Regulation of Immune Responses by Natural Killer T Cells

SHAYAN SHARIF and TERRY L. DELOVITCH*

Autoimmunity/Diabetes Group, The John P. Robarts Research Institute, and Departments of Microbiology and Immunology, and of Medicine, University of Western Ontario, London, Ontario N6G 2V4, Canada

Abstract. Natural killer T (NKT) cells, which comprise a minor population of T cells in primary and secondary lymphoid organs, possess phenotypic characteristics of both NK and T cells. NKT cells respond to various external stimuli by an early burst of cytokines, including IL-4 and IFN-γ. Thus, a key immunoregulatory role has been attributed to them. Autoimmune diseases, especially type I diabetes (TID), may be caused by dysregulation of the immune system, which leads to hyporesponsiveness of regulatory T helper 2 (Th2) cells and promotion of autoimmune Th1 cells. Furthermore, several lines of evidence exist to support the notion that an NKT cell deficiency in individuals at risk of TID may be causal to TID. As a result, targeting NKT cells using immunotherapeutic agents may prove beneficial in the prevention or recurrence of TID. Indeed, our data demonstrate that stimulation of NKT cells with a specific ligand prevents the onset and recurrence of TID in nonobese diabetic (NOD) mice.

Key words: immunoregulation; autoimmunity; type I diabetes; α-galactosylceramide.

Introduction

The role of natural killer T (NKT) cells in the induction and regulation of immune responses has been the focus of many investigations. Due to their peculiar immunoregulatory characteristics, these cells may be a potential target for novel immunotherapeutic interventions aimed at the prevention or treatment of infectious disease, cancer and autoimmune disease. The main focus of this review is the role of NKT cells in autoimmune type I diabetes (TID).

Phenotypic Characteristics and Development of NKT Cells

NKT cells were first discovered during the characterization of KLH-specific mouse suppressor T cell hybridomas, which were found to express an invariant T cell receptor (TCR)α chain consisting of the Vα14-Jα281 gene segments. Subsequently, a subset of CD4–CD8– thymocytes was identified as having a bias in their TCRβ gene usage, as they frequently used the Vβ8 followed by Vβ7 and Vβ2 gene segments. Further characterization of the phenotypic markers of these cells revealed the expression of various NK cell-related surface markers, including NK1.1. Therefore, the term NKT cell was coined to delineate a cell population with both T and NK cell surface phenotypic characteristics. NKT cells may be detected in primary (thymus) and secondary (spleen) lymphoid organs, and are abundant in the liver and bone marrow. Cells with similar phenotypic characteristics have also been described in humans, preferentially using the invariant Vα24-JαQ TCR gene segments.

Phenotypic markers expressed on the surface of NKT cells closely resemble those presented on acti-
vated T cells, such as CD122 (IL-2Rβ), CD44 and CD69. The majority of NK1.1+ T cells are either CD4+ or CD4–CD8–. Although CD8– NK1.1+ T cells were reported to be undetectable, recent evidence, using a pan-NK cell marker (DX5), suggests the presence of CD8– NK T cells. The splenic DX5+TCRβ+ subpopulation may differ from the conventional Vα14-Jα281 TCRβ+ T cells, since transgenic expression of CD8 on T cells inhibits the development of Vα14-Jα281+ NK T cells.

The fetal development of Vα14 NK T cells starts at day 9 of gestation in mice, before the fetal thymus is formed, and their development can occur independently of the yolk sac. NK T cells appear to pass through a CD4+CD8– double positive stage, as evidenced by the presence of a precursor cell population in day 12 fetal thymic cultures that differentiates into NK1.1+CD4+CD8– T cells. The number of NK T cells is very low at birth; subsequently, they accumulate in various tissues, including primary and secondary lymphoid organs, and become most abundant in liver and bone marrow. The dependence of NK T cell development on the thymus is presently unclear. Some NK T cell subpopulations appear to be thymus-dependent, whereas other subpopulations may develop extra-thymically, e.g. in the liver.

The origin of NK T cells is controversial, as it is not known whether NK T cells are derived from a unique, committed precursor cell or a subset of conventional T cells which, during some stages of their normal development, branch off to become NK T cells. Nevertheless, it is evident that these cells comprise a unique subset of T cells whose development is differentially regulated from mainstream T cells. This notion is supported by the observation that deletion of the Vα14 gene segment, which is involved in the formation of the NK T invariant TCR, selectively depletes the formation of NK T cells, but all other T cells remain virtually intact.

### NKT-CD1 Interactions

Although natural ligands of NK T cells are not well characterized, NK T cell restriction to the CD1 molecule, whose structure resembles that of the major histocompatibility complex class I (MHC-I), is well established. Unlike expression patterns of MHC-I, the CD1 molecule is primarily expressed on hematopoietic cells such as B cells, macrophages and dendritic cells. The development of NK T cells is CD1-dependent, as the number of NK T cells is markedly reduced in CD1 gene knock-out mice. The absent NK T cells in CD1–– mice are those that preferentially use the invariant Vα14-Jα281 TCR, whereas another subpopulation of NK T cells may develop in the absence of CD1, which possesses a diverse TCR repertoire as well as phenotypic characteristics of naive T cells. The former population is predominantly present in the thymus and liver and the latter population is present in the spleen and bone marrow. The CD1 crystallography findings indicate the presence of a deep, narrow and hydrophobic antigen-binding groove in this molecule, which can accommodate lipid antigens. The natural ligand of CD1-restricted NK T cells appears to be a glycosphatidylinositol (GPI). Recently, a glycolipid, α-galactosylceramide (AGC), was identified by Kirin Brewery (Japan) from marine sponges as a potential anti-cancer compound. Further investigations determined that this compound is a ligand for both human and mouse NK T cells. Presentation of AGC by the CD1 molecule is transporter-associated protein (TAP)-independent and NK T cells respond vigorously to this compound by proliferation, expression of activation molecules, increase in their cytotoxic activity and secretion of various cytokines.

### Function of NK T Cells

Upon stimulation, NK T cells exert various functions, ranging from cytotoxic activity to cytokine production. These cells have a unique property that enables them to produce large amounts of cytokines upon primary stimulation. For instance, production of a mixture of T helper 1 (Th1) and Th2 cytokines is detectable in splenocytes harvested from mice treated in vivo with an anti-CD3 mAb 90 min earlier, and the responsible cell population has the phenotypic characteristics of NK T cells. More recently, the CD1-restricted NK T cell response to AGC was shown to be phylogenetically conserved, as human and mouse CD1 molecules can bind to AGC and activate NK T cells from both species. NK T cells respond after interaction with CD1-bearing dendritic cells loaded with AGC. This interaction requires cell-cell contact through CD40-CD40L. Secretion of Th1 or Th2 type cytokines appears to be differentially regulated by the number of in vivo doses of treatment with AGC. A single in vivo dose of AGC encourages expression of IFN-γ and other characteristics of a Th1 response, a multiple-dose regime will promote a Th2 response. It is noteworthy that, although NK T cells are primarily triggered by AGC, bystander cells, including NK, CD8, CD4 and B cells,
may also become non-specifically activated as a consequence of NKT cell activity. The peculiar property of NKT cells, which enables them to produce large amounts of Th1- and Th2-type cytokines very early after stimulation, renders them ideal candidates for the regulation of an immune response.

**Effector activity**

Due to the wide range of activities, NKT cells may play effector or regulatory roles in different immunological settings. AGC-activated human NKT cells confer anti-tumor activity via an elevated perforin-dependent cytotoxicity. Similarly, mouse NKT cells activated with AGC-pulsed dendritic cells inhibit the metastasis of experimental melanoma. NKT cells may also have effector functions in response to various pathogenic microorganisms, e.g. resistance to infection with *Toxoplasma gondii* and *Plasmodium yoelii*. Thus, it is tempting to speculate that NKT cells are intimately involved in providing the first layer of host immunity against invading pathogens. However, paradoxically, inhibition of CD1-NKT interaction in *Listeria monocytogenes*-infected mice reduced the expression of TGF-β2 and, as a result, the survival of these mice was significantly increased. This suggests a regulatory function for NKT cells under certain circumstances in the course of a bacterial infection. NKT cells may also possess other effector functions during the development of concanavalin A-induced hepatitis, abortion in mice and hyperacute graft-versus-host-disease (GVHD) in humans.

**Regulatory activity**

The role of NKT cells in the regulation of an immune response has received much attention (Fig. 1). Initially, two Vα14-Jα281 TCRαβ KLH-specific T cell hybridomas were described that could each secrete a soluble suppressor factor. The suppressor factor from one hybridoma induced the other hybridoma ("inducible acceptor suppressor") to exert its KLH-specific functions. More recently, DNP-specific T suppressor hybridomas were identified, one of which has a TCR Vα14-Jα281 rearrangement. These hybridomas also produce a soluble suppressor factor, which may be a soluble TCR. However, these soluble suppressor factors could also be cytokines, such as TGF-β, IL-4 and IL-10, or other immunomodulators that enhance one type of immune response and suppress another type. In fact, early investigations pointed to the crucial role of NKT cells in triggering Th2 type responses. Despite these findings, several groups have argued that NKT cells have a dispensable role in the development of

![Fig. 1. Model of NKT activation and function. NKT cells are stimulated by CD1-bearing antigen-presenting cells (APC) that are loaded with AGC. NKT cells receive stimulatory signals from APC (via IL-12) and, in return, stimulate them by secretion of IFN-γ. Upon stimulation, NKT cells exert effector (e.g. cytotoxicity via Fas-Fasl. and perforin pathways) and immunoregulatory (e.g. secretion of cytokines: IL-4, TGF-β, TGF-β1 and IFN-γ) functions. The early burst of cytokine secretion by NKT cells may be critical in the differentiation of Th0 cells to Th1 or Th2 cells. B cells and NK cells may also become activated by NKT cells.](image-url)
a Th2 response. The degree of NKT cell involvement in the development of a Th2 versus a Th1 response likely depends on the particular immunological setting. For example, NKT cells potentiate a Th1 response by mediating IL-12-induced IFN-γ production. Dendritic cells are stimulated by ligand-activated NKT cells through a direct CD40-CD40L interaction that increases their production of IL-12. Subsequently, the secreted IL-12 induces NKT cells to increase their surface expression of IL-12R as well as production of IFN-γ. The AGC-induced IL-12 production is inhibited in Vα14-Jα281 knock-out mice, which selectively lack NKT cells, or by in vivo administration of an anti-CD40L mAb. Thus, an intimate interaction between NKT cells and antigen-presenting cells (APCs) is critical for the development of a Th1 or a Th2 response.

Several additional immunomodulatory roles of NKT cells have been reported. During allogeneic GVHD, NKT cells can ameliorate disease. In this model, conventional T cells (TCRβ+CD4+ or TCRβ-CD8+) derived from bone marrow or blood elicit acute GVHD, whereas NK1.1+CD4+, NK1.1+CD8+ or NK1.1+CD4-CD8-T cells suppress GVHD. Suppression of disease by NK1.1+ T cells is dependent on expression of IL-4, as NK1.1+ T cells from IL-4−/− mice lose their modulatory activity. Paradoxically, in a hyperacute case of human allogeneic GVHD, the frequency of NKT cells in peripheral blood was very high and this result implicated their effector role in this response. An immunomodulatory role of NKT cells in immune privilege was recently described in an elegant study involving a model of anterior chamber-associated immune deviation (ACAIĐ). Induction of regulatory T cells in the spleen, which inhibit proliferation or the effector functions of CD4+, may initiate ACAID. However, induction of ACAID is severely perturbed in strains of mice, including SJL/J and β2m knock-out mice, which have developmental and functional deficits in their NKT cell population. Further investigations proved more conclusively that ACAID is dependent on CD1 expression, as evidenced by the absence of ACAID in CD1−/− mice and by an in vivo blockade of CD1-NKT interaction using anti-CD1 antibodies.

Other immunoregulatory functions of NKT cells may involve the induction of tolerance. In the trinitrobenzenesulfonic acid (TNBS)-induced model of colitis in mice, NK1.1+ liver-associated lymphocytes were found to be important in the induction of oral tolerance against the development of colitis. Tolerized mice had significantly higher serum concentrations of IL-4 and TGF-β1, while the serum concentration of IFN-γ was significantly reduced. Furthermore, intra-graft NKT cells may be important in the induction of tolerance in recipients of hepatic allografts.

**NKT Cells and Autoimmunity**

A potential role for NKT cells in the regulation of autoimmunity was initially recognized in SJL mice that are susceptible to experimental allergic encephalomyelitis (EAE), a Th1-mediated disease. SJL mice are deficient in their Th2-mediated responses, and their NKT cells are significantly reduced in number and function (TCR-stimulated rapid burst in cytokine production) in various tissues. A functional deficit in NKT cells, especially in the production of IL-4 and other regulatory cytokines, in autoimmune disease-prone mouse strains can lead to a dominant pathogenic autoreactive Th1 response. NKT cells have also been implicated in the regulation of susceptibility to autoimmune disease in humans. Patients with systemic sclerosis have a reduced frequency of peripheral blood NKT (Vα24-JαQ) cells. Similarly, the number of NKT cells in the peripheral blood of patients with rheumatoid arthritis is reported to be significantly decreased.

**NKT cells and type I diabetes**

The strongest evidence in support of a role for NKT cells in the regulation of autoimmunity stems from studies of TID in humans and nonobese diabetic (NOD) mice. NOD mice spontaneously develop a form of TID that shares many genetic, immunologic and pathologic features with the human disease. Pancreatic islet β cell destruction is mediated by infiltrating T cells, and both CD4+ and CD8+ T cells are required for the development of TID. Although islet β cell destruction and the onset of TID is attributed to the presence of autoimmune CD4+ Th1 effector cells, an important role for CD8+ T cells in islet β cell destruction has also been identified.

An intricate balance between autoimmune destructive T cells and immunoregulatory T cells regulates the development of TID. A consensus opinion suggests that the conversion from a non-destructive insulitis to destructive insulitis involves a disruption of the balance between destructive Th1 and regulatory Th2 autoimmune cells. Non-destructive insulitis is associated with higher pancreatic levels of IL-4, whereas destructive insulitis is associated with a higher intra-pancreatic concentration of IFN-γ. We have observed deficiencies in NOD T cells that may be causal to the occurrence of TID, including T cell proliferative hypore-
responsiveness following TCR stimulation and reduced IL-4 and IL-2 production upon T cell activation. Stimulation with IL-4 fully restores proliferative hyporesponsiveness and a normal level of IL-4 production by NOD T cells, and treatment of prediabetic NOD mice in vivo with IL-4 prevents insulitis and onset of TID. In addition, young, 2-week-old, NOD mice treated with IL-4 for 10 weeks show a reduced incidence of TID, which is attributed to the prevention of Th1 autoreactive T cells from entering the pancreas as well as the promotion of regulatory Th2 cell activity in the thymus, spleen and pancreatic islets. Taken together, these findings support the notion that immunoregulatory CD4+ Th2 cells mediate protection of NOD mice from TID. Thus, it is conceivable that the proliferative hyporesponsiveness of regulatory Th2 cells promotes a dominant Th1 environment in the pancreas that leads to the breakdown of peripheral self tolerance.

Co-stimulatory pathways, such as those mediated by CD28-B7 interactions, may also contribute to NOD T cell hyporesponsiveness and, consequently, to the loss of tolerance and onset of TID. Indeed, administration of an activating anti-CD28 mAb to young NOD mice of 2–4 weeks of age restores IL-2 and IL-4 secretion of thymic and splenic T cells and also completely protects them from TID. These results and those highlighted above further support the idea that immune dysregulation is a principal causal factor of autoimmune TID. Accordingly, correction of this state of immune dysregulation by an immunomodulatory agent that elicits minimal in vivo adverse effects offers a promising approach to prevent occurrence of TID in high risk subjects and to inhibit recurrence of TID in patients receiving islet transplants.

NKT cells provide a potentially interesting target for such an immunomodulator(s). As previously discussed, the critical role of NKT cells in the development of several Th2 responses is widely accepted. Interestingly, there exist age-dependent deficiencies in the number and function of NKT cells in NOD mice and human TID patients. In NOD mice between 3 and 5 weeks of age, the proportion of NKT-like thymocytes is reduced and TCR cross-linking-induced IL-4 production is significantly impaired. Note that these quantitative and qualitative deficiencies first appear at an age that represents an important checkpoint in the progression to TID, i.e. immediately prior to the development of insulitis in 3– to 4-week-old mice. Subsequently, these deficiencies become virtually undetectable in 12- to 15-week-old NOD mice.

Deficient IL-4 production by NOD NKT-like thymocytes can be corrected by stimulation with IL-7, raising the possibility that the developmental deficiency of NOD NKT thymocytes might be related to an insufficient availability of IL-7. The finding that the development of NKT-like thymocytes in the NOD thymus is impaired was later confirmed by Godfrey et al., who also noted that transfer of thymocytes enriched for CD4+CD8− TCRβ+ cells (i.e. NKT cells) into young NOD recipients prevents TID. The protection conferred by these thymic NKT cells is mediated by IL-4 and IL-10, as neutralization of these cytokines abrogates the protective effects of NKT cells. Analyses of different lines of transgenic NOD mice that express variable levels of the TCR Vα14-Jα281 chain have also confirmed a pivotal role for NKT cells in protection from TID. The most protection from TID was achieved in the mouse line that possessed the greatest number of NKT cells, highest level of IL-4 secretion by NKT cells induced by TCR stimulation, and best capacity to transfer protection with splenic T cells. We have shown that NKT cells in the thymus and spleen of NOD mice secrete normal amounts of IFN-γ but are deficient in IL-4 production in response to in vitro stimulation with ACG. In contrast, TCR- or IL-12-stimulated NOD NKT cells were previously reported to be severely deficient in IFN-γ but not IL-4 production. In the latter studies, purified NOD NKT cells supplemented with IL-7 not only did not prevent TID, but rather accelerated the onset of TID. Further experimentation is required to resolve these apparent discrepancies.

A role for NKT cells in the pathogenesis of human TID was recently reported. In studies of a group of twin/triplet sets discordant for TID, the diabetic siblings had a lower frequency of CD4+CD8+ TCRVα14JQ+ NKT cells in their peripheral blood in comparison with their non-diabetic non-progression siblings. More importantly, CD4+CD8+Vα14JQ+ T cell clones from these discordant sets differed appreciably in their production of IL-4 and IFN-γ. Whereas all clones from diabetic siblings only produced IFN-γ, 76 of 79 clones derived from at risk non-progression siblings simultaneously produced IL-4 and IFN-γ. Thus, it is conceivable that correction of quantitative and qualitative deficiencies of NKT cells in individuals at risk for TID may prevent occurrence of the disease. Alternatively, the up-regulation of NKT cell number and function may prevent/reduce the recurrent autoimmune destructive response and, as a result, prolong islet graft survival in newly transplanted diabetic patients.

Since AGC is a ligand for CD1-restricted mouse NKT cells and is a potent stimulator of these cells, we investigated whether treatment of NOD mice in vivo with AGC protects against TID. Our results indicate
that female NOD mice are significantly protected against the onset of cyclophosphamide (CY)-induced TID when treated with AGC on days 0, 2, 4, 6 and 8 relative to the time of CY challenge. Moreover, when female NOD mice are treated with AGC at 10, 11 and 14 weeks of age, the spontaneous development of TID is delayed by 4–5 weeks and the incidence is reduced by 50%. A more comprehensive treatment regime beginning at 4 weeks of age also affords partial protection and a significant delay in disease onset. Our data indicate that these different modes of AGC-induced protection from TID in NOD mice are mediated by the activation of NKT cells.

We have also explored whether AGC can modulate the onset of a recurrent autoimmune destructive response in overtly diabetic NOD recipients of syngeneic pancreatic islet transplants. In this model, engraftment is normal and euglycemia is restored for about 7–10 days post-transplantation, following which grafts generally fail owing to a recurrent autoimmune response. More recently, prolongation of pancreatic duodenal graft survival in non-recurrent spontaneous diabetic BB rats was associated with the proliferation of donor-derived NKT cells in hepatic and splenic tissues and higher serum levels of IL-4. We have obtained evidence in support of the hypothesis that stimulation of NKT cells by AGC prolong islet graft survival by down-modulation of the recurrent autoimmune response. Our data demonstrate that multiple doses of AGC administered pre- and post-transplantation in recipients prolong islet graft survival in newly diabetic NOD mice from the typical 7 days to as long as 22 weeks. Ongoing studies will determine whether NKT cells play a direct or indirect role in enhanced islet graft survival and homeostasis.

**Conclusions**

Failure of immune regulation in NOD mice or human subjects at risk of TID, which stems from deficient T cell-mediated regulation, and Th2 anergy may be causal to the onset of autoimmune TID and, conceivably, correction of this immune dysregulation may protect at risk individuals against TID. Indeed, we have shown that cytokine-based (IL-4) and mAb-based (anti-CD28) immunotherapies can protect NOD mice from TID. NKT cells, which are quantitatively and qualitatively deficient in diabetes-prone NOD mice and humans, provide an excellent target for future immunotherapeutic approaches aimed at the prevention or reversal of human TID. Since these cells play a major role in the early induction of Th1 and Th2 immune responses, appropriate activation of NKT cells, e.g. towards a Th2 response, may correct immune dysregulation and prevent the pathogenesis of TID. The newly discovered specific ligand for NKT cells, AGC, has already been tested in phase I clinical anti-cancer trials and its lack of toxicity has been confirmed. Thus, it seems feasible to use this compound to protect individuals who are genetically and environmentally at risk for TID. Alternatively, this immunomodulator may be used in diabetic patients that have received an islet transplantation to protect them from the recurrence of TID.

**Acknowledgment.** This work was supported by grants from the Juvenile Diabetes Foundation International (JDFI) and an MRC of Canada/JDFI Diabetes Centre of Excellence. S. Sharif is the recipient of a Canadian Diabetes Association Post-doctoral Fellowship.

**References**


2. **Ballas Z. K. and Rasmussen W. (1990): NK1.1<sup>+</sup> thymocytes. Adult murine CD4<sup>+</sup>, CD8<sup>+</sup> thymocytes contain an NK1.1<sup>+</sup>, CD3<sup>+</sup>, CD5<sup>+</sup>, CD44<sup>+</sup>, TCR-V<sub>D</sub> 8<sup>+</sup> subset. J. Immunol., 145, 1039–1045.**


9. **Burdin N., Brossay L. and Kronenberg M. (1999): Immu-


34. Kitamura H., Ohi T., Sekimoto M., Satoh M., Iwakabe K., Nakai M., Yahata T., Meng H., Koda T., Nishimura S.,


Mendiratta S K, Martin W D, Hong S, Boestedt A, Joyce S, and Van Kerk L. (1997): CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. Immunity, 6, 469–477.


Yanagihara Y, Shizawa K, Takai M, Kyogoku M, and


Received in March 2000
Accepted in May 2000