNKT cell therapy and distinguish responder and nonresponder patients would be useful. With the use of a cDNA microarray-based gene expression analysis of PBMCs, we recently found several surrogate biomarkers that can be used to evaluate the responses of α-GalCer-pulsed APCs (Okita et al. in press). Two candidate genes, *LTB4DH* and *DPYSL3*, were selected, and these genes were associated with the responsiveness of IFN-γ production. In the next step, we would like to find the pretreatment biomarkers that can predict appropriate candidate cases for iNKT cell therapy based on the findings of a substantial number of patients. This will enable the development of a better protocol that produces better clinical outcomes.

The use of other molecularly defined reagents that activate adaptive antitumor immunity is preferable to induce more stringent antitumor effects in patients with malignant diseases. In the various mouse models, α-GalCer enhanced the adaptive immune responses induced by a tumor-associated antigenic peptide or whole tumor protein (Fujii et al. 2003; Hermans et al. 2003; Shimizu et al. 2007). To date, numerous tumor specific antigens have been identified, and these tumor antigenic peptides or proteins have already been applied to the clinical trials (Rosenberg et al. 2004). Therefore, it may be possible to design a combination therapy aimed at the augmentation of the activation of tumor-specific $CD8^+$ T cells using adjuvant effects induced by iNKT cell-based immunotherapy. In addition, combinations with monoclonal antibody-based immunotherapy may be interesting approaches for further investigation (Takeda et al. 2007).

In order to elicit the potent antitumor immune responses by using the direct and indirect adjuvant effects of iNKT cells, new combination approaches should be considered. These immune-adjuvant combination therapies, targeting the renewal of iNKT cell immune system could therefore provide a new axis for cancer treatment.

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Index

A
Acquired immune system, 2
Acute myeloid leukemia (AML), 161–162
Adaptive immunity, 55, 56, 64
Adjuvant activity, NKT cells
  advanced lung cancer, 7–8
  DCs, 6
  FN-γ, 6
  functional cells, generation, 9
  α-GalCer, 5
  immunity, 6, 7
  iPS-derived, 10
Adjuvant effects
  in vivo, activated iNKT cells, 98–100
  therapeutic approaches, activated iNKT cells
    tumor antigen Co-administrative therapy, α-GalCer, 103–104
    vector cells, 104–106
Adjuvant tumor vaccines, α-GalCer
  anti-tumor adaptive immune response, 117
  CD1d ligands, 122
  harnessing NKT cell, 117
  immunization strategies, 116
  intranasal and oral administration, 118
  NKT cell activation, 117
Antigen presentation/processing
  lipid exchange, 72
  molecules, 15–16
  protein kinase C δ, 85
  TGF-β inhibition, 84
Antigen presenting cells (APCs)
  innate lymphocytes, 97
  mature DCs, 96
Antigens (Ags), iNKT cells
  endogenous lipid
    cell hybridoma, 25–26
    GPI, 25
    GSLs, 26
    isoglobotrihexosyl ceramide (iGb3), 26–27
    lyso-phosphatidylcholine (lyso-PC), 27
  human CD1 genes, 16
  MHC-encoded class I and II molecules, 15–16
  natural microbial lipid
    BbGL-II, 24–25
    Borrelia burgdorferi, 23–24
    cholesterol-containing antigen, 25
    GSL4A, 23
    GSLs, 22–23
    Sphingomonas bacteria, 23
  structural basis, lipid recognition
    α-anomeric linkage, sugar, 28
    binding, GSL ligands, 29
    CD1d, 28
    cross-species reactivity, 30–31
    fatty acids, 30
    α-GalCer, 29–30
    phosphatidylcholine (PC), 29
  synthetic lipid
    3′ and 4′-hydroxyl groups, phytosphingosine base, 19–20
    CD1d complexes, 21–22
    disaccharide compound, 19
    α-GalCer, 17, 18
    hydrocarbon chains, 20
    in vivo production, 20–21
    mammalian cells, 17–18
    mouse CD1d binding groove, 19

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Antigens (Ags), iNKT cells (*cont.*)
surface plasmon resonance measurements, 18
Th1 and Th2 cytokine skewing, 21
Th2 cytokines, 20
T cell antigen receptors (TCRs), 16, 17
vertebrates, 15
APCs. *See* Antigen presenting cells

B
B-cell lymphoma
antitumor response, 78, 81
NKT cells, 160
Bone marrow transplantation (BMT), 162–163

C
Cancer
immunotherapy
α-GalCer-pulsed APCs, 190–192
malignant tumors, α-GalCer-pulsed DCs, 193
KRN7000 administration
autologous in vitro expanded iNKT cell, 177–178
α-GalCer immature monocyte derived DC, 174, 176
α-GalCer mature monocyte derived DC, 176–177
α-GalCer pulsed APC fractions, 177
iNKT-cells, 179–181
intravenous administration, 172–174
CD1
CD1d
βGalCer loaded, 18
binding groove, 19
Borrelia infection control, 23
C-αGalCer–CD1d complexes, 21
human, 25
PIM-loaded, 22
reactive T cells, 16
structure, 28
tetramers, 16
hematopoietic cells, 139
human genes, 16
induce IFNg production, 141
murine and human prostate tumors, 133
polypeptides, 16
CD8 cytotoxic T cells
MHC^+ tumor cells elimination, 6
OVA-specific, 10
CD1d
CD1d-negative tumors, 83
$CD1d^+$ tumor cells, antitumor activity
APCs activation, 82–83
cytokine secretion, 81–82
direct cytolytic activity, 81
ectopic expression, 79
mechanisms, 81
$CD1d^+$ and $CD1d^-$ tumors regulation
antitumor activity, NKT cells
APCs activation, 82–83
cytokine secretion, 81–82
direct cytolytic activity, 81, 82
neuroblastomas, 83
immunity regulation, 75–79
NKT cells
$D4^+$ and $CD8^+$/DN, 75
IL-17-producing, 75
type I, 73–74
type II, 74
CD1d-reactive T cells
tumor microenvironment, 142–143
type II NKT cells, 130
CD1d-restricted T cell populations
cancer patient
CD1d-restricted T cells, 135
organ iNKT cells, 134
quantitative defects, iNKT, 132
selective qualitative defects
proliferative defects, 133
soluble/matrix-bound factors, 134
tumor-induced tolerogenic DCs, 133
Clinical trials
iNKT cells
active immunotherapy, 189–190
adoptive immunotherapy, 193–194
combination immunotherapy, 194–195
α-GalCer-pulsed APCs, 190–193
α-GalCer-pulsed DCs, malignant tumors, 193
KRN7000, advanced cancer
autologous in vitro expanded iNKT cell, 177–178
immature monocyte derived DC, 174, 176
intravenous administration, 172–174
mature monocyte derived DC, 176–177
pulsed APC fractions, 177
Cross-regulation, 63
Cytokines
anti-tumor efficacy, α-GalCer, 115
iNKT, 130
NKT cells, 114, 115
rapid production, 112
responses, tumor-bearing individuals
cancer-bearing mice, 138

CD1d, 139
TRAMP mice, 138
Th1 and Th2, 134

D
Dendritic cells (DCs)
adjuvant vector, uses
α-GalCer-loaded tumor model, 105
T cell-mediated antitumor responses, 104, 105
tumor antigen delivery problem, 104
tumor/Gal, 104, 105
conventional and plasmacytoid, 96
α-GalCer-pulsed, 7, 193
human NKT cells activation, 5
immature, 6, 187
iNKT targeting therapy
IFN-γ, 98
in vivo *vs.* in vitro loading, α-GalCer, 97
type I IFN/IL–12 and IL–18, 97
maturation, 6, 96
monocyte-derived (moDCs), 189
NKT cells
activated iNKT cell-mediated DC cross-presentation, T cells, 101–103
in vivo adjuvant effect, activated iNKT, 98–100
iNKT cell-induced fully functional maturation, 103
mechanism, 100–101
tumor antigen co-administrative therapy, α-GalCer, 103–104
types, immune, 95

E
Embryonic stem (ES) cells, 8

G
α-Galactosylceramide (α-GalCer)
activated type I NKT cells, 62
antigen stimulation, 10
antimetastatic activity, 170
antitumor effect, 57–58
clinical trials, 59
crystal structure, trimolecular complex, 5
description, 41
drug, 5, 7
IL-12 production, 6, 58–59
iNKT cells
activation, 42, 46
harnessing, 47–48
and stimulation, 186
targeting therapy, α-GalCer-loaded DC, 97–98
intranasal administration, pulsed APC fractions, 177
intravenous administration
pulsed APC fractions, 177
pulsed immature monocyte derived DC, 174–176
pulsed mature monocyte derived DC, 176–177
mouse tumor models, 47
NKT
cell activation, 4–5
cell-targeted adjuvant therapy, 8
reactivity, 10
pulsed APCs
active immunotherapy, 189–190
combination immunotherapy, in vitro activated iNKT cells, 194–195
head and neck cancer, 192–193
lung cancer, 190–192
malignant tumors, 193
soluble, solid tumors
immunological responses and antitumor effects, 188
KRN7000 administration, 187
tumor antigen co-administrative therapy, 103–104
α-GalCer. *See* α-Galactosylceramide
Glycosylphosphatidylinositol (GPI), 25
Graft-versus-host disease (GVHD), 162–163

H
Hematologic malignancies, NKT cells
AML, 161–162
B-cell lymphoma, 160
BMT and GVHD, 162–163
CD1d-restricted T cells, antitumor immunity, 155
EBV-related, 163
MDS, 160–161
MM
antigenic targets, CD1d-restricted T cells, 158–159
CD1d, role, 156–157
iNKT cells, human, 159–160
NKT/CD1d axis
immune regulation, 155
myeloma, 156
relative frequency, mice *vs.* human, 163

I
IL-13. *See* Interleukin 13
Immune regulation tumor immunity, NKT cells. *See* Tumor immunity and regulation, NKT cells
Immune suppression
  balance, immunoregulatory axis, 64
  type II NKT cells
    definition, 59–60
    graft-versus-host disease and tumor models, 60
    humans, 60–61
    Jα18KO and CD1dKO mice, 61
    15–12RM fibrosarcoma model, 61
    sulfatide, 60
Immunesurveillance
  adaptive immune system, 112
  elimination phase, 111–112
  natural NKT cell activity
    anti-tumor responses, 112
    heterogeneous population, 113
    p53 tumor suppressor gene mutations, 113
    SEREX, 113
    sulfatide activator, 114
Immunoregulatory axis, 62–64
Induced pluripotent stem (iPS) cells
  adjuvant activity, 10
  embryonic stem (ES) cells, 8
  functional cells, 9
  generation, NKT cells, 9
  in vivo function, 10
  reprogramming activity, 9
iNKT cells. *See* Invariant natural killer T cells
Innate
  and adaptive anti-tumor immunity, 118, 119
  immune system, 2, 7, 112
  immunity, 55, 64
  lymphocyte population, 112
Interferon-γ (INF-γ), 96, 114, 121, 161
Interleukin 13 (IL–13)
  binding, 61
  production, 62–63
Invariant natural killer T (iNKT) cells. *See also* Antigens (Ags), iNKT cells
  activated iNKT cell-mediated DC cross-presentation, T cells, 101–103
  activation
    agonists and overstimulation, 42
    endogenous and exogenous lipid ligands, 40–41
    α-GalCer, 41
    in vivo adjuvant effect, 98–100
    TCRs and MHC molecules, 40
  adaptive antitumor immunity, 196
  adjuvant effects, activated tumor antigen co-administrative therapy, α-GalCer, 103–104
    uses, vector, 104–106
  adoptive immunotherapy, activated
    in vitro expanded cell therapy, 194
    regeneration, functional, 193–194
  antigen-presenting cells, active immunotherapy, 189–190
  autologous in vitro expanded cell fractions, 177–178
  based cancer immunotherapy, 181
  clinical trials, 47–48
  combination immunotherapy, 194–195
  CXCL16 and CXCR6 interaction, 46–47
  dendritic and B-cell, 39–40
  depletion, 179
  description, 186
  α-GalCer and OVA, 46
  α-GalCer-pulsed antigen
    head and neck cancer, 192–193
    lung cancer, 190–192
  α-GalCer-pulsed DCs, malignant tumors, 193
  induced fully functional maturation, DCs, 103
  innate and adaptive immunity, 187
    B-cell activation, 44
    $CD8^+$ T-cell priming, 45
    dendritic and B-cell maturation, 42–43
    function modulation, 45
    hepatic lymph nodes, 44–45
    immunosurveillance and SAA–1, 43
    MDSC suppressive activity, 43–44
  intravenous and subcutaneous routes, 47
  mouse tumor models, 47
  PBMCs, 195
  peripheral blood
    cancer patients, 173
    reduction, KRN7000 injection, 175
  pool, 178–179
  soluble α-GalCer administration, solid tumors
    immunological responses and antitumor effect, 188
    KRN7000 administration, 187
  stimulation, 186
  targeting therapy, ex vivo α-GalCer-loaded DC, 97–98
  ThrCer *vs.* α-GalCer, 48
Invariant Vα14 antigen receptor
  hybridomas, 3
  identification, 2
  TCRα repertoire, 3
  unprimed normal mice, RNase protection assay, 3

K
KRN7000
APC fractions, α-GalCer-pulsed, 177
autologous in vitro expanded iNKT cell fractions, 177–178
chemical formula, 170
α-GalCer pulsed immature monocyte derived DC
open-label phase 1 study, 174
serum IFN-γ levels, 176
α-GalCer-pulsed mature monocyte derived DC, 176–177
iNKT-cells
activation, 180
based cancer immunotherapy, 181
depletion, 179
pool, 178–179
intravenous administration
GM-CSF and TNF-α serum levels, 174, 175
iNKT cell numbers, 173
maximum tolerated dose (MTD), 172
patient characteristics, 172, 173
lenalidomide, 179
pre-clinical models, 171
Vγ9Vδ2-T cells, 180

L
Lipid antigen
glycolipid, 16, 21
mediated stimulation, iNKT, 22
Lung cancer, α-GalCer-pulsed APCs
cell processing and stimulation procedures, 191
IFN-γ producing cells, 190, 191
phase I dose-escalation study, 190
survival curve, 192

M
Major histocompatibility complex (MHC)
CD1d molecule, 2
class I, 101, 102
class II and co-stimulatory molecules, 97
injected, mismatched mice, 105
molecules, 40, 44
β-Mannosylceramide (β-ManCer)
antitumor immunity, 57
type I NKT cell-mediated tumor protection, 59
MDSCs. *See* Myeloid derived suppressor cells
MHC. *See* Major histocompatibility complex
Multiple myeloma (MM)
antigenic targets, CD1d-restricted T cells
autoreactivity, 158
inflammatory responses, 159
lyso-phosphatidylcholine (LPC), 158–159
CD1d and NKT function alteration
antitumor properties, iNKT cells, 157
precursor lesion, 156
iNKT cells, human, 159–160
Myelodysplastic syndrome (MDS), 160–161
Myeloid derived suppressor cells (MDSCs)
activation, NKT cells, 63
definition, 62
influenza A infection, 43–44
TGF-β production, 62–63

N
Natural killer T (NKT) cells. *See also* Hematologic malignancies, NKT cells
abnormal signal transduction pathways, 85
activated iNKT cell-mediated DC cross-presentation, T cells
CTL, 102
LP-DC, 102
MHC class I, 101–102
adenosine receptors role, 142
adoptive transfer
anti-cancer agent, 121
anti-tumor activity, 120
immunotherapy, 120
vaccination strategies, 120
antitumor activity
CD1d-negative tumors, 83
CD1d-postive tumor cells, 79–83
$CD4^+$ and $CD8^+$/DN, 75
CD1d mAb based-therapy, 142
CD1d-restricted T cell populations
antitumor activity, 132
cancer patient organ iNKT cells, 134
selective qualitative defects, 133–134
specific quantitative defects, 132
tumor growth inhibition, 131
CD1d-restricted T cell-related clinical trials
Graft-versus-Leukemia (GvL) activity, 137
immunological responses, 135
inflammatory responses, 136
potent antitumor effects, 135
therapeutic approach, 136
CD1d-restricted T cell subsets
human T cells, 130
physiological endogenous ligands, 130

Natural killer T (NKT) cells. *See also* Hematologic malignancies, NKT cells (*cont.*)
cell-mediated adjuvant activity, anti-tumor responses
advanced lung cancer, 7–8
DCs, 6
FN-γ, 6
functional cells, generation, 9
α-GalCer, 5
immunity, 6, 7
cytokine responses, tumor-bearing individuals
activation, prostate tumors, 139–141
IL-12 Restores iNKT cell activation, 141
suppression defects model, 138, 139
discovery
clonal expansion, lymphocyte, 3
complementary DNAs (cDNAs), 2
hybridomas, 3–4
Vα14 receptor, 3
disruption, TCR-signaling, 85–86
diversity
antigen recognition, 153
innate and adaptive immunity, 154
subsets, 154
type I cells, 154
down-regulation, CD1d expression, 84
endogenous iNKT ligands, 141
in vivo adjuvant effect, activated iNKT cells
DCs, 98
α-GalCer link, innate and adaptive immunity, 99
OVA, 99–100
IL-17, 75
immunotherapy, 142
induced pluripotent stem cells
adjuvant activity, 10
embryonic stem (ES) cells, 8
generation, functional, 9
in vivo function, 10
reprogramming activity, 8–9
iNKT cells (*see* Invariant natural killer T cells)
adjuvant and clinical trials, 137–138
induced fully functional maturation, DCs, 103
ligand
alanine mutagenesis, 4–5
amino acids, 5
dendritic cells (DCs), 5
α-GalCer, 4
lipid shedding
glycolipids, 84
multiple myeloma patients, 85
mechanism, NKT cell-induced DC maturation
CD27/CD70 interactions, 101
factors, 100–101
TNF-α and IFN-γ, 100
model, polarization of NKT, 142–143
peak levels, 176, 178
prototypic Th1 immune responses, 131
therapeutic approaches, 143–144
tumor cells interaction, 143
tumor immunity regulation
growth, preclinical/clinical models, 76–77
iNKT cells, antitumor effects, 78
MDSCs, 79, 80
type I, 75, 80
type II, 75, 79
type I (*see also* Type I NKT cells)
Epstein-Barr virus (EBV), 74
iNKT cells, 73
murine models, 73
SLAM-associated protein (SAP), 73–74
type II (*see also* Type II NKT cells)
α-GalCer, 74
Vα24, 74
Nitric oxide synthase (NOS), 58
NKT cell agonist, 57
NKT cell antigens
α-GalCer, 56
lyso-phospholipids, 57
structure function studies, 56–57
sulfatide, 60
NKT cell-targeted adjuvant cell therapy, 7–8
NOS. *See* Nitric oxide synthase

O

OVA. *See* Ovalbumin
Ovalbumin (OVA)
antigen plus α-GalCer, 100
dying cells, 99
expressing tumor, 99
injection, 100
mouse tumor models, 47
specific T-cell expansion, 46

P

Prostate tumors
iNKT cells
activation, 141
anergic-like state, 140
IL-12, 139–141
murine prostate tumor cells, 141
murine and human, 133
TRAMP mice, 139

R
Regulatory T cells (Tregs)
immune responses, 64
interaction, NKT cells, 64

T
T-cell receptor (TCR)
iNKT cells
antigen-specific, 40
CD1d complexes, 41
ligation, 43
recognition, CD1d-antigen
Ag orientation, 28
α-anomeric linkage, sugar, 28
α-GalCer, 17, 18
GSL4A, 23
T lymphocytes, 16
TGF-β. *See* Transforming growth factor-β
Therapeutic approaches utilizing NKT cells
anti-tumor immunity
immune surveillance, 112–114
induction, exogenous TCR stimulus, 114–116
elimination phase, 111–112
harnessing NKT cells
adoptive transfer, 120–121
CD1d monoclonal antibody-based therapy, 121–122
α-GalCer, tumor vaccines, 116–118
tumor cells, α-GalCer administration, 118–119
heterogeneous composition, 123
immunoediting, 111
immune system, 111
Threitolceramide (ThrCer), 48
TNF-α. *See* Tumor necrosis factor-α
Toll-like receptors (TLRs)
DCs, 96
ligands and iNKT cell ligands, 96
Transforming growth factor-β (TGF-β)
antibody blockade, 61
production, 62–63
Tregs. *See* Regulatory T cells
Tumor immune evasion
abnormal signal transduction pathways, 85
disruption, TCR-signaling, 85–86
down-regulation, CD1d expression, 84
lipid shedding, 84–85
Tumor immunity and regulation, NKT cells
definition, 55–56
interaction
CD40/CD40L, 62
MDSCs, 62–63
Tregs, 64
type I cells
β-linked glycosylceramides, 57
counterregulation, 61–62
cytokine secretion and IL–12 production, 58–59
α-GalCer, 57–59
Ja18KO mice and β-ManCer, 59
liver and lung metastasis, 58
lyso-phospholipids, 57
mice and human, 56
structure, antigens, 56–57
type II cells
counterregulation, 61–62
immune suppression, 59–61
Tumor immunity regulation
growth, preclinical/clinical models, 76–77
iNKT cells, antitumor effects, 78
MDSCs, 79
type I cells, 75
type II, 75, 79
Tumor necrosis factor-α (TNF-α)
binding, 61
tumor protection, 58
Type II NKT cells
counterregulation, 61–62
immune suppression
definition, 59–60
graft-versus-host disease and tumor models, 60
humans, 60–61
Jα18KO and CD1dKO mice, 61
15–12RM fibrosarcoma model, 61
sulfatide, 60
Type I NKT cells
counterregulation, 61–62
immune regulation
β-linked glycosylceramides, 57
cytokine secretion and IL-12 production, 58–59
α-GalCer, 57–59
Ja18KO mice and β-ManCer, 59
liver and lung metastasis, 58
lyso-phospholipids, 57
mice and human, 56
structure, antigens, 56–57

U
Unprimed normal mice, 3

V
Vα14 receptor, 3
Vγ9Vδ2-T cells, 180