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NKT Cells and Autoimmune Diseases: Unraveling the Complexity

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Abstract CD1d-restricted invariant natural killer T (NKT) cells emerge as unique lymphocyte subsets implicated in the regulation of autoimmunity. Abnormalities in the numbers and functions of NKT cells have been observed in patients with diverse autoimmune diseases as well as in animal models of autoimmune diseases. NKT cells recognize glycolipid antigens presented by the nonpolymorphic MHC class I-like protein CD1d and participate in various kinds of immunoregulation due to a potent ability to produce a variety of cytokines. In this review, we examine the potential roles of NKT cells in the regulation and pathogenesis of autoimmune disease and the recent advances in glycolipid therapy for autoimmune disease models.

1 Introduction

Autoimmunity is not forbidden in the healthy immune system and may target peptide or lipid antigens. However, in most healthy individuals, autoimmunity does not manifest its dangerous nature, but rather it plays an essential role in maintaining the immunological homeostasis as a physiological regulator. As

evidenced by a number of studies of autoimmune disease models, "dangerous" autoimmunity appears to be controlled by "protective" autoimmunity in the physiological immune network [1, 2].

Although the functional dichotomy of autoimmunity has been documented mostly in peptide and MHC-reactive T cells, it may also hold true for lipid-specific T cells. In fact, opposing functions mediated by lipid-reactive, CD1-restricted T cells have been documented in recently published studies on autoimmune disease models [3–8] (Table 1). Namely, CD1d-restricted invariant $V\alpha14^{+}$ NKT cells (*NKT*) play a protective role in experimental autoimmune encephalomyelitis (EAE) or type I diabetes in NOD mice, whereas they appear to be involved in mediating the inflammatory pathology of arthritis models. Whatever mechanism may be operative in polarizing *NKT* cells toward being protective or pathogenic, it is obvious that autoimmunity to lipid antigens is not always beneficial for our health. Here we review the recent publications on lipid-reactive T cells, particularly CD1d-restricted *NKT* cells and autoimmune diseases.

2
The Role of NKT Cells in Animal Models of Autoimmune Diseases

2.1
NKT Cells in Experimental Autoimmune Encephalomyelitis

After transgenic mice overexpressing or lacking $V\alpha14$–$J\alpha18$ T cell receptors (TCRs), which define invariant NKT cells, were established, the role of CD1d-restricted *NKT* cells was studied in depth in various autoimmune conditions. Amongst these, EAE is a prototypical model for multiple sclerosis (MS) mediated by Th1 autoimmune cells, which can be induced by immunization with central nervous system (CNS) antigens such as myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP) in mycobacterium-containing adjuvant. Approximately 2–3 weeks after sensitization, susceptible mice would manifest MS-like ascending limb paralysis due to inflammatory lesions within the brain and spinal cord. However, as seen with human MS, EAE mice usually recover from paralysis, which is thought to involve elaborate immune regulatory processes. Several research groups have investigated the possible involvement of *NKT* cells in the regulation of EAE using transgenic or gene knockout mice. In TCR $V\alpha14$–$J\alpha18$ transgenic NOD mice, bearing an increased number of *NKT* cells, MOG-induced EAE was significantly suppressed in association with inhibition of antigen-specific IFN-γ production in the spleen [9]. Consistent with this, another study showed that $CD1d^{-/-}$ mice developed a more severe EAE compared to C57BL/6 mice [10], suggesting the

Table 1 iNKT cells in autoimmune disease models

The role of iNKT cells in autoimmune disease models		Effect of glycolipid ligand on disease	
Type I diabetes			
NOD mice		NOD mice	
Protection by transfer of NKT enriched thymocytes	[27]	Protection by α-GalCer	[24, 25, 29, 30]
Protection in Vα14 Jα18Tg	[28]	Protection by OCH	[31]
Protection in CD1Tg	[26]		
Exacerbation in CD1 KO	[23–25]		
Transfer of BDC2.5 T cells		Transfer of AI4 T cells	
Protection by increase of iNKT cells	[33]	Protection by α-GalCer	[34]
Transfer of AI4 T cells			
Exacerbation by increase of iNKT cells	[35]		
Experimental autoimmune encephalomyelitis (EAE)		MOG-induced EAE in C57BL/6	
Protection in Vα14 Jα18Tg/NOD	[9]	Protection by OCH	[15]
Exacerbation in CD1 KO	[10]	Protection by α-GalCer co-immunization	[11–13]
No difference in CD1 KO	[11, 12]	MBP-induced EAE in B.10PL	
No difference in Jα18 KO	[12]	Protection by α-GalCer pretreatment	[13]
Protection in CD1 KO(C57BL/6,B10.PL.)	[11]	MBP-induced EAE in PL/J	
		Protection by α-GalCer co-immunization	[11]
		MBP-induced EAE in SLJ/J	
		Exacerbation by α-GalCer co-immunization	[11, 13]

Table 1 (continued)

The role of iNKT cells in autoimmune disease models		Effect of glycolipid ligand on disease	
Arthritis models			
Collagen-induced arthritis		CIA in C57BL/6	
Protection in Jα18 KO	[37, 39, 40]	Protection by OCH	[39]
Protection by anti-CD1d mAb	[37]	CIA in SJL	
Antibody-induced arthritis		Protection by OCH	[39]
Protection in Jα18 KO	[37, 41]		
Protection in CD1 KO	[37]		
Lupus models			
MRLlpr/lpr		Dermatitis in MRLlpr/lpr	
Exacerbation of dermatitis in CD1 KO	[50]	Protection by α-GalCer	[52]
No difference in dermatitis in CD1 KO	[51]	Nephritis in MRLlpr/lpr	
No difference in nephritis in CD1 KO	[50, 51]	No effect by α-GalCer	[52]
Pristane-induced nephritis		Pristane-induced nephritis in Balb/c	
Exacerbation in CD1 KO	[49]	Protection by α-GalCer	[53]
		Pristane-induced nephritis in SJL/J	
		Exacerbation by α-GalCer	[53]
Nephritis in (NZBxW)F1		Nephritis in (NZBxW)F1	
Protection by anti-CD1d mAb	[56]	Exacerbation by α-GalCer	[56]
Inflammatory bowel disease model			
Oxazalone-induced protection in Jα18 KO		Dextran sodium sulfate-induced colitis	
Protection in CD1 KO or Jα18 KO	[59]	Protection by α-GalCer	[57]
		Protection by OCH	[58]

KO, knockout mice; *Tg*, transgenic mice

immunoregulatory role of NKT cells. We could not rule out the possibility that NKT cells take the space of pathogenic MHC-restricted T cells. Other groups, however, reported that there was no difference in disease course between wild type and mice lacking all CD1d-restricted T cells (CD1d$^{-/-}$) or selectively lacking invariant NKT cells (Jα18$^{-/-}$) [11, 12] in ameliorating disease in C57BL/6. CD1d$^{-/-}$ and B10.PL.CD1d$^{-/-}$ mice [13]. Although the basis for these inconsistencies is not clear, it is possible that the role of *NKT* cells in each EAE system is critically determined by various ill-defined factors such as non-MHC genes that result from different levels of back-crossing to the C57BL/6 background, the breeding environment (cleanliness, serenity), or dose and quality of adjuvant used for EAE induction.

After α-galactosylceramide (α-GalCer) was identified as the efficacious glycolipid ligand for *NKT* cells, this glycolipid was widely used as a pharmacological reagent to explore the potential for *NKT* cells to regulate autoimmunity. The results obtained from α-GalCer treatment of EAE also generated conflicting results. Intraperitoneal injection of α-GalCer before immunization led to suppression of EAE in B.10 PL mice induced with a peptide from MBP [13]. Co-immunization of α-GalCer with an encephalitogenic MOG peptide ameliorated EAE induced in C57BL/6 mice, as compared to MOG immunization without α-GalCer [13]. This co-immunization protocol was adopted by others and proved effective in suppressing EAE in MBP-immunized PL/J mice [11, 12]. However, exacerbation of EAE was observed by a similar co-immunization with α-GalCer in MBP-immunized B10.PL and SJL/L mice [11, 12].

Accompanying ex vivo analysis showed that the protective effect of α-GalCer seemed to correlate with the differential abilities of the mouse strains to produce IL-4 upon stimulation with α-GalCer. For, example, protection was seen in strains secreting higher levels of IL-4 in response to α-GalCer. Moreover, the protective effect of α-GalCer in EAE was not observed in IL-4- or IL-10-deficient mice [11], whereas disease was ameliorated in IFN-γ-deficient mice [13, 14]. Taken together, this suggests that EAE protection by α-GalCer is mediated by Th2 cytokines produced by *NKT* cells, although one study demonstrated that IFN-γ but not IL-4 is critical for the disease protection by α-GalCer in C57BL/6 mice [13]. Another line of evidence to support Th2-mediated protection is that in vivo injection of α-GalCer-pulsed antigen-presenting cells (APCs) with CD86 blockade (treated with anti-B7.2 antibodies does not only polarize *NKT* cells toward a Th2-like phenotype but mediates concomitant suppression of EAE, whereas α-GalCer presented by anti-CD40 activated APC induces a bias of *NKT* cells toward a Th1-like phenotype and exacerbated EAE [14].

Further support for Th2-mediated suppressive effect on EAE by *NKT* cells has been shown using OCH, a sphingosine truncated analog of α-GalCer,

which preferentially induces IL-4 from NKT cells [15–18]. OCH has been shown to be more effective than α-GalCer in preventing EAE in C57BL/6 mice, and it possesses some efficacy even when treatment was initiated several days after EAE induction. OCH was also effective when administrated orally, which is the favored treatment route for humans. The protective effect by OCH was abrogated by neutralization of IL-4 [15].

Taken together, NKT cells appear to work as a regulatory cells in EAE and a proper activation of NKT cells could lead to amelioration of EAE.

2.2
NKT Cells in Diabetes Models

Nonobese diabetic (NOD) mice develop spontaneous autoimmune diabetes that is similar to the human disease, insulin-dependent type 1 diabetes mellitus. In parallel with effector cells such as Th1 type $CD4^+$ cells and $CD8^+$ T cells, regulatory cells including *NKT* cells have been suggested to inhibit the development of diabetes. While a deficit in the number and function of *NKT* cells has been indicated in NOD mice [19–22], further deletion of *NKT* cells by genetic ablation expression accelerated onset and increased incidence of diabetes [23–25]. Protection against diabetes in transgenic NOD mice overexpressing CD1d molecules within the pancreatic islets further supports a CD1d-dependent regulatory function of *NKT* cells [26].

Consistent with the hypothesis that NKT cells are protective in experimental models of diabetes, NOD mice were also protected against diabetes by increasing the number of *NKT* cells either by infusion of *NKT* cell-enriched thymocyte preparations [27] or by the introduction of the Vα14 Jα18 gene into NOD mice [28]. Moreover, activation of *NKT* cells with synthetic glycolipid ligands such as α-GalCer or OCH has been shown to prevent the development of diabetes in NOD mice [24, 25, 29–31].

In many studies, protection from diabetes by *NKT* cells is associated with the induction of Th2 response to islet autoantigens. Neutralization of IL-4 and IL-10 abolished the protection from diabetes by transferred $CD4^-CD8^-$ $TCR\alpha\beta^+$ (NKT) thymocytes [27]. In Vα14 Jα18 transgenic mice, the response to the pancreatic autoantigen, glutamic acid decarboxylase (name autoantigen) response was shifted toward Th2 phenotype and neutralization of IL-4 abrogated protection from cyclophosphamide-induced diabetes [32]. Th2 polarization was also observed in mice treated with glycolipid ligands for NKT cells [24, 25, 29–31]. Treatment with α-GalCer ameliorated cyclophosphamide-induced diabetes in an IL-4-dependent manner [32], though the role of IL-10 is controversial [30, 32]. Th2-independent mechanisms underlying *NKT* cell-mediated suppression have been reported in a dif-

ferent system using TCR transgenic mice. The BDC2.5 TCR was cloned from an MHC class II-restricted T cell clone specific to an islet-derived antigen. Diabetogenic CD4 T cells from BDC2.5 NOD mice were unable to induce diabetes when transferred into mice with increased *NKT* cell numbers [33]. The presence of *NKT* cells inhibited the differentiation of BDC2.5 T cells into IFN-γ producing cells without a Th2 shift. BDC2.5 T cells were initially activated in pancreatic lymph nodes and then became anergic. The regulation of BDC2.5 T cells by *NKT* cells was not dependent on IL-4, IL-10, IL-13, or TGF-β [34]. A similar mechanism was implied in the transfer of diabetogenic transgenic $CD8^+$ AI4 T cells expressing the β cell-autoreactive TCR to sublethally irradiated NOD mice when *NKT* cells had been activated with α-GalCer [35]. Activation of *NKT* cells enhanced the apoptosis and induced anergy of AI4 T cells. Even though several different mechanisms might be involved, NKT cell seems to work as regulatory cells in diabetes models. Recently however, the opposing effect of *NKT* cells on $CD8^+$ MHC-restricted T cells was reported. In diabetes induced by the transfer of $CD8^+$ T cells specific for the influenza virus hemagglutinin into mice expressing the hemagglutinin antigen in pancreatic cells, a high frequency of *NKT* cells exacerbated diabetes by enhancing CD8 T cell activation, expansion, and differentiation into effector cells producing IFN-γ [36]. Acceleration of disease by the presence of *NKT* cells was also observed in other autoimmune models such as arthritis.

2.3 NKT Cells in Arthritis Models

Collagen-induced arthritis (CIA) is an animal model for human rheumatoid arthritis induced by immunizing susceptible mouse strains with type II collagen (CII) with adjuvant. When mice develop arthritis, the proportions of *NKT* cells in liver and peripheral blood have been reported to be increased, even though it is not clear what stimulates *NKT* cells to proliferate or if *NKT* cells are generated in situ or recruited from other organs [37]. Since it has been shown that activation of *NKT* cells can be enhanced by non-TCR stimulation such as IL-12 and possibly other cytokines [38], it might be possible that *NKT* cells proliferate in CIA by stimulation of inflammatory cytokines elevated in this model. The protective effect of blocking anti-CD1 monoclonal antibody on CIA has revealed that *NKT* cells contributed to the development of arthritis [37]. Furthermore, amelioration of arthritis is seen in mice lacking all *NKT* cells ($CD1d^{-/-}$) or invariant NK T cells ($J\alpha18^{-/-}$), further supported the important role of *NKT* cells in the development of arthritis [37, 39, 40]. The reduction of disease severity in $J\alpha18^{-/-}$ mice was associated with a decrease in IL-10 or IL-1β production in response to antigen stimulation, suggesting that NK T cells directly or indirectly control the levels of these cytokines [37, 40].

Recent advances in the use of anti-inflammatory drugs such as anti-TNF reagents reminded us of the importance of the later occurring inflammatory phase in the pathogenesis of arthritis in which cytokines amplify local tissue destruction. The K/BxN serum transfer model of arthritis allows arthritis to be induced in a manner that bypasses the initial T cell and B cell interactions necessary to promote autoantibodies and instead measures the downstream events involved in antibody-induced, cytokine-mediated joint destruction. To more specifically investigate the more distal inflammatory phase of arthritis, K/BxN serum transfer arthritis or anti-CII antibody-induced arthritis preferable to CIA [42, 43]. The proportion of *NKT* cells was increased in the lymph nodes and peripheral blood of mice receiving K/BxN serum similar to CIA [39]. Arthritis induced either by injection of K/BxN serum or CII antibody in *NKT*-deficient mice was ameliorated [39, 41]. Regarding the mechanisms that *NKT* cells contribute to the development of arthritis in K/BxN serum transfer model, production of IFN- γ and IL-4 from *NKT* cells has been implicated in the suppression of TGF-β1 [41]. TGF-β is known to inhibit arthritis, especially when administrated systemically [44–46]. TGF-β inhibits T cell proliferation and downregulates the expression of IL-1 receptor, which may result in the suppression of arthritis. Even though *NKT* cells appear to contribute to the pathogenesis of arthritis, activation of *NKT* cells by synthetic glycolipid ligands protected mice against CIA [37]. Repeated injections of OCH, the Th2 polarizing form of an αGalCer, inhibited the clinical course of CIA, whereas α-GalCer administration exhibited a mild suppression of disease. Interestingly, OCH treatment suppressed CIA in SJL mice that have defects in numbers and functions of *NKT* cells. Moreover, OCH treatment ameliorated disease even after arthritis had developed. Suppression of arthritis was associated with the elevation of the IgG1:IgG2a ratio, suggesting a Th2 bias of CII-reactive T cells in OCH-treated mice. Furthermore, neutralization of IL-10 or IL-4 by monoclonal antibody reversed the beneficial effect of OCH treatment. In contrast to CIA, the treatment of α-GalCer has been shown to exacerbate disease in K/BxN serum transfer arthritis [41]. In our study, however, administration of α-GalCer efficiently inhibited K/BxN serum transfer arthritis by a Th2-independent mechanism [75]. The protective effect of arthritis by synthetic glycolipid ligands in arthritis models seems inconsistent with the reduction of disease severity in *NKT*-deficient mice. It is possible that activation of *NKT* cells by synthetic glycolipid is different from the physiological activation of *NKT* cells with endogenous ligands under pathogenic conditions such as arthritis.

2.4
NKT Cells in Lupus Models

Early studies in mice strains which spontaneously develop lupus-like disease such as MRL lpr/lpr mice, C3H gld/gld mice, and (NZBxNZW)F1 mice demonstrated a decrease in *NKT* cell number before the onset of disease, suggesting a preventive effect of *NKT* cells, although NKT cells were not well defined in these studies [47, 48]. The studies using *NKT*-deficient mice revealed a functional role for *NKT* cells in lupus animal models. CD1d deficiency led to exacerbation of pristine-induced nephritis in Balb/c mice [49] and skin disease in MRL lpr/lpr mice without inducing significant differences in nephritis and production of autoantibodies to nuclear antigens [50]. Another group, however, demonstrated that CD1d deficiency neither accelerated skin disease nor ameliorated kidney disease in MRL/lpr mice [51]. Stimulation of *NKT* cells with α-GalCer ameliorated dermatitis in MRL lpr/lpr mice in association with expansion of *NKT* cells and increased Th2 responses, while treatment with α-GalCer had no effect on kidney disease and serum anti-DNA antibody level [52]. In pristine-induced nephritis models, the effect of α-GalCer differs, depending on the strains of mice used. In Balb/c mice, treatment with α-GalCer promoted Th2 responses and protected mice against nephritis. Conversely, treatment with α-GalCer promoted Th1 responses and exacerbated disease in SJL/J mice [53]. The difference in the effect of α-GalCer seems to correlate with the ability to produce Th2 cytokines by activated *NKT* cells, similar to the phenomenon observed in EAE models.

Enhancement of disease rather than protection was observed in (NZB x NZW)F1 mice. In wild-type mice, *NKT* cells increased in number after the onset of disease [54, 55], and the transfer of NK1.1$^+$ T cells from diseased mice to young F1 mice (before the onset of renal failure) induced proteinuria and swelling of the glomeruli [56]. Moreover, treatment with anti-CD1d monoclonal antibody augmented Th2-type responses, increased serum levels of IgE, decreased levels of IgG2a and IgG2a anti-double-stranded DNA (dsDNA) antibodies, and ameliorated lupus [56]. Consistent with these results, activation of *NKT* cells with α-GalCer accelerated nephritis in correlation with enhancement of Th1 responses [56]. Despite the differences in the outcome of disease treatment following *NKT* cell activation, one consistent finding in the above studies is that *NKT* cell-driven Th1 responses lead to disease exacerbation, whereas *NKT* cell-driven Th2 responses lead to disease amelioration. One future direction may be to concentrate on which strain-dependent factors promote *NKT* cell-induced Th1 or Th2 responses.

2.5 NKT Cells in Colitis

Dextran sodium sulfate (DSS) -induced colitis is an experimental model for Crohn's disease mediated by Th1 cells. Activation of *NKT* cells with α-GalCer or OCH has been shown to protect mice against DSS-induced colitis [57, 58]. While treatment with both glycolipids induced higher amounts of IFN-γ and IL-4 than controls, the IFN-γ:IL-4 ratio was decreased compared to the control group [58]. The level of IL-10 in the supernatants of colon organ cultures after the injection of OCH was increased [58]. Treatment with OCH induced higher IL-10 production than did α-GalCer, which is consistent with the stronger inhibitory effect of OCH in this model of colitis [58]. Conversely, *NKT* cells have been proposed to act as effector cells in oxazalone-induced colitis [59]. Oxazalone-induced colitis is an experimental colitis model of human ulcerative colitis and is dependent on IL-13. This model of colitis was effectively blocked by neutralizing the IL-13 or depleting *NKT* cells. Moreover, the colitis did not develop in mice deficient in *NKT* cells, indicating the crucial pathological role of *NKT* cells in this model, similar to asthma models which *NKT* cells play an important role in the pathogenesis through IL-13 production.

3 NKT Cells in Human Autoimmune Diseases

Previous studies have documented a reduced number of *NKT* cells in the peripheral blood of patients suffering from systemic sclerosis [60], type 1 diabetes [61,62], multiple sclerosis (MS) [63–65], and other autoimmune disease conditions [66–68]. However, in type 1 diabetes, inconsistent results (decreased [61, 62], normal [69] or increased [70] number of *NKT* cells) were obtained by three independent groups, which led to considerable argument on the role of *NKT* cells in autoimmunity. To identify *NKT* cells, recent studies used α-GalCer-loaded CD1d tetramers, the most reliable tool for staining *NKT* cells, and confirmed a reduced number of *NKT* cells in the peripheral blood from untreated MS patients in remission [64] compared to healthy subjects. Unexpectedly, the number of *NKT* cells tended to increase in the relapse phase of MS and that the deficiency of *NKT* cells may become normalized in the patients treated with low doses of oral corticosteroids (unpublished results). These results imply that disease activity as well as prescribed medications would greatly influence on the number of *NKT* cells, which may provide some clue to designing future studies.

Production of Th2 cytokines was previously described as a cardinal feature of *NKT* cells. Now it is widely accepted that anti-inflammatory Th2 cytokines are secreted from $CD4^+$ *NKT* cells, but not from $CD4^-$ *NKT* cells [71, 72]. Namely, $CD4^+$ *NKT* cells are able to produce both Th1 and Th2 cytokines, whereas $CD4^-$ *NKT* cells selectively produce proinflammatory Th1 cytokines TNF-α and IFN-γ. Given this important dichotomy of *NKT* cells, we have re-analyzed the number and functions of *NKT* cells from untreated MS patients after sorting the cells into $CD4^+$ and $CD4^-$ populations [64]. $CD4^-$ *NKT* cells were greatly reduced in the peripheral blood of the untreated patients in remission, whereas a reduction of $CD4^+$ *NKT* cells was only marginal. Moreover, $CD4^+$ *NKT* cells from the patients were Th2-biased, as evidenced by enhancement of IL-4, whereas $CD4^-$ *NKT* cells were not. As both of the observed changes, Th2 bias of $CD4^+$ and a great reduction of $CD4^-$ *NKT* cells, could be regarded as disease stabilizing changes, we interpreted that this finding to suggest that the role of *NKT* cells in remission of MS is protective against disease development. In contrast, Th1 bias, as evidenced by loss of IL-4 secretion, was confirmed in *NKT* cell clones derived from peripheral blood [61] as well as draining lymph nodes [73] of type 1 diabetes. Although it remains unclear why *NKT* cells from MS and type 1 diabetes are biased toward opposing directions, it is possible that it may reflect the differential disease activity at the time of examination or due to differences in disease pathogenesis.

4 Concluding Remarks and Future Research

The research of the last decade has firmly established that *NKT* cells have the potential to either drive or suppress autoimmune conditions, although we know very little about the precise rules of how the regulation of *NKT* cells in vivo. Despite previous controversies, it now seems that numerical or functional changes of NKT cells may be associated with certain stages of autoimmune diseases, as has been revealed in MS [64] Therapeutic effects of glycolipid ligands for *NKT* cells have indicated that these lymphocytes bridging innate and adaptive immunity may be an excellent target for immune intervention. In particular, the design of "altered" CD1 glycolipid ligands has been shown in many models to alter the outcomes of disease. Meanwhile, it is much less clear how *NKT* cells regulate unwanted autoimmunity taking place in the context of the physiological immune network. It is likely that *NKT* cells produce Th2 cytokines to achieve this, and that the trigger of cytokine production may be an encounter of *NKT* cells with an endogenous ligand bound to CD1d. However, one known endogenous ligand, iGb3, seems to provoke

Th1 cytokines from *NKT* cells [74]. In this regard, there is room for a seeking Th2-inducing natural ligand. Not mutually exclusive to this is the theory that cytokines produced in the inflammatory site together with endogenous antigen and TCR-mediated signals may play a key role in conducting *NKT* cells [38] to secrete Th2 cytokines (Sakuishi et al., unpublished data). To gain deeper insights into the role of *NKT* cells in autoimmunity, the details of natural regulation needs to be clarified in the future.

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Invariant $V_\alpha 19i$ T cells regulate autoimmune inflammation

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T cells expressing an invariant $V_\alpha 19$-$J_\alpha 33$ T cell receptor α-chain ($V_\alpha 19i$ TCR) are restricted by the nonpolymorphic major histocompatibility complex class Ib molecule MR1. Whether $V_\alpha 19i$ T cells are involved in autoimmunity is not understood. Here we demonstrate that T cells expressing the $V_\alpha 19i$ TCR transgene inhibited the induction and progression of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Similarly, EAE was exacerbated in MR1-deficient mice, which lack $V_\alpha 19i$ T cells. EAE suppression was accompanied by reduced production of inflammatory mediators and increased secretion of interleukin 10. Interleukin 10 production occurred at least in part through interactions between B cells and $V_\alpha 19i$ T cells mediated by the ICOS costimulatory molecule. These results suggest an immunoregulatory function for $V_\alpha 19i$ T cells.

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Two distinct mouse T cell subsets express invariant TCRα chains: $V_\alpha 14$-$J_\alpha 18$ ($V_\alpha 14i$; ref. 1) and $V_\alpha 19$-$J_\alpha 33$ ($V_\alpha 19i$; ref. 2). Although conventional T cells recognize peptide antigens presented by polymorphic major histocompatibility complex class Ia molecules, $V_\alpha 14i$ 'invariant' T cell populations recognize nonpeptide antigens[3,4] presented in the context of the nonpolymorphic major histocompatibility complex class Ib molecule CD1d. MR1 may be able to present glycolipids *in vitro* to $V_\alpha 19i$ T cells[5], but the identity or type of endogenous ligand recognized by $V_\alpha 19i$ T cells *in vivo* is unknown. However, antigen recognition is essential for the development of T cells expressing $V_\alpha 14i$ and $V_\alpha 19i$ TCR chains, as these subsets are absent from *Cd1d1*$^{-/-}$ and *Mr1*$^{-/-}$ mice, respectively[6,7]. Similar invariant T cell subsets are present in humans[8,9]. Many of these cells also express natural killer (NK) cell markers on their surface (such as mouse NK1.1). Consequently, CD1d-restricted invariant T cells have traditionally been referred to as 'NKT cells' ($V_\alpha 14i$ NKT cells)[10].

Transgenic overexpression of the $V_\alpha 14i$ TCR chain protects against the development of mouse models of type I diabetes[11] and multiple sclerosis[12], suggesting that $V_\alpha 14i$ NKT cells may be involved in regulating autoimmunity. In addition, susceptibility to type I diabetes is linked to quantitative and functional deficiencies in $V_\alpha 14i$ NKT cells[13]. Mechanistic studies suggest that $V_\alpha 14i$ NKT cells may down regulate autoimmunity by increasing the production of T helper type 2 (T_H2) cytokines[14–19]. However, in other conditions, NKT cells may promote the exacerbation of autoimmune disease. $V_\alpha 14i$ NKT cell–deficient mice show ameliorated arthritis compared with that of their wild-type counterparts[18,20,21].

The immune function of MR1-restricted invariant T cells remains less clear than that of CD1d-restricted lymphocytes. MR1-restricted invariant T cells were first identified among human peripheral blood CD4$^-$CD8$^-$ T cells as a clonally expanded population expressing an invariant $V_\alpha 7.2$-$J_\alpha 33$ TCR chain ($V_\alpha 7.2i$ T cells)[22]. Subsequent studies identified clonally expanded T cells expressing the highly homologous invariant $V_\alpha 19$-$J_\alpha 33$ TCR chain in mice and cattle[9]. $V_\alpha 19i$ T cell development has been found to depend on the nonpolymorphic major histocompatibility complex class Ib molecule MR1 and on the presence of B cells[7]. The $V_\alpha 19i$ TCR is uniquely overexpressed in the gut lamina propria and $V_\alpha 19i$ T cell development depends on the presence of commensal gut flora, indicating potential involvement of these cells in gut immunity[2,7]. As MR1 molecules are thought to be retained in the endoplasmic reticulum, intestinal flora might provide exogenous ligands for the $V_\alpha 19i$ TCR, or a cellular 'stress' signal, that enables transit of MR1 from the endoplasmic reticulum to the cell surface[2,7].

Human $V_\alpha 7.2i$ T cells[2] but not mouse gut $V_\alpha 19i$ T cells express NKT cell markers[7]. In contrast, the $V_\alpha 19i$ TCR is expressed by most T cell hybridomas derived from liver NK1.1$^+$ T cells from *Cd1d1*$^{-/-}$ mice[23]. Furthermore, 25–50% of $V_\alpha 19i$ cells from $V_\alpha 19i$ transgenic mice on a *Tcra*$^{-/-}$ background express NK1.1 (ref. 24). Those divergent results regarding NK1.1 expression remain unclear, but may be due to differences among mouse genetic backgrounds. Alternatively, as with CD1d-restricted T cells, a subpopulation of MR1-restricted T cells may lack NK1.1 expression. Based on their predominant distribution in the gut, MR1-restricted T cells are often referred to as 'mucosal-associated invariant T cells'[2,7]. To avoid confusion, we subsequently use the term '$V_\alpha 19i$ T cells' to describe $V_\alpha 19i$ T cells expressing NK1.1.

The $V_\alpha 7.2i$ TCR is over-represented in central nervous system (CNS) lesions from multiple sclerosis autopsy samples[25], whereas the $V_\alpha 24i$ TCR is mostly absent[26]. Those findings led us to speculate that MR1-restricted T cells may 'preferentially' migrate to CNS lesions,

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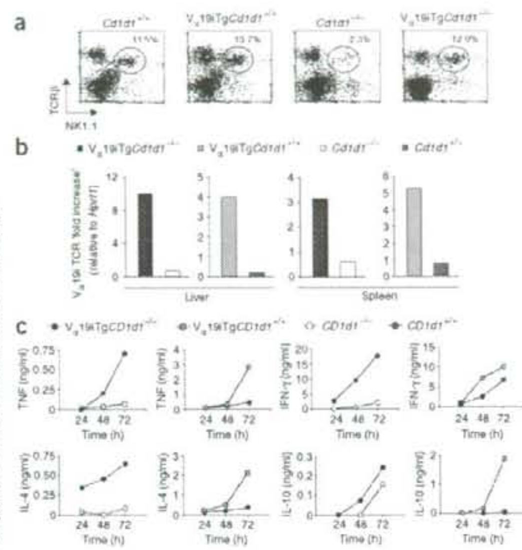

Figure 1 Characterization of NK1.1$^+$ T cells from V_α19iTg mice. (**a**) Flow cytometry of liver NK1.1$^+$ T cells 48 h after anti-asialo-GM1-mediated depletion of NK cells (mouse genotypes, above plots). Numbers above gated regions indicate the percentage of NK1.1$^+$TCRβ$^+$ cells. (**b**) Real-time RT-PCR of V_α19i TCR mRNA expression in liver or spleen NK1.1$^+$ T cells (mouse genotypes, key). Data are presented as 'fold increase' over expression of *Hprt1*. (**c**) Cytokines in the supernatants of sorted liver NK1.1$^+$ T cells (mouse genotypes, key) stimulated by immobilized anti-CD3 *in vitro*, measured at 24, 48 and 72 h after stimulation. Data are representative of two separate experiments (**a**,**b**) or the mean of two replicate values from two separate experiments (**c**).

where they regulate CNS inflammation. We designed this study to address the function of MR1-restricted T cells in experimental autoimmune encephalomyelitis (EAE)[14,17], a mouse model of multiple sclerosis. Here we report that over-representation of V_α19i T cells decreased the severity of EAE, whereas depletion of V_α19i T cells exacerbated EAE. Furthermore, V_α19i T cells exerted an influence on the phenotype and functions of autoimmune T cells in the draining lymph nodes and spleens of mice. In particular, over-representation of V_α19i T cells reduced the production of proinflammatory cytokines and increased the production of interleukin 10 (IL-10), which may account for V_α19i T cell–mediated suppression of autoimmune disease. Finally, interactions between V_α19i T cells and B cells mediated by the ICOS costimulatory molecule increased B cell IL-10 production and may therefore represent a mechanism by which V_α19i T cells regulate inflammation.

RESULTS

Characterization of transgenic V_α19i T cells

An antibody specific for the V_α19i TCR chain does not exist, and wild-type mice have very few MR1-restricted V_α19i T cells. Therefore, to circumvent those experimental hurdles and to evaluate the function of V_α19i T cells *in vivo*, we used V_α19i TCR–transgenic (V_α19iTg) mice[5], which were originally generated by injection into C57BL/6 mouse oocytes of a transgenic construct encoding a V_α19-J_α33 TCR construct driven by the endogenous *Tcra* promoter. We crossed the transgenic line with *Cd1d1*$^{+/+}$ and *Cd1d1*$^{-/-}$ C57BL/6 mice for seven to nine generations. First we compared numbers of liver NK1.1$^+$ T cells present in *Cd1d1*$^{+/+}$, *Cd1d1*$^{-/-}$, V_α19iTg*Cd1d1*$^{+/+}$ and V_α19iTg*Cd1d1*$^{-/-}$ mice (**Fig. 1a**). TCRβ$^+$NK1.1$^+$ T cells comprised 11.5% of total liver lymphocytes in *Cd1d1*$^{+/+}$ mice but only 2.3% of total liver lymphocytes in *Cd1d1*$^{-/-}$ mice. Therefore, most (about 80%) of NK1.1$^+$ T cells in *Cd1d1*$^{+/+}$ mice corresponded to CD1d-restricted V_α14i NKT cells, whereas about 20% were probably MR1 restricted[23]. Notably, V_α19iTg*Cd1d1*$^{-/-}$ mice had many NK1.1$^+$ T cells (12.0%), indicating that overexpression of the V_α19i TCR in *Cd1d1*$^{-/-}$ mice compensated for the reduction in NK1.1$^+$ T cells caused by CD1d deficiency. In contrast, the number of NK1.1$^+$ T cells was only slightly higher in V_α19iTg*Cd1d1*$^{+/+}$ mice, which had normal numbers of V_α14i NKT cells. To confirm that the NK1.1$^+$ T cell population in V_α19iTg mice was enriched in cells expressing the V_α19i TCR chain, we measured V_α19i mRNA transcripts in NK1.1$^+$ liver cells and splenocytes by real-time RT-PCR (**Fig. 1b**). V_α19i mRNA expression was much greater in liver and splenic NK1.1$^+$ T cell populations from V_α19iTg*Cd1d1*$^{+/+}$ or V_α19iTg*Cd1d1*$^{-/-}$ mice than in those from nontransgenic littermates (**Fig. 1b**). In V_α19i T cells, the V_α19i TCR chain 'preferentially' associates with TCRβ chains containing V_β8 or V_β6 segments[24]. Approximately 60–70% of liver NKT cells from V_α19iTg*Cd1d1*$^{-/-}$ or V_α19iTg*Tcra*$^{-/-}$ mice expressed either V_β8 or V_β6, compared with 30–40% of conventional T cells in the same mice (unpublished observations). These observations collectively demonstrate that NK1.1$^+$ T cell populations in V_α19iTg mice are highly enriched in cells expressing V_α19-J_α33 TCR chains and V_β6 or V_β8 TCR chains. Next we compared the ability of NK1.1$^+$ T cells from V_α19iTg and nontransgenic mice to produce immunosuppressive cytokines. To obtain V_α19i T cells, we depleted V_α19iTg*Cd1d1*$^{-/-}$ mice of NK cells by injecting antibody to

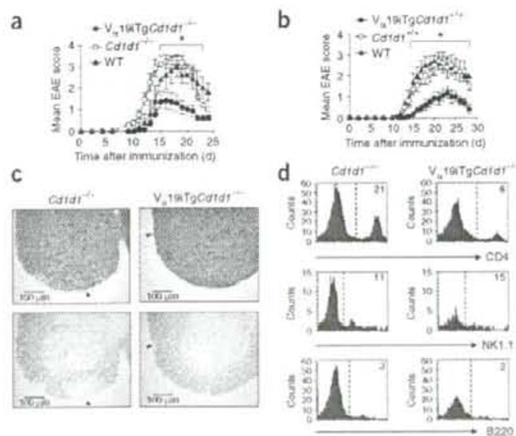

Figure 2 V_α19i T cells in EAE. (**a**,**b**) Clinical EAE scores of mice immunized with MOG(35–55). WT, wild-type. Data represent mean score ± s.e.m. from three independent experiments (n = 10–22 mice). (**c**) Monocyte infiltration and demyelination (arrowheads) of the lumbar spinal cord during EAE (day 15). (**d**) Quantification of spinal cord cellular infiltrates by flow cytometry. Areas to the right of dashed lines indicate positive cellular staining; numbers in histograms indicate percentage of CD4$^+$, NK1.1$^+$ (gated on CD3$^+$) or B220$^+$ cells. *, $P < 0.05$ (Mann-Whitney U-test). Data are representative of three separate experiments.

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Table 1 V_{α}19i T cells in EAE

Group	Mice with EAE	Group score	EAE score	Day of onset
Wild-type	10 of 10	3.3 ± 0.3	3.3 ± 0.3	13.6 ± 0.7
Cd1d1$^{-/-}$	18 of 18	3.4 ± 0.2	3.4 ± 0.2	11.7 ± 0.5
V_{α}19iTg*Cd1d1*$^{-/-}$	13 of 22	1.3 ± 0.3***	2.2 ± 0.2**	14.3 ± 0.6**
Wild-type	7 of 7	3.6 ± 0.2	3.6 ± 0.2	13.6 ± 0.5
Cd1d1$^{+/+}$	11 of 11	3.3 ± 0.4	3.3 ± 0.4	14.8 ± 0.7
V_{α}19iTg*Cd1d1*$^{+/+}$	9 of 13	1.3 ± 0.3**	1.9 ± 0.4*	18.6 ± 1.2**
NK1.1$^-$ AdTx	10 of 10	3.6 ± 0.3	3.6 ± 0.3	11.6 ± 0.5
V_{α}19i AdTx	8 of 10	2.2 ± 0.4*	2.8 ± 0.3	15.8 ± 0.6***
Mr1$^{+/+}$	10 of 10	3.0 ± 0.2	3.0 ± 0.2	13.9 ± 0.5
Mr1$^{-/-}$	8 of 8	4.0 ± 0.0**	4.0 ± 0.0*	11.5 ± 0.5***

Clinical outcome of mice immunized with MOG(35–55) to induce EAE. Data represent number of mice with EAE (of total mice in group); mean group EAE score (± s.e.m.); mean EAE score excluding mice without evidence of EAE (± s.e.m.); and mean day of onset (± s.e.m.). In one experiment, mice received adoptive transfer (AdTx) of V_{α}19i T cells or NK1.1$^-$ cells as a control. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared with control groups (Mann-Whitney U nonparametric test).

asialo-GM1 (anti-asialo-GM1). We then sorted NK1.1$^+$ cells from the liver. When activated by plate-bound anti-CD3, NK1.1$^+$ T cells from *Cd1d1*$^{+/+}$ mice secreted more interferon-γ (IFN-γ), tumor necrosis factor (TNF) and interleukin 4 (IL-4) than did those from *Cd1d1*$^{-/-}$ mice, confirming that CD1d-restricted T cells are a chief source of cytokines (**Fig. 1c**). However, NK1.1$^+$ T cells from V_{α}19iTg mice secreted more T_H1 cytokines (IFN-γ and TNF) and T_H2 cytokines (IL-4 and IL-10) than did NK1.1$^+$ T cells from nontransgenic littermates (**Fig. 1c**). During subsequent experiments, we used V_{α}19iTg*Cd1d1*$^{-/-}$ mice as a source of V_{α}19i T cells.

V_{α}19i T cells in EAE

To determine if an abundance of V_{α}19i T cells could modulate autoimmune disease, we analyzed the development and progression of EAE in V_{α}19iTg mice. We induced EAE by immunizing mice with a peptide of amino acids 35–55 of myelin oligodendrocyte glycoprotein (MOG(35–55)). The presence of the V_{α}19i transgene suppressed the development and progression of EAE, regardless of whether CD1d-restricted NKT cells were present (**Fig. 2a,b** and **Table 1**). The onset of EAE was delayed in V_{α}19iTg mice, and the incidence and severity of clinical EAE was reduced.

Histological examination of the lumbar (L3) region of the spinal cord 15 d after EAE induction showed less monocyte infiltration and demyelination (assessed by luxol fast blue staining) in V_{α}19iTg*Cd1d1*$^{-/-}$ mice than in *Cd1d1*$^{-/-}$ mice (**Fig. 2c**). In agreement with the histology, spinal cords of *Cd1d1*$^{-/-}$ mice contained three times more infiltrating cells than did those from V_{α}19iTg*Cd1d1*$^{-/-}$ mice (0.09×10^6 and 0.03×10^6 cells respectively, pooled from three mice). Flow cytometry showed fewer CD4$^+$ T cells infiltrating the CNS at an active stage of EAE (day 15) in V_{α}19iTg*Cd1d1*$^{-/-}$ mice (6%) than in nontransgenic littermates (21%; **Fig. 2d**). Moreover, 11% and 15% of CNS-infiltrating CD3$^+$ T cells expressed NK1.1$^+$ in *Cd1d1*$^{-/-}$ and V_{α}19iTg*Cd1d1*$^{-/-}$ mice, respectively, and NK1.1$^+$ T cells comprised between 1% and 2% of total CNS-infiltrating cells (**Fig. 2d**). Also, few B cells trafficked into the CNS during EAE (3% and 2% in *Cd1d1*$^{-/-}$ and V_{α}19iTg*Cd1d1*$^{-/-}$, respectively, **Fig. 2d**). To determine potential mechanisms of reduced CNS infiltration, we analyzed the expression of chemokine receptors and adhesion molecules necessary for T cell migration into the CNS. TCRβ$^+$CD4$^+$ T cells isolated from the CNS, lymph nodes and spleens of V_{α}19iTg*Cd1d1*$^{-/-}$ and *Cd1d1*$^{-/-}$ mice on day 18 after EAE induction had similar surface expression of CCR1 and CCR2 (data not shown). However, V_{α}19iTg*Cd1d1*$^{-/-}$ mice had fewer CD44$^+$ and CD49d$^+$ TCRβ$^+$ splenocytes than did *Cd1d1*$^{-/-}$ mice (**Supplementary Fig. 1** online).

Next we examined recall responses of MOG(35–55)-primed T cells by *ex vivo* rechallenge with MOG(35–55) on day 10 after disease induction. Compared with nontransgenic cells, lymph node cells from MOG(35–55)-primed V_{α}19iTg*Cd1d1*$^{-/-}$ mice produced less pro-inflammatory cytokines (IFN-γ, TNF, IL-2 and IL-17) and more immunosuppressive IL-10 ($P < 0.05$; **Fig. 3a**). IL-4 and IL-5 were below the limits of analysis detection (less than 5 pg/ml).

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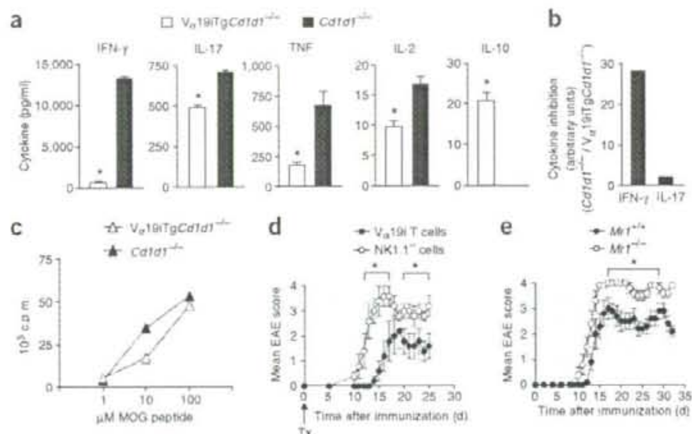

Figure 3 Inhibition of EAE is associated with decreased T_H1 cytokine production. (**a**) Cytometric bead assay of cytokines in the supernatants of MOG-specific lymph node cells (1×10^6) isolated from mice on day 10 after EAE induction and rechallenged with 100 µM MOG(35–55) *in vitro*, measured 72 h after rechallenge. Data represent the mean ± s.e.m. of duplicate samples from three separate experiments. *, $P < 0.05$ (two-tailed Student's *t*-test). (**b**) Inhibition of IFN-γ or IL-17 in V_{α}19iTg*Cd1d1*$^{-/-}$ mice versus *Cd1d1*$^{-/-}$ mice from **a**, presented as 'fold inhibition' of cytokine, calculated as the cytokine concentration from *Cd1d1*$^{-/-}$ mice divided by the cytokine concentration from V_{α}19iTg*Cd1d1*$^{-/-}$ mice. (**c**) T cell proliferation of cell preparations identical to those in **a** from lymph nodes (mouse genotypes, key) rechallenged for 72 h with varying doses of MOG(35–55), assessed by [^{3}H]thymidine incorporation. Data represent the mean of triplicate samples from three separate experiments. (**d**) Clinical EAE scores of wild-type nontransgenic mice ($n = 10$) that received 1×10^6 sorted V_{α}19i T cells or an equal number of NK1.1$^-$ TCRβ$^+$ liver cells from V_{α}19iTg*Cd1d1*$^{-/-}$ mice on the day of immunization with MOG(35–55). Tx indicates the day of adoptive transfer of cells. (**e**) Clinical EAE scores of *Mr1*$^{-/-}$ and *Mr1*$^{+/+}$ mice ($n = 8$–10) immunized with MOG(35–55). Data are representative of triplicate samples from three separate experiments.