previous reports showing that $CD4^-$ NKT cells are necessary for, or that type II NKT cells are sufficient for, the suppression of tumor immunosurveillance (10–13, 16). Type I NKT cells, in contrast, have been described in different models as enhancing tumor immunosurveillance (20–23). Consistent with those reports, we also showed that the lack of type I NKT cells accelerates tumor growth and the selective stimulation of type I NKT cells induces protection. In contrast, here for the first time we found that the stimulation of type II NKT cells (by sulfatide, which appears to act selectively through this NKT cell subset) enhances tumor growth. Furthermore, we found that the simultaneous activation of both type I and type II NKT cells results unexpectedly in a clear suppressive effect of type II NKT cells over type I NKT cell stimulation, with the down-regulation of type I NKT cell activation in vitro and diminished protection against tumors in vivo in two different tumor models. These findings identify a novel immunoregulatory axis between the two subsets of NKT cells with opposite functions.

Although little is still known about the physiological roles of type II NKT cells, recently several groups have succeeded in characterizing a role for this NKT cell subset in different immune responses, including infectious and autoimmune diseases (9, 30–32, 39). These studies raised attention to a little-studied NKT cell subpopulation, which can potentially play a role in a wider range of immune responses. However, in the context of tumor immunity, beyond our initial observation (12) there are no reports on the potential roles of type II NKT cells. In this study, for the first time, we directly investigated the activity of the little-studied type II NKT cell in the regulation of tumor immunosurveillance. Recently, Jahng et al. (9) characterized the myelin-derived lipid Ag sulfatide as a selective ligand for a proportion of the type II NKT cells. The in vivo activation of a non-αGalCer-reactive type II NKT cell with sulfatide suppressed pathological autoimmune responses in a murine model (9, 40). In our study, we took advantage of the activation of a proportion of type II NKT cells by sulfatide to study them in our tumor models. In both NKT cell-intact WT mice and type I NKT cell-deficient Jα18KO mice, treatment with sulfatide increased the number of lung nodules. Tumor growth in CD1dKO mice was not affected by sulfatide treatment, confirming that sulfatide is specifically activating type II NKT cells and is not exerting nonspecific effects or toxicity. Although we have not yet identified the specific type II NKT cell Ag in our tumor models, these findings suggest that the suppressive cells are sulfatide-reactive and their activation by sulfatide directly demonstrates their ability to down-regulate tumor immunosurveillance, although it does not imply that sulfatide is the physiologic ligand. Indeed, similarly αGalCer itself is not a physiologic type I NKT cell ligand and is not expressed in humans or other mammals at all, but it has been widely used a tool to study type I NKT cell activity. Sulfatide is the simplest member of a class of acidic glycolipids containing sulfate esters that are found in many tissues as well as many tumors (41, 42). In humans, different classes of CD1 molecules have been shown to present members of the sulfatide family (43). We plan to investigate whether any of these tumor-derived lipids are involved in type II NKT cell activation in our tumor models.

Over the past years many studies have investigated the role of NKT cells in tumor immunosurveillance, mainly focusing on type I NKT cells, and have implicated this cell population primarily in the promotion of tumor immunosurveillance (21–23). We previously reported that type I NKT cell-deficient Jα18KO mice would eventually develop lung nodules as well as WT mice (12). To examine the role of type I NKT cells over the course of tumor growth, we compared the tumor growth in their presence (WT mice) or absence (Jα18KO mice) at an early stage of tumor growth. At a very early time point, Jα18KO mice are more susceptible to tumor growth and have no CTL immune response to tumor Ag-pulsed cells in contrast to WT mice, indicating, in accordance with a previous study (21), that type I NKT cells contribute to the natural tumor immunosurveillance during early tumor growth. The observation of a weak anti-tumor CTL response in WT mice but not in Jα18KO mice was made at the same early tumor stage in which a higher susceptibility to tumor growth was observed in Jα18KO mice compared with WT mice. The two observations seems reasonably correlated: we therefore reason that such a weak immune response in WT mice, although too weak to mediate significant protection against tumor growth, is the result of less suppression of the immune system by type II NKT cells in the presence of type I NKT cells. To further examine the protective role of the type I NKT cell, we stimulated this population in vivo. In accordance with previous observations (20, 44), the in vivo activation of type I NKT cells with the strong agonist αGalCer completely protected the mice from tumor growth. Further, we found that the OCH analog of αGalCer, shown to preferentially induce Th2 cytokines in type I NKT cells and to suppress Th1-induced autoimmune disease (36, 45), strongly suppressed tumor growth as well. This suggests a protective role for type I NKT cells within the range of the cytokine profiles we could test. Although this result makes less likely a role of type I NKT cell-secreted Th2 cytokines in the suppression of tumor immunosurveillance, the OCH ligand does not completely skew the immune response toward Th2 and induces a reasonable level of IFN-γ production, although at lower levels compared with αGalCer. It would be of interest to further investigate the clinical effect of a complete Th2 skewing of type I NKT cell activation.

Finally, we investigated whether type I and type II NKT cells could potentially cross-talk when both cell populations were stimulated simultaneously. Although the stimulation with sulfatide is much weaker than the stimulation with αGalCer in terms of the induction of proliferation and cytokine release in vitro, surprisingly, when both types of NKT cells were stimulated at the same time the αGalCer-induced (Fig. 5, *A*, *C*, and *D*) or OCH-induced (Fig. 5*D*) proliferation was reduced in vitro and in vivo (E. Ambrosino, M. Terabe and J. A. Berzofsky unpublished observations). Moreover the αGalCer-induced cytokine production was reduced and skewed toward a higher IL-13/IFN-γ (Fig. 5*F*) ratio in vitro, and a similar trend was observed in vivo (Fig. 6*B*). The same counteractive effect was observed even when type II NKT cells were stimulated 15–30 min later than type I NKT cells or when APC were independently pulsed with αGalCer or sulfatide and then mixed (Fig. 5*C*), ruling out a possible competition by sulfatide for αGalCer binding to CD1d molecules or a direct antagonistic effect of sulfatide on the same cell as αGalCer upon the stimulation of type I NKT cells independently of type II NKT cells. The lesser degree of suppression in the culture in which APC were pulsed with αGalCer or sulfatide and then mixed to stimulate $CD4^+$ cells compared with that in the culture in which soluble Ags were added may be due to the lower affinity of sulfatide for CD1d molecules compared with αGalCer (9). Thus, it is unlikely that the suppression of type I NKT activation by type II NKT cells is a result of competition for CD1d binding. Most importantly, the clinical protective effect of αGalCer treatment was either reversed or reduced when sulfatide was coadministrated in vivo, in that the protection induced by type I NKT cell stimulation was partially or completely lost, depending on the tumor model (Fig. 7). In vivo, the expression of CD1d is so widespread that these molecules could not be anywhere near saturation by αGalCer or sulfatide at the doses administrated, again ruling out direct competition of

these ligands for CD1d. Also, we found no evidence of a nonspecific cytotoxic effect of sulfatide on APCs, type I NKT cells, or conventional T cells either in vitro or in vivo (data not shown) by evaluating cell numbers and proportions of the different populations (T cell subsets, B cells, myeloid dendritic cells, and plasmacytoid dendritic cells) remaining, their surface markers, and their propidium iodide staining after culture in sulfatide or vehicle. Also, the lack of effect of sulfatide in CD1dKO mice (Fig. 4*C*) or in mice depleted of $CD4^-$ T cells (Fig. 4*A*) excludes a nonspecific or toxic effect as the mechanism of tumor growth enhancement. For all of these reasons taken together we believe that sulfatide most likely acts directly on type II NKT cells, which recognize the sulfatide presented by CD1d, and, therefore, that it is the type II NKT cells that mediate the downstream effects; however, we cannot absolutely exclude more complex mechanisms involving other cells not tested in these studies.

In view of the central role of IL-13 in mediating the suppressive activity of NKT cells in tumor immunosurveillance (10, 11, 13, 46) in the tumor models used in this study, the tendency toward an increase in IL-13 secretion in vivo when type II NKT cells were simultaneously stimulated with type I NKT cells may contribute to the suppression of protection in mice treated with both αGalCer and sulfatide. The difference between the complete reversal of protection in the 15-12RM model and the partial reversal of protection in the CT26 model may relate to the greater sensitivity of the CT26 lung metastasis model to IFN-γ and NK cells activated by IFN-γ. Because the suppression of αGalCer-induced IFN-γ production by sulfatide was incomplete, the residual cytokine may be sufficient to partially protected in the lung metastasis model, but not in the s.c. 15-12RM tumor model. Nevertheless, the results demonstrate in two different models a novel suppressive effect of type II NKT cells on the ability of type I NKT cells to protect against cancer.

In conclusion, in this study we have defined a complex regulatory pathway of tumor immunosurveillance in which both subsets of NKT cells are involved and play opposite roles, forming a novel immunoregulatory axis. Furthermore, our data suggest a cross-talk between them, resulting in a counter-regulation of functions. Because we could not directly examine whether the interaction between type I and type II NKT cells occurs naturally in vivo, as for most studies, we had to stimulate the different cell populations to examine their activity. Nevertheless, our results clearly show that the described interaction has biologic significance in vivo in two different tumor models. At the moment we do not have detailed information about the mechanism through which type II NKT cells inhibit type I NKT cell activation. Also, we cannot distinguish a direct suppressive effect from one mediated through an intermediate cell such as a dendritic cell (47). The evidence that medium from type II NKT cell-activated cultures, when added to type I NKT cell-activated cultures, was not sufficient to inhibit their proliferation and that blocking soluble factors (such as IL-13 and TGFβ; E. Ambrosino, M. Terabe and J. A. Berzofsky unpublished observations) did not inhibit the suppressive activity of a type II NKT cell suggests that the mechanism of suppression could be by cell-to-cell contact rather than by soluble factors. Further studies will be performed to test either hypothesis. The final result is a balance between the type I and type II NKT cell activities regulating tumor immunosurveillance.

Because one of the mechanisms that may limit the effectiveness of immunotherapy of cancer is the active suppression of immune responses by lymphocytes, the blockade or elimination of these regulatory cells may represent a strategy for improving antitumor vaccines (48, 49). In this context, our studies suggest that the alteration of the balance between the protective type I and the suppressive type II NKT cell may be exploited for therapeutic intervention in cancer.

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Disclosures

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Review article

Understanding the behavior of invariant NKT cells in autoimmune diseases

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Abstract

Invariant NKT (iNKT) cells are a unique subset of lymphocytes that recognize glycolipid antigens presented by a monomorphic glycoprotein CD1d. Numerous works have shown that iNKT cells may serve as regulatory cells in autoimmune diseases including multiple sclerosis (MS). However, recent studies have revealed that the presence of iNKT cells accelerates some inflammatory conditions, implying that their protective role against autoimmunity is not predetermined. Here we review recent information concerning the mechanism of how iNKT cells intervene or promote autoimmune inflammation. Although iNKT cells are thought to be specific for a limited set of glycolipids, they may cross-react to self and non-self ligands. Regarding the response to non-self, it is now known that iNKT cells produce enormous amounts of proinflammatory cytokines during the course of infectious diseases, which is triggered by TCR ligation by microbial lipids, cytokines produced from APCs or both. Whereas the strongly activated iNKT cells play a beneficial role in combating environmental pathogens, they could play a deleterious role in autoimmunity by producing disease-promoting cytokines. However, iNKT cells in the steady state would retain an ability to produce anti-inflammatory cytokines, which is needed for terminating the ongoing inflammation. Though an initial trigger for their regulatory responses remains elusive, our recent work indicates that iNKT cells may start regulating inflammation after sensing the presence of IL-2 in addition to recognizing a ubiquitous endogenous ligand. Understanding of how iNKT cells regulate autoimmunity should lead to a more sophisticated strategy for controlling autoimmune diseases.

Keywords: NKT cells; iNKT cells; Multiple sclerosis

Contents

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1. Introduction

Invariant NKT (iNKT) cells are regulatory T lymphocytes reactive to lipid antigen presented by a monomorphic glycoprotein CD1d (Bendelac et al., 2007; Kronenberg, 2005; Taniguchi et al., 2003). Many previous reports have documented that the number or function of iNKT cells is altered in patients with autoimmune diseases such as multiple sclerosis (MS) (Araki et al., 2003; Illés et al., 2000; Kojo et al., 2001; van der Vliet et al., 2001b; Wilson et al., 1998). Studies using transgenic mice that over- or under-express iNKT cells have basically supported the involvement of iNKT cells in the pathogenesis of autoimmune diseases (Miyake and Yamamura, 2007a). Moreover, stimulating iNKT cells with synthetic glycolipids has proven effective for preventing experimental autoimmune encephalomyelitis (EAE) (Miyamoto et al., 2001; Pál et al., 2001) or spontaneous type 1 diabetes (T1D) in NOD mice (Naumov et al., 2001; Sharif et al., 2001), indicating the important role of iNKT cells in controlling pathogenic autoreactivity and maintaining immune homeostasis (Miyake and Yamamura, 2007b). However, more recent studies have shown that iNKT cells may augment inflammatory conditions in models of arthritis (Chiba et al., 2005; Kim et al., 2005; Ohnishi et al., 2005), $CD8^+$ T cell-mediated diabetes (Griseri et al., 2005), experimental colitis (Ronet et al., 2005; Ueno et al., 2005) and airway hypersensitivity reactions (Akbari et al., 2003; Meyer et al., 2007). These results indicate that unlike $CD4^+$ $CD25^+$ regulatory T cells that appear to be a faithful regulator of unwanted immune responses (Sakaguchi and Sakaguchi, 2005), iNKT cells' help is only conditional and would occasionally take part in augmentation of harmful inflammation. How activation of iNKT cells manifests such opposing results and what is an initial trigger for the regulatory iNKT cell responses has remained to be unanswered. Here we review recent advances in the research of iNKT cells that may be relevant for understanding the "Janus-like" behavior of iNKT cells (Wilson and Delovitch, 2003). Our ultimate goal is to seek ways for making iNKT cells serve as a reliable guardian for our health.

2. General properties of iNKT cells

Although iNKT cells express T cell receptor (TCR) α- and β-chains, their TCR diversity is very limited owing to their expression of a single α-chain (Vα14-Jα18 in mice, Vα24-Jα18 in human) coupled with a β-chain rearranged with a limited Vβ gene segments (Vβ8.2, Vβ2 and Vβ7 in mice, Vβ11 in human). Unlike conventional T cells, they constitutively express memory/activated T cell phenotype and are capable of producing enormous amounts of pro- and anti-inflammatory cytokines shortly after TCR engagement (Bendelac et al., 2007; Kronenberg, 2005; Miyake and Yamamura, 2005; Taniguchi et al., 2003). The cytokine burst following iNKT cell activation then triggers a maturation process in downstream cells such as NK cells, dendritic cells (DCs), B cells and T cells, leading to subsequent alteration of a broad range of adaptive immune responses. It is widely accepted that they could behave very much like innate lymphocytes rather than conventional T cells (Mempel et al., 2002), and owing to the rapidity with which they respond to various stimuli, they play an important role in bridging innate and adaptive arms of immune response.

The ability of iNKT cells to produce regulatory cytokines is so outstanding that they could efficiently alter an adaptive immune response. Mouse iNKT cells can produce interferon-γ (IFN- γ), IL-2 (Jiang et al., 2005), -3 (Leite-de-Moraes et al., 2002), -4, -5, -13, -17, -21 (Coquet et al., 2007), GM-CSF (Leite-de-Moraes et al., 2002), and osteopontin (Diao et al., 2004) after an optimal engagement of TCR. However, it does not mean that iNKT cells would purposefully use all the listed cytokines. In fact, it can be assumed that except for extreme conditions (like stimulation with strong agonists), iNKT cells may produce only a set of Th1 or Th2 cytokines in physiological conditions. We support this postulate because the TCR engagement by an endogenous ligand is likely to be modest or suboptimal in most situations (Sakuishi et al., 2007). With regard to their role in balancing immune homeostasis, an organized production of Th1, Th2 or Th17 cytokines is probably required for iNKT cells to conduct meaningful jobs.

3. Exogenous glycolipids stimulatory for iNKT cells

Since a marine sponge-derived glycosphingolipid, α-galactosylceramide (α-GalCer), was discovered as a potent ligand for iNKT cells (Kawano et al., 1997), a synthetic α-GalCer has widely been used for study of iNKT cells as a surrogate ligand (Fig. 1). It is now established that two lipid chains of α-GalCer are inserted to hydrophobic grooves of the CD1d glycoprotein expressed by antigen presenting cells (APCs) (McCarthy et al., 2007), whereas the α-linked sugar moiety is accessible and recognized by the TCR of iNKT cells. Recently, the crystal structure of the invariant TCR and CD1d loaded with α-GalCer has shown a very unique orientation of TCR towards CD1d (Borg et al., 2007), which allows a selective involvement of the invariant α-chain for recognition of the α-linked sugar.

Comparison of α-GalCer with its structurally altered analogues has provided important insights into how iNKT cells may differentially respond to glycosphoingolipids with lipid tail variants (Brutkiewicz, 2006; Miyake and Yamamura, 2007b). As a representative example, we showed previously that an α-GalCer analogue called OCH (Miyamoto et al., 2001; Oki et al., 2004, 2005), with a shorter sphingosine chain (Fig. 1), would selectively stimulate IL-4 production from iNKT cells, whereas α-GalCer stimulation induces both IL-4 and IFN-γ. Accordingly, OCH stimulation of iNKT cells favors a Th2 bias of immune responses *in vivo*, as compared to α-GalCer stimulation.

α-linked sugars such as α-GalCer are not recognized as a product of mammalian cells, implying that α-GalCer is not a physiological ligand for iNKT cells. Currently, it is well recognized that iNKT cells can be activated during infectious diseases (Tupin et al., 2007). Interestingly, it has been reported that α-GalCer-like glycosphingolipids are rather ubiquitously found in the environment, indicating that α-GalCer may be actually derived from bacteria residing with the marine sponge. Whether or not α-GalCer is derived from bacteria, we may ask a number of questions as to whether infectious diseases may influence on autoimmune disease via activation of iNKT cells

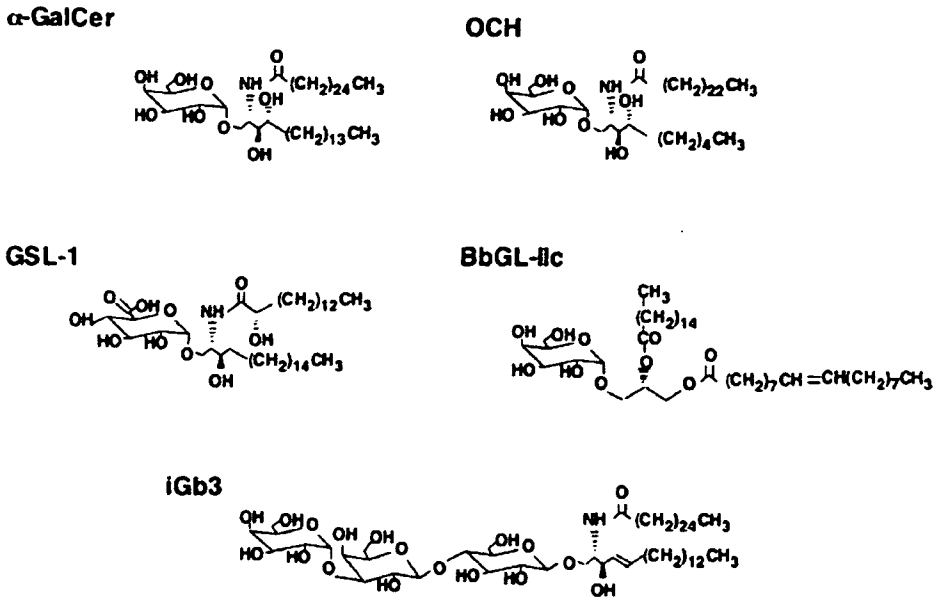

Fig. 1. Structure of glycolipid ligands for iNKT cells. Shown here are the structure of NKT cell agonists: α-galactosylceramide (α-GalCer) (Kawano et al., 1997), an α-GalCer analog called OCH, bearing a shorter sphingosine chain (Miyamoto et al., 2001), *Sphingomonas*-derived glycosphingolipid GSL-1 (Kinjo et al., 2005), *Borrelia burgdorferi*-derived diacylglycerol glycolipid BbGL-IIc (Kinjo et al., 2006), and isoglobotryhexosylceramide (iGb3) (Zhou et al., 2004).

(Godfrey and Berzins, 2006). Although multiple pathways are operative for iNKT cell activation in facing microbial challenge, it has been shown that glycosphingolipids from LPS-negative α-Proteobacteria such as *Sphingomonas* (Fig. 1) could stimulate a proportion of iNKT cells (Kinjo et al., 2005; Mattner et al., 2005). They also found that diacylglycerol glycolipids, extracted from *Borrelia burgdorferi*, stimulate at least 25% of iNKT cells (Kinjo et al., 2006; Kinjo et al., 2005). It is currently thought that arthritis and carditis found in Lyme disease following *B. burgdorferi* infection may be mediated by an autoimmune process. Whether iNKT cells activated by the diacylglycerol lipids may contribute to the pathogenesis of Lyme disease is an interesting question to be addressed. Likewise, an interesting idea is that relapse of MS following infection may be triggered by iNKT cells that are activated in response to microbial stimuli. Of note is that iNKT cells may produce osteopontin, which is reported to trigger relapses of EAE by promoting the survival of activated T cells in the inflammatory site (Hur et al., 2007).

4. Endogenous ligand for iNKT cells: search is not over

Search for an endogenous ligand of iNKT cells has led to the identification of lysosomal glycosphingolipid isoglobotryhexosylceramide (iGb3) (Fig. 1), a β-linked sugar capable of stimulating iNKT cells as a potential endogenous ligand for mouse and human iNKT cells (Mattner et al., 2005; Zhou et al., 2004). With regard to the role of iGb3 in adaptive immune responses, Mattner et al. reported that Gram-negative, LPS-positive *Salmonella typhimurium* activates NKT cells through the recognition of iGb3, presented by LPS-activated dendritic cells. However, very recent works have cast doubt on the meaning of the iGb3 discovery (Porubsky et al., 2007; Speak et al., 2007). The study by Zhou et al. (2004) indicated that iGb3 presented by CD1d-expressing $CD4^+CD8^+$ thymocytes should be involved in the thymic positive selection of iNKT cells. Porubsky et al. has then generated iGb3 synthetase deficient mice and examined if iNKT cells are really missing in the mice lacking expression of iGb3. They found that the number and function of iNKT cells were as normal as those seen in wild-type mice. Using highly sensitive HPLC assay, Speak et al. sought for the presence of iGb3 in various mouse and human tissues. The only tissue containing iGb3 was the dorsal root ganglion of mice. No iGb3 was detected in any human tissue (Porubsky et al., 2007; Speak et al., 2007). These new findings do not support the idea that iGb3 is central in the selection of iNKT cells and re-opened the search for endogenous ligands for iNKT cells.

With regard to the pathogenesis of MS, it is interesting to know if brain-derived lipids may stimulate iNKT cells. Although such ligands have not been identified yet for iNKT cells, sulfatide derived from the myelin appears to be a ligand for non-invariant NKT cells or type II NKT cells (Godfrey et al., 2004) that bear diverse TCR repertoire although restricted by CD1d glycoprotein (Jahng et al., 2004; Zajonc et al., 2005). This interesting finding leaves room for exploring presence of myelin-derived ligands for iNKT cells that may play a role in the pathogenesis of MS.

5. Human iNKT cells and autoimmune diseases

iNKT cells' recognition of CD1d ligand is well known for its evolutionary conservation across species barriers as indicated by the fact that both mouse and human iNKT cells share a highly homologous CDR3 of TCR α-chain and would cross-recognize α-GalCer (Spada et al., 1998). However, iNKT cells from mouse and human significantly differ in population size in lymphoid organs and peripheral blood (mouse ≫ human). In addition, a clear functional dichotomy for $CD4^+$ and $CD4^-$

populations is found in human (Gumperz et al., 2002; Lee et al., 2002) but not in mouse (Kronenberg and Gapin, 2002). A lower number of the iNKT cells has led to repeated questions about the actual role of iNKT cells in human. However, studies have shown that human iNKT cells show an outstanding ability to proliferate after *in vitro* (van der Vliet et al., 2001a; Yanagisawa et al., 2002) or *in vivo* stimulation with α-GalCer (Chang et al., 2005). Moreover, patients with rare genetic diseases associated with the absence of iNKT cells are reported to suffer from serious viral infections (Levy et al., 2003; Rigaud et al., 2006). These results support a vital role for iNKT cells in maintaining the human health.

The $CD4^+/CD4^-$ dichotomy of human iNKT cells (Gumperz et al., 2002; Lee et al., 2002) is widely appreciated at present. In brief, $CD4^+$ iNKT cells could produce both pro- and anti-inflammatory cytokines after proper stimulation, indicating their ability to balance immune homeostasis. In contrast, $CD4^-$ iNKT cells predominantly produce proinflammatory cytokines such as TNF-α and IFN-γ, but little Th2 cytokines, which is reminiscent of NK cells rather than T cells. A number of studies have addressed the difference between $CD4^+$ and $CD4^-$ iNKT cells in human disease conditions (Araki et al., 2003; Illés et al., 2000; Takahashi et al., 2003). A striking reduction of the total number of iNKT cells in the peripheral blood from remission state MS has been reported from us in previous studies (Araki et al., 2003; Illés et al., 2000). When the $CD4^+$ and $CD4^-$ iNKT cells were analyzed separately, we again noted a remarkable reduction of $CD4^-$ iNKT cells in MS. However, a reduction of $CD4^+$ iNKT cells was only modest. Furthermore, we generated long-term $CD4^+$ iNKT cell lines from MS and healthy subjects and compared their ability to produce IFN-γ and IL-4. We found that the $CD4^+$ NKT cells from subjects with MS produce much more IL-4 than those from healthy subjects, whereas production of IFN-γ was not significantly different. The data collectively support that Th2 biased $CD4^+$ NKT cells may somehow contribute to maintaining the remission state of MS. In contrast, a Th1 bias of iNKT cells has been reported in human type I diabetes (Kent et al., 2005; Wilson et al., 1998). This bias is characterized by the inability to produce IL-4. A similar Th1 bias was also confirmed by using iNKT cell clones derived from draining lymph nodes of affected pancreas from T1D patients (Kent et al., 2005). As such, Th2 bias of iNKT cells during remission of MS seems to be purposeful, whereas the Th1 bias found in T1D could contribute to enhancing pathogenic autoimmunity.

6. iNKT cells regulate autoimmunity in response to exogenous ligands

By using mice lacking CD1d or TCR Jα18 gene that is required for development of iNKT cells, a number of works have proven the role of iNKT cells in self-tolerance and prevention of autoimmunity. Yet, how iNKT cells actually contribute to maintaining self-tolerance remains largely unknown. Earlier works have mainly asked how an exogenous therapeutic ligand such as OCH would modulate autoimmune disease processes. A single injection of OCH protects against development of EAE. However, a simultaneous injection of anti-IL-4 antibody abrogated the preventive effect of OCH. Moreover, disease protective effects of OCH could not be seen in IL-4 knockout mice, indicating that IL-4 produced from iNKT cells is involved in the disease suppression (Miyamoto et al., 2001). Thus, a single NKT cell stimulation with OCH probably inhibits EAE in an Ag-nonspecific mechanism. In contrast, it has been shown by others that repeated injections of α-GalCer would suppress T1D by promoting differentiation and recruitment of tolerogenic DCs in draining lymph nodes (Chen et al., 2005; Gillessen et al., 2003; Naumov et al., 2001). It is possible that presentation of a tissue-specific antigen by tolerogenic DCs may induce Ag-specific regulatory $CD4^+$ T cells secreting IL-10, which accounts for the protection against diabetes.

Without applying an exogenous glycolipid, Lehuen and colleagues have recently shown that iNKT cells could prevent a T cell-transfer model of diabetes by inducing an anergic state of the pathogenic, islet-specific T cells. In contrast to other related works, this suppression did not require Th2 cytokines but was dependent upon direct cell-cell contact (Beaudoin et al., 2002). Subsequent studies showed that the cellular interaction does not involve CD1d recognition by NKT cells (Kent et al., 2005; Novak et al., 2007). Although the mechanism of iNKT cell-mediated regulation in this model remains unclear, it is reminiscent of our work showing that a newly recognized NKT cells (MR1-restricted Vα19 NKT cells) would mediate immune regulation via direct contact with B cells through ICOS-ICOSL interaction independent of TCR recognition (Croxford et al., 2006).

7. Cytokines instruct iNKT cell response towards Th1 or Th2

Although iNKT cells could conduct a tremendous job following stimulation with exogenous ligands or via direct cellular contact, recent studies on the behavior of iNKT cells during *S. typhimurium* infection have highlighted the importance of iNKT cell recognition of an endogenous CD1d ligand in combating against microbial pathogen (Brigl et al., 2003). The work by Brigl et al. showed that iNKT cells would respond to *S. typhimurium* by producing IFN-γ, when co-cultured with DCs. Interestingly, even stimulation with LPS from *S. typhimurium* could similarly induce the IFN-γ production, indicating the involvement of TLRs rather than TCR engagement by bacterial components. Subsequent experiments showed that this IFN-γ production critically required IL-12 that was derived from DCs via TLRs in a MyD88-dependent way. However, IL-12 was not sufficient to cause the iNKT cell production of IFN-γ. It was thought that the production of IFN-γ would require recognition of endogenous CD1d ligand, as anti-CD1d antibodies proved to block the response. Whether or not iGb3 is involved is still not clear, but these results clarified that iNKT cells would exert a decisive effector function (such as a predominant IFN-γ production) when iNKT cells recognize an endogenous ligand in the presence of an exogenous cytokine.

We have recently explored if cytokines other than IL-12 may induce an effector function of iNKT cells. For this aim, human $CD4^+$ iNKT cell clones were stimulated with various cytokines in the presence of DCs. None of the clones co-cultured with

DCs exhibited any noticeable response in the absence of exogenous cytokines. However, 7 out of 27 clones examined produced a large amount of IL-5 and IL-13 when IL-2 was added to the NKT-DC co-cultures. The amount of IL-5 and IL-13 was comparable to that induced with the most potent ligand α-GalCer. However, α-GalCer never induces such a biased response but stimulates production of a broad spectrum of pro- and anti-inflammatory cytokines. Remarkable production of IL-5 and IL-13 but not of other cytokines was also confirmed by conducting DNA microarray analysis. This surprising result raises two points: 1) human $CD4^+$ iNKT cells may comprise functionally distinct populations, including such IL-5/-13 producing clones, and 2) IL-2 may be a critical factor that induces a physiological Th2 response of iNKT cells. Further analysis showed that the production of Th2 cytokines was dependent on the TCR recognition of CD1d ligand. Indeed, addition of anti-CD1d antibody blocked the response, and CD1d lacking APCs could not induce the response. Furthermore, the combination of IL-2 with a weak TCR stimulus by suboptimal concentration of anti-CD3 antibody has reproduced a similar Th2 cytokine production. These results indicate that IL-2 could play a major role in instructing the iNKT cell population to selectively produce Th2 cytokines (Sakuishi et al., 2007). Taking all these into consideration, we propose that sensing the presence of cytokines is probably one of the most fundamental abilities for the iNKT cells that are to be given only a weak TCR signal *in vivo*.

IL-12 induced production of IFN-γ (Brigl et al., 2003; Mattner et al., 2005) as well as IL-2 induced production of IL-5 (Sakuishi et al., 2007) depends upon the recognition of endogenous ligand via TCR. However, iNKT cells could also produce a large amount of cytokine in response to cytokine signals independently of TCR signals. It has been shown that iNKT cells can be activated by *Escherichia coli* LPS, and produce IFN-γ, but not IL-4. Nagarajan and Kronenberg have shown that the production of IFN-γ was dependent upon LPS-induced IL-12 and IL-18 from APC, but did not require CD1d-mediated presentation of an endogenous Ag. Furthermore, they showed that exposure to a combination of IL-12 and IL-18 sufficiently activated the iNKT cells (Nagarajan and Kronenberg, 2007). TCR-independent production of Th1 cytokine strongly indicates the innate lymphocyte-like property of iNKT cells.

8. Antigen presenting cells for iNKT cells

To evaluate reactivity of iNKT cells, previous works have mostly used dendritic cells (DCs) or unseparated lymphoid cells as APCs. Recently, two groups have used non-professional APCs for stimulating iNKT cells, and obtained interesting results (Bezbradica et al., 2005; Im et al., 2006). The study by Bezradica et al. has compared the ability of DCs, B cells, hepatocytes, and macrophages to present α-GalCer to mouse NKT cells. Whereas presentation with DCs induced a remarkable production of IFN-γ and IL-4 from NKT cells, α-GalCer-loaded hepatocytes or

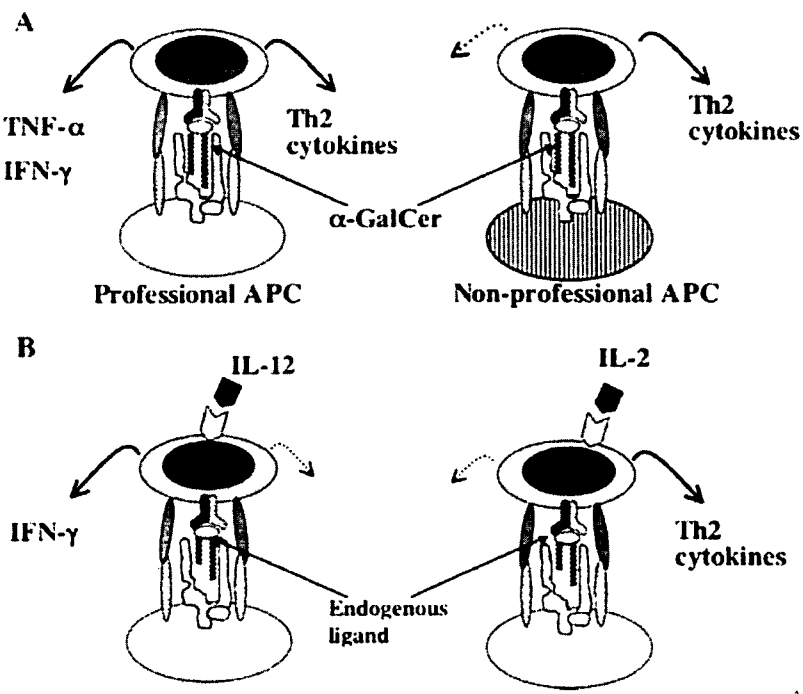

Fig. 2. Activation of iNKT cells by unconventional ways leading to functional bias. A: APC-dependent functional bias of NKT cells. Stimulating NKT cells with α-GalCer presented by professional APCs leads to production of both pro- and anti-inflammatory cytokines (left). However, when non-professional APCs such as Schwann cells (Im et al., 2006) are used, α-GalCer could induce a preferential production of Th2 cytokines from NKT cells. B: Cytokine-dependent functional bias of NKT cells recognizing endogenous ligand/CD1d. NKT cells usually exhibit only a marginal response in response to endogenous ligand bound with CD1d. However, when cytokines are added exogenously, the cells that recognize the endogenous ligand would produce a large amount of selected cytokines. For example, IL-12 induces production of IFN-γ (left) (Brigl et al., 2003), whereas IL-2 provokes IL-5 and IL-13 (right) (Sakuishi et al., 2007).

macrophages did not appear to induce iNKT cells responses. Interestingly, NKT cell stimulation with α-GalCer presented by B cells induced a weak cytokine response characterized by a low production of IL-4. Porcelli and his colleagues have examined the ability of human Schwann cells to present α-GalCer to NKT cells (Im et al., 2006). They showed that iNKT cells produced much lower amounts of proinflammatory cytokines (TNF-α and IFN-γ) but predominantly produced Th2 cytokines (IL-5 and IL-13) when Schwann cells were used as APCs. Although these studies did not examine the NKT cell reactivity to self-CD1d ligand, the results indicate that non-professional APCs tend to provoke production of Th2-associated cytokines from iNKT cells, allowing us to speculate that iNKT cell responses may greatly vary in different organs and tissues resided with different types of APCs.

9. Concluding remarks

Although most previous works have used α-GalCer or anti-CD3 antibody for stimulating iNKT cells to evaluate their functions, recent works have identified various alternative ways by which iNKT cells could be properly and differentially activated (Fig. 2). It is of particular note that iNKT cells exert polarized regulatory functions when exposed to an endogenous CD1d ligand in the presence of cytokines such as IL-12 and IL-2. We speculate that cytokine-triggered activation of iNKT cells should reflect a number of physiological or pathological conditions that could take place in the maintenance of immune homeostasis. Occurrence of Th1 polarization for iNKT cells or robust production of proinflammatory cytokines such as IFN-γ and osteopontin in response to infectious stimuli indicates a new mechanism for exacerbating autoimmune diseases preceded by an infection. Very interestingly, a growing number of potential agonists for iNKT cells have been identified from relatively common pathogens (Tupin et al., 2007). This opens a new possibility that environmental pathogens may play an active role in maintaining the population size and functions of iNKT cells in healthy conditions. Given that the frequency of iNKT cells in the peripheral blood greatly varies among healthy populations, this is an interesting question to be addressed experimentally. This new idea and a prevailing view about the major influence by genetic factors on iNKT cells are not mutually exclusive. Consequently, new approaches exploiting the role of iNKT cells in autoimmunity should probably consider their relation to pathogenic bacteria as well as non-pathogenic microbes.

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The Complementarity Determining Region 2 of BV8S2 (Vβ8.2) Contributes to Antigen Recognition by Rat Invariant NKT Cell TCR[1]

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Invariant NKT cells (iNKT cells) are characterized by a semi-invariant TCR comprising an invariant α-chain paired with β-chains with limited BV gene usage which are specific for complexes of CD1d and glycolipid Ags like α-galactosylceramide (α-GalCer). iNKT cells can be visualized with α-GalCer-loaded CD1d tetramers, and the binding of mouse CD1d tetramers to mouse as well as to human iNKT cells suggests a high degree of conservation in recognition of glycolipid Ags between species. Surprisingly, mouse CD1d tetramers failed to stain a discrete cell population among F344/Crl rat liver lymphocytes, although comprised iNKT cells are indicated by IL-4 and IFN-γ secretion after α-GalCer stimulation. The arising hypothesis that rat iNKT TCR recognizes α-GalCer only if presented by syngeneic CD1d was then tested with the help of newly generated rat and mouse iNKT TCR-transduced cell lines. Cells expressing mouse iNKT TCR reacted to α-GalCer presented by rat or mouse CD1d and efficiently bound α-GalCer-loaded mouse CD1d tetramers. In contrast, cells expressing rat iNKT TCR responded only to α-GalCer presented by syngeneic CD1d and bound mouse CD1d tetramers only poorly or not at all. Finally, CD1d-dependent α-GalCer reactivity and binding of mouse CD1d tetramers was tested for cells expressing iNKT TCR comprising either rat or mouse AV14 (Vα14) α-chains and wild-type or mutated BV8S2 (Vβ8.2) β-chains. The results confirmed the need of syngeneic CD1d as restriction element for rat iNKT TCR and identified the CDR2 of BV8S2 as an essential site for ligand recognition by iNKT TCR. *The Journal of Immunology*, 2006, 176: 7447–7455.

The hallmark of invariant NKT cells (iNKT cells)[5] is the expression of a TCR with characteristic invariant α-chain rearrangement and limited BV (Vβ) usage which recognizes glycolipids like α-galactosylceramide (α-GalCer) in a CD1d-restricted manner (1). Mouse iNKT TCR α-chains rearrange the variable gene 14 (*AV14*) and joining gene 18 (*AJ18*), which pair with β-chains of high CDR3 variability comprising BV8S2 and to lesser extent to BV7 or BV2 (2–4). The human iNKT TCR is composed of AV11/AJ18 (homolog of mouse AV14) α-chains paired with BV11 (homolog of mouse BV8) β-chains (2, 5). In the rat, homologous α-chain rearrangements have also been found (6). A contribution of iNKT cells in the control of tumors, infections, and autoimmune diseases (reviewed in Refs. 7–9) has been demonstrated in many mouse models and by clinical observations in humans.

iNKT TCR ligands are endogenous or microbial glycolipids that are presented by the nonpolymorphic MHC class I-like molecule CD1d. Crystal structures of complexes of CD1d and glycolipid Ags have been reported very recently (10–12). Natural ligands are isoglobotrihexosylceramide (13) and α-anomers of various glycosphingolipids, which have been isolated from α_4-proteobacteria (14, 15). Other ligands activate only small subpopulations of iNKT cells. Their features are reviewed in Ref. 16. Still the most thoroughly characterized Ag of iNKT cells is the α-anomer of galactosylceramide (α-GalCer), which was originally isolated from a marine sponge. Essentially all iNKT cells respond to α-GalCer, and they can be visualized with α-GalCer-loaded CD1d oligomers (reviewed in Ref. 17). Of special importance to our study is the observation that α-GalCer-loaded mouse CD1d oligomers bind to human iNKT cells (18–20) and human CD1d tetramers stain mouse iNKT cells. Thus, it appears that ligand recognition is highly conserved throughout evolution (21).

The rat also expresses genes for CD1d (22, 23) and the AV14, AJ18 (6), and BV8S2 (24) gene segments, which are highly similar to those of the mouse (>80% sequence similarity of the translated products). Peculiar to the rat is the existence of a multimember AV14 gene family and the organ-specific preferences of certain AV14AJ18 rearrangements. Within the BN/SsNHsd genome, 10 *AV14* genes have been identified (25), and analysis of F344/Crl rearrangements identified five AV14 family members, which based on CDR2 sequence similarity, have been divided into the type I and type 2 genes. Rearrangements of type I genes (*AV14S1*, *AV14S2*, *AV14S4* (a pseudogene); and *AV14S8*, described in this paper) have been reported to be predominant within intrahepatic

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[5] Abbreviations used in this paper: iNKT cells, invariant NKT cells; α-GalCer, α-galactosylceramide; IHL, intrahepatic lymphocytes; MFI, mean fluorescence intensity.

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lymphocytes (IHL), whereas rearrangements of the type II gene *AV14S3* are more frequently found in spleen, bone marrow lymphocytes, and thymocytes (6, 25). In all cases, either a G or an A have been found at position 93, located at the VJ junction (6), which is similar to the mouse, where mostly a G but also rarely A, V, or I are found in this region (2, 26).

Despite this information on the genetics, knowledge of the phenotype, function, and Ag recognition by rat iNKT TCR-bearing cells is rather limited. The comparison of NKR-P1A-positive rat T lymphocytes (6) with mouse NKT cells has elucidated some differences in terms of phenotype and functions. First of all, NKR-P1A (rat homolog of mouse NK1.1)-positive T cells found in spleen and liver (6, 27, 28) were of CD8$\alpha\beta$ phenotype and showed no preferential BV usage. This is in stark contrast to the mouse, in which most of the NK1.1-positive T cells, and nearly all iNKT cells, are $CD4^+$ or $CD4^-CD8^-$. Secondly, NKR-P1A-positive rat T cells produce IFN-γ but not IL-4 upon in vitro CD3 stimulation (28). Thus, it appears that these cells are not the equivalent of mouse iNKT cells.

Nevertheless, there is also evidence that favors the existence of typical iNKT cells in the rat. Matsuura et al. (6) showed that coculture of F344/Crl IHL with CD1d-transduced hepatocytes leads to the accumulation of cells with AV14 transcripts, and they identified AV14AJ18 rearrangements in a NKR-$P1A^{high}$ subset of intrahepatic T lymphocytes of LEC rats (29). Additionally, another group has reported the generation of $CD4^+$ or $CD4^-CD8^-$ NKR-$P1A^+$ T cell clones from PVG rats that home to the liver and produce Th1 and Th2 cytokines (30). However, to our knowledge, α-GalCer reactivity of rat T lymphocytes or binding of α-GalCer-loaded CD1d oligomers have not been described yet. Both groups reported staining of the presumed rat iNKT cells by the BV8-specific mAb R78 (6, 30), which depending on the *Tcrb* haplotype binds to different rat homologs of mouse BV8S2. In F344/Crl rats (*Tcrb*^{a} haplotype), R78 reacts with BV8S4 (BV8S4A2) but not with BV8S2 (BV8S2A2), whereas in PVG rats (*Tcrb*^{l} haplotype) BV8S4 (BV8S4A1) is not functional and R78 Ab stains positively BV8S2 (BV8S2A1) (24, 31).

This paper describes our attempts to characterize F344/Crl rat iNKT cells, their phenotype, and their α-GalCer reactivity. Although like mouse NKT cells, the lymphocytes isolated from F344/Crl rat liver secreted cytokines upon α-GalCer in vitro stimulation, they could not bind α-GalCer-loaded mouse CD1d tetramers (mouse CD1d tetramers). To test the species specificity of recognition of α-GalCer-CD1d complexes, mouse and rat iNKT TCR were cloned, and a panel of cell lines expressing mouse and rat, wild-type or mutated iNKT TCR were generated. The $AV14^+$ lines were tested for rat vs mouse CD1d-restricted α-GalCer recognition and for binding of mouse CD1d tetramers. The results confirmed the hypothetical species specificity of CD1d-dependent α-GalCer recognition by rat iNKT cells and allowed, for the first time, definition of the important role of the CDR2β as a germline-encoded TCR region for this recognition.

Materials and Methods

Animals

C57BL/6 mice and LEW/Crl rats were bred in the animal facilities of the Institute for Virology and Immunology, University of Würzburg, Würzburg, Germany. F344/Crl rats were obtained from Charles River Wiga. All animals were maintained under specific pathogen-free conditions and were used at 6–10 wk of age.

Cell preparation and culture

Mouse and rat IHL were isolated using discontinuous Percoll (Pharmacia Biotech) gradients (40%/70% or 40%/80%) as described in Ref. 32. In both cases, liver was perfused with complete medium (via the portal vein) until it became opaque. Then the organ was homogenized by passing through a metal mesh, and cells were washed with medium. Cells were resuspended in 40% isotonic Percoll solution and underlaid with 70 or 80% isotonic Percoll solution. After 25 min of centrifugation at 900 × *g* at room temperature, mononuclear cells were isolated from the interface. Remaining erythrocytes were removed from the cell pellet by lysis with TAC buffer (Tris-ammonium chloride, 20 mM Tris (pH 7.2), 0.82% NH_4Cl). Thymocytes were isolated by passing the organ through a metal sieve followed by washing with complete medium. Primary cells and cell lines were cultured at 37°C with 5% CO_2 and H_2O-saturated atmosphere. Almost all cell types were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 5 or 10% FCS, 100 mM sodium pyruvate, 0.05% (w/v) glutamine, 10 mM nonessential amino acids, and 100 μM 2-ME (Invitrogen Life Technologies). DMEM with the same supplements was used for transfection of 293T cells, conducted to produce retroviruses for gene transfer.

Cloning and expression of rat and mouse iNKT TCR

Rat AV14S8 α-chain was directly cloned from F344/Crl IHL cDNA, whereas the rat AV14S1 α-chain construct was generated using molecular biology methods. For the F344/Crl AV14S8 α-chain, RNA was isolated from cytoplasmic extracts of ~10^6 IHL following the protocol of the RNeasy MiniKit (Qiagen). The cDNA was synthesized according to the manufacturer's RT-PCR protocol supplied with a First Strand cDNA Synthesis Kit (MBI Fermentas). PCR was performed with HotStar DNA polymerase (Qiagen), using AV14-specific primers: (MWG-Biotech) rVα14/1,2,3-Fow (5′-TTT GGG GCT AGG CTT CTG-3′), RCαend-STOP-Rev (5′-TCA ACT GGA CCA CAG CCT TAG CG-3′). PCR products were cloned into TOPO cloning vector (Topo pCR2.1-TOPO-TOPO TA Cloning Kit; Invitrogen Life Technologies) and sequenced using an ABI sequencer. Subsequently, rat AV14S8 α-chain DNA was ligated into *Eco*RI sites of pczCGZ5 IEGZ retroviral vector.

An AV14S1 α-chain with a V domain amino acid sequence described by Matsuura et al. (6) was generated using molecular biology methods. F344/Crl genomic DNA was amplified by PCR with rVα14*Eco*RI-Fow (5′-GGG CTA GAA TTC TGC AGA AAA ACC ATG GGG AAG C-3′) and r/mVα14Rev (5′-CAC CAC ACA GAT GTA GGT GGC AG-3′) primers. This DNA was digested with *Eco*RI and *Esp* enzymes and gel purified. The resulting fragment, which encoded the leader and the first 72 aa of mature V region peptide, was coligated with a *Esp*I-F344/Crl cDNA-*Bam*HI fragment (encoding the JC terminus of another liver-derived α-chain) into the *Eco*RI/*Bam*HI sites of pczCGZ5 IEGN vector, and the insert was sequenced. The generation of the rat BV8S2 β-chain and its mutants has been described elsewhere (33).

A mouse type I NKT cell TCR was cloned by RT-PCR from mouse KT12 hybridoma (34) using α-chain (mVα14-*Eco*RI-Fow: 5′-GGG GAA TTC AAC CAT GAA AAA GCG CC-3′; and mCα14-*Eco*RI-Rev: 5′-CCC GAA TTC CTC AAC TGG ACC ACA GCC-3′) and β-chain (mVβ8.2-*Bam*HI: 5′-CGG GAT CCT GAG ATG GGC TCC AGG CTC TTC-3′; and mCβend-*Bam*HI: 5′-GGG GGA TCC TCA GGA ATT TTT TTT CTT GAC C-3′)-specific primers. Mouse AV14S1A2 α-chain DNA was ligated into the *Eco*RI site of pczCGZ5 IEGN (containing genes for neomycin resistance and enhanced green fluorescence protein), and mouse BV8S2 β-chain DNA was ligated into *Bam*HI sites of pczCGZ5 IEGZ (containing genes for zeozin resistance and enhanced green fluorescence protein) retroviral vectors (35).

Rat TCR α-chains (AV14S8 and AV14S1) as well as mouse AV14SA2-TCR α-chain were expressed together with C57BL/6 mouse or rat BV8S2 TCR β-chains in BW58r/mCD28 cells using a transient three-plasmid expression system. BWr/mCD28 cells are BW58 TCR^- mouse hybridoma transduced with chimeric rat/mouse CD28 molecule (36). These cells are especially suitable for the analysis of Ag presentation by CD80-positive APC (33). Expression of transduced α-chains was estimated from the green fluorescence of the reporter gene. Cell surface expression of transduced TCR was analyzed by staining with anti-mouse CD3 mAb. When necessary to obtain similar levels of TCR expression, cell lines were sorted using a FACSVantage (BD Biosciences) machine or by coculture in selection medium containing 1 mg/ml neomycin (Invitrogen Life Technologies) or 250 μg/ml zeozin (CAYLA), alternatively.

Cloning and expression of rat and mouse CD1d

P80rCD80 cells were transduced with mouse or rat CD1d. P80rCD80 cells are P80 cells (P815 mouse mastocytoma transduced with rat CD80; Ref. 37) which, to increase rat CD80 expression, were additionally infected with pczCGZ5IZ or pczCGZ5IEGZ retroviral vectors expressing genes for rat CD80 and zeozin resistance. These have been generated by RT-PCR from the CD80-containing BCMGSC vector (37) and subsequently cloned into the *Eco*RI sites of both retroviral vectors. P80rCD80 cells transduced with

rat CD1d are designated as P80rCD80rCD1d, those transduced with mouse CD1d as P80rCD80mCD1d.

Mouse CD1d was cloned from A20mCD1d cell line (38) by RT-PCR using the following primers: mCD1d-*Eco*RI-Fow (5′-GGG GAG AAT TCC GGC GCT ATG CGG TAC CTA CC-3′); and mCD1d-*Eco*RI-Rev (5′-GGT GGA ATT CAG AGT CAC CGG ATG TCT TGA TAA G-3′). The sequence of the insert showed a complete overlap with the mouse CD1d sequence available in the gene bank under X13170 (39). Rat CD1d cDNA was obtained by RT-PCR using RNA isolated from F344/Crl rat bone marrow as a template and CD1d-specific primers: N366 (5′-TCG GAG CCC AGG GCT GTG TAG A-3′); and rCD1dRev (5′-TTC TGA GCA GAC AAG GAC TGA-3′). PCR product was cloned into TOPO cloning vector and sequenced. The sequence was identical with rat CD1d (GenBank accession number AB029486) published by Katabami et al. (23). Mouse and rat CD1d DNA were cloned into *Eco*RI site of pczCGZ5IZ and pczCGZ5 IEGZ vectors, respectively, and were further used for retroviral infection of P80rCD80 cells.

The expression of mouse CD1d was tested with the CD1d-specific mAb 1B1 (BD Pharmingen), whereas expression of rat CD1d was assessed from the green fluorescence of the *EGFP* reporter gene. Surface expression of rat CD1d was also confirmed with a novel rat CD1d-specific mAb (E. Pyz and T. Herrmann, unpublished observations). The Ag-presenting cell lines were enriched for CD1d expression by cell sorting or selection with antibiotics.

Stimulation with α-GalCer in vitro

α-GalCer was generated as described (40). The reactivity of mouse and rat IHLs to α-GalCer was tested by culture of IHL (1×10^5 cells/well of a 96-well round-bottom plate) in the presence of α-GalCer (100 ng/ml), vehicle (DMSO), or complete medium for 24 h at 37°C. The level of IL-4 and IFN-γ released into culture supernatants was determined using ELISA kits (BD Pharmingen).

To analyze the α-GalCer reactivity of TCR-transduced cell lines, mouse and rat thymocytes (1×10^6 cells/well), or CD1d-transduced cells (P80mCD1drCD80, P80rCD1drCD80, 5×10^4 cell/well) used as APC were loaded with either α-GalCer (1–100 ng/ml) or vehicle (DMSO) for 1–2 h before the addition of responder cells. As a positive control, TCR-positive cell lines were stimulated with plate-bound anti-mouse CD3 mAb 145C11. After 24 h of culture, supernatants were taken, and the secreted mouse IL-2 was quantified using a commercial ELISA Kit (BD Pharmingen).

Immunofluorescence and flow cytometry

For the staining, 2×10^5 cells were diluted in 100 μl of FACS buffer (PBS (pH7.4), 0.1% BSA, 0.02% NaN_3) and were treated for 10 min at 4°C with normal mouse Ig (Sigma-Aldrich) or mouse FcγR-specific 2.4G2 Ab to block unspecific binding or binding to Fc receptors. Subsequently, cells were stained for 30 min with labeled mAbs, washed, and stained with another mAb or analyzed with a FACScan or FACSCalibur flow cytometer (BD Biosciences).

All mouse and rat mAbs were obtained from BD Pharmingen and are given with their clone names: mouse Vβ8.1, 8.2, 8.3 (F23.1); mouse CD3 ε-chain (145-C11); mouse CD1d (1B1); mouse CD4 (GK1.5); mouse CD8α (53-6.7); NK1.1 (PK136); BV8S4A1 and BV8S4A2, Vβ8.2 of LEW rats and Vβ8.4 of F344/Crl rat (R78); rat TCR β-chain (R73); rat CD4 (W3/25); rat CD4 (OX35); rat CD8β (3.4.1.); rat NKR-P1A (10-78). Abs were usually FITC or PE labeled. Biotinylated mAbs, when used, were visualized with streptavidin-CyChrome. Unconjugated Abs, used in indirect immunofluorescent staining, were detected by using fluorochrome-conjugated Abs: PE- or Cy5.5-conjugated $(Fab')_2$ fragment of donkey anti-mouse IgG or goat anti-hamster IgG FITC obtained from Dianova or Serotec).

Staining with α-GalCer-loaded mouse CD1d-PE tetramer

α-GalCer-loaded or control mouse CD1d-PE tetramers were generated as described in Ref. 18. Tetramer staining of mouse/rat IHL- or TCR-transduced cell lines was performed as normal FACS staining, but with incubation for 1 h at room temperature. Tetramer concentrations were 350 (high tetramer) or 35 ng/50 μl cell suspension (low tetramer).

Results

Phenotype and α-GalCer response of rat IHL

In mouse and human, the highest proportion iNKT cells can be found among intrahepatic lymphocytes. In an attempt to identify the corresponding population in rat, IHL of F344/Crl rats and C57BL/6 mice were compared for cell surface phenotype (Fig. 1*A*), binding of α-Gal-loaded mouse CD1d tetramers (Fig. 1*B*), and α-GalCer-induced cytokine production (Fig. 1*C*). In agreement with published data, about one-third of mouse IHL coexpressed NK1.1 (mouse homolog to rat NKR-P1A) and TCR. More than 20% of IHL coexpressed NK1.1 and CD4, but very few coexpressed NK1.1 and CD8αβ. As shown in Fig. 1*B*, ~27% of IHL show costaining of α-GalCer-loaded CD1d tetramer and anti-CD3, with the tetramer positive cells having a lower or intermediate level of expression of CD3. 25.5% of IHL were costained by tetramer and anti-NK1.1 (data not shown) and 20.4% by tetramer and anti-CD4, whereas only very few (0.38%) stained with CD1d tetramer and CD8-specific mAb (data not shown).

The phenotypes of rat and mouse IHL differed considerably. First of all, <5% of rat IHL coexpressed NKR-P1A and CD3, and most of these cells were positive for CD8αβ but not for CD4, and they did not express intermediate CD3 levels. Secondly, in contrast to results found in mice, only a very small number of $CD3^+$ rat IHL were stained with α-GalCer-loaded mouse CD1d tetramer;

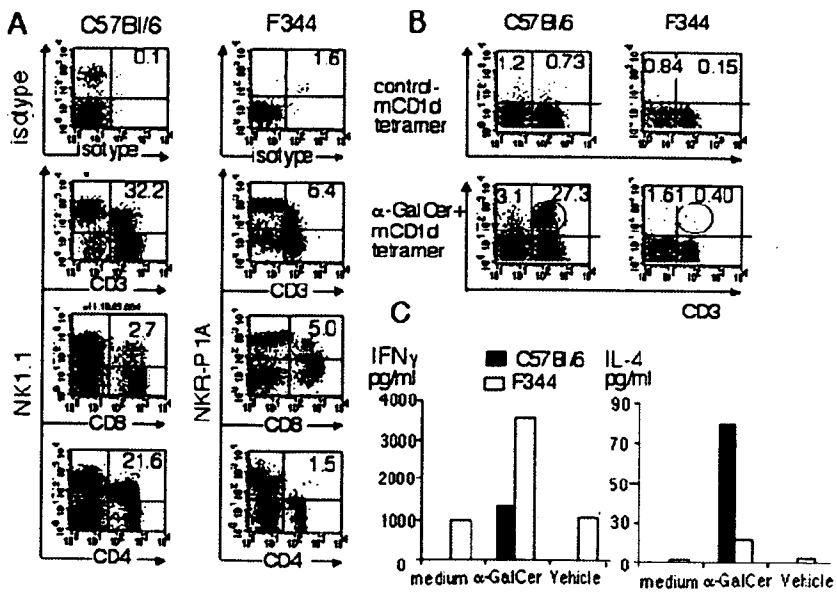

FIGURE 1. Phenotypic and functional analysis of typical iNKT cell features of C57BL/6 mouse and F344/Crl rat IHL. *A*, Two-color flow cytometry for coexpression of NK1.1 or NKR-P1A and indicated T cell markers. Percent of positive cells are indicated by numbers in the *upper right quadrant*. *B*, Two-color flow cytometry for binding of α-GalCer-loaded or unloaded mouse CD1d tetramers to $CD3^+$ positive (*upper right quadrant*) or $CD3^-$ (*upper left quadrant*) C57BL/6 mouse or F344/Crl rat IHL. Percentages of tetramer-positive cells are given in the respective quadrants. *C*, IFN-γ or IL-4 secretion during 24-h stimulation of 1×10^5 rat or mouse IHL with 100 ng/ml α-GalCer dissolved in DMSO, vehicle (DMSO alone), or medium alone. *Ordinate*, Cytokine concentration in picograms per milliliter.

0.4% of IHL were stained with α-GalCer-loaded tetramer and 0.15% with control tetramer. Even higher proportions of $CD3^-$ lymphocytes were stained by α-GalCer-loaded tetramers (1.61%) or control tetramers (0.84%), which made it likely that (much of) the tetramer staining of $CD3^+$ rat IHL was unspecific.

To test whether the lack of binding of mouse CD1d tetramer to rat IHL was due to the absence of α-GalCer-specific cells in F344/Crl rat liver, the α-GalCer reactivity of F344/Crl and C57BL/6 IHL (Fig. 1*C*) was compared. After 24 h of stimulation with α-GalCer (100 ng/ml), mouse and rat liver lymphocytes produced both IFN-γ and IL-4. The amount of rat IL-4 reached ~15% of that secreted by mouse cells. The IFN-γ production by rat IHL exceeded that of mouse IHL, but rat IHL showed also a high level of background IFN-γ production.

The α-GalCer-induced activation of cytokine production in conjunction with the detection of AV14AJ18 rearrangements in rat IHL strongly support the existence of an iNKT cell population in F344/Crl rats, although these cells could not be detected by mouse CD1d tetramer. This could be a consequence of 1) an extremely low frequency of rat iNKT cells and/or 2) a requirement for presentation of α-GalCer by syngeneic CD1d (species specificity), which finally would result in a lack of binding of mouse CD1d tetramers to rat iNKT TCR. To test the latter hypothesis, iNKT TCRs were cloned and expressed in TCR-negative BW58r/mCD28 cells and tested for mouse CD1d tetramer binding. In addition, these lines as well as lines expressing iNKT TCR variants were tested for reactivity to α-GalCer presented by mouse or rat CD1d.

Cloning and transduction of mouse and rat CD1d and iNKT TCR

Cloning, transduction, and quantification of surface expression of iNKT TCR was performed as described in *Materials and Methods*. Three AV14 α-chains were cloned into a retroviral vector carrying an *EGFP* as reporter gene. Two of them comprised V-encoded amino acid sequences identical with that of rat AV14S1 and rat AV14S8. The mouse AV14S1A2-chain was cloned from the α-GalCer-reactive mouse C57BL/6-derived iNKT cell hybridoma KT12. All AV14 α-chains were coexpressed with different mouse or rat BV8S2 β-chains, the properties of which will be discussed later in this section.

The sequences of the tested iNKT TCR α-chains are compared in the upper part of Fig. 2. Both rat AV14S1 and rat AV14S8 α-chain comprise type 1 AV14 sequences. The V domain of the rat AV14S1 α-chain is identical with sequences previously found by Matsuura et al. in F344/Crl rat liver (6). The rat AV14S8 α-chain sequence was directly cloned from F344/Crl IHL, as described in *Materials and Methods*. AV14S8 has not yet been described for F344/Crl rats, but an identical sequence has been found in the BN/SsNHsd rat genome, where it has been named AV14S8 (25). A peculiarity of the AV14S8-comprising α-chain used in this study may be the valine located at position 93 of the VJ junction which corresponds to the adult type of AV14AJ18 rearrangements (26). Otherwise, the mature Vα domains of the two rat TCRs differed by the following substitutions: K1R, Q15E, and K51T.

The middle part of Fig. 2 aligns the sequences of the TCR β-chains used in this study. The BV8S2-positive mouse β-chain was originally isolated from the iNKT T cell hybridoma KT12. The rat BV8S2 (BV8S2A1 or Tcrb-V8.2^l)-comprising β-chain used in this study was derived from the rat T cell hybridoma 35/1, which was generated with an encephalitogenic cell line of LEW/Crl origin as fusion partner. The 35/1 TCR is $RT1B^l$-restricted $gpMBP_{68-88}$ specific and reacts also with the superantigens of *Yersinia pseudotuberculosis* and *Mycoplasma arthritidis* and the staphylococcal enterotoxins B and C1 (33). As previously described in some detail (33), replacement of the CDR2 and/or the CDR4/HV4 of the BV8S4A2 with those of F344/Crl rats had distinct effects on (super)Ag reactivity. Changes in the CDR2 abolished reactivity for peptide Ag and staphylococcal enterotoxins B and C1, whereas mutation of the HV4/CDR4 affected only the response to staphylococcal enterotoxins (33). The β-chain containing the mutations within CDR2 and CDR4 is, with exception of a lacking L14K substitution, identical with the BV8S4A2 of F344/Crl rats. It lost specificity for the peptide Ag, staphylococcal enterotoxins and the superantigen of *M. arthritidis* (33).

The lower part of Fig. 2 presents the amino acid sequence of the α-1 and α-2 domains of rat and mouse CD1d. The α-helical parts

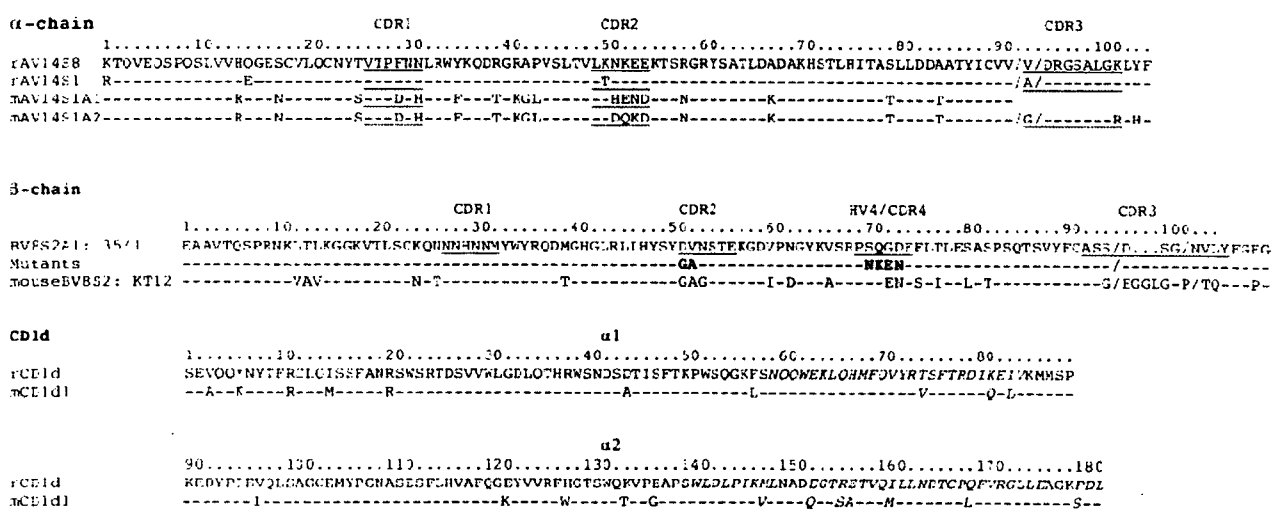

FIGURE 2. Alignment of amino acid sequences of the mature peptides TCR chain proteins (α-chain and β-chain) and CD1d molecules used or discussed in this study. Underlined parts of the TCR sequences indicate localization of CDRs. Parts of CD1d sequences in italics indicate α-helical regions. Amino acid sequences were deduced from the nucleotide sequences, the accession numbers of which can be found in GenBank: rAV14S8 α-chain, DQ340291; rAV14S1 α-chain, DQ340293; mAV14S1, AY158221; mAV14S1A2 (KT12 hybridoma), DQ340292; BV8S2A1 TCR35/1 β-chain, AY228549. Mutants entry indicates localization of the CDR2 and CDR4/HV4 substitutions introduced in the TCR35/1 β-chain, which are highlighted by bold letters. mBV8S2 TCR KT12, DQ340294; mCD1d (mouse CD1d), X13170.1; rCD1d (rat CD1d), AB029486.

of CD1d are marked. The α helices of the α1 domains differ in 3 aa. Visualization of the of the PDB files 1ZHN (10) and 1Z5L (12) of the mouse CD1d crystal structure by Swiss-PDB-viewer (http://swissmodel.expasy.org/SM_TOPPAGE.html) shows that T74 points upwards and K81 outwards, defining them as theoretical contact sites with the TCR. I83 points into the binding groove. The α-helical parts of the α-2 domain differ by 7 aa. With exception of the R157S, side chains of all substitutions show upwards and provide possible contacts for the TCR. In contrast to the differences in potential TCR contacts, those amino acids shown to provide H bonds with α-GalCer are conserved (12). Both *CD1d* genes were expressed in P815 cells (P80rCD80) overexpressing rat CD80 as described in *Materials and Methods*.

Species specificity of CD1d restriction in Ag recognition by rat iNKT TCR

First, we tested three responder cell lines for their α-GalCer reactivity and their capacity to bind mouse CD1d tetramers. The lines were BWr/mCD28 cells expressing: 1) as positive control, mouse iNKT TCR isolated from the KT12 hybridoma which consisted of a mouse AV14S1A2 α-chain and mouse BV8S2 β-chain; 2) rat AV14S1 α-chain with the CDR2+4 β-chain mutant; 3) the rat AV14S8 α-chain with the same β-chain mutant. The BV8S4-like CDR2+4 β-chain mutant was used, because there is circumstantial evidence that in F344/Crl rats, iNKT cells express the BV8S4-comprising β-chains (6). The two rat TCR lines expressed very similar levels of TCR, whereas expression of the mouse TCR was considerably lower (Fig. 3). Cell lines were tested three to five times for their α-GalCer-induced IL-2 secretion. IL-2 levels after CD3 ligation were quite similar, with the exception of sometimes considerably lower IL-2 production by the mouse iNKT TCR-transduced line (data not shown). Fig. 3 shows data from one representative assay of α-GalCer-induced IL-2 secretion. The APC-type thymocytes vs CD1d-transduced P80 cells and the origin of the transduced CD1d (rat vs mouse) considerably affected the outcome of the assay. Generally, with CD1d-transduced P80rCD80 cells as APC, Il-2 production was much higher than with thymocytes. This may reflect the differences in the level of CD1d and CD80 surface expression in primary vs CD1d-transduced cells (data not shown). In assays with mouse thymocytes as APC, some background IL-2 production was found, even if TCR-negative BW58 cells were used as responders, suggesting that IL-2 was secreted by α-GalCer-stimulated thymocytes (Figs. 3 and 5). With regard to a possible species specificity in CD1d-restricted α-GalCer recognition, all three lines responded to α-GalCer presented by rat CD1d-expressing cells, whereas α-GalCer mouse CD1d complexes stimulated only mouse iNKT TCR responder cells. In addition, the stimulation of the line expressing the rat AV14S1 α-chain was considerably stronger than of the rat AV14S8 α-chain-expressing line.

The differences in the response to α-GalCer presented by mouse CD1d correlated with the pattern of mouse CD1d tetramer staining as is shown in Fig. 3 Binding of mouse CD1d tetramers was normalized by dividing mean fluorescence intensity (MFI) of tetramer staining, through MFI of CD3 staining. After normalization, tetramer staining of the mouse iNKT TCR-expressing line was 24-fold, respectively, 8-fold stronger than that of the rat AV14S8 α-chain-expressing line or the rat AV14S1 α-chain-expressing line.

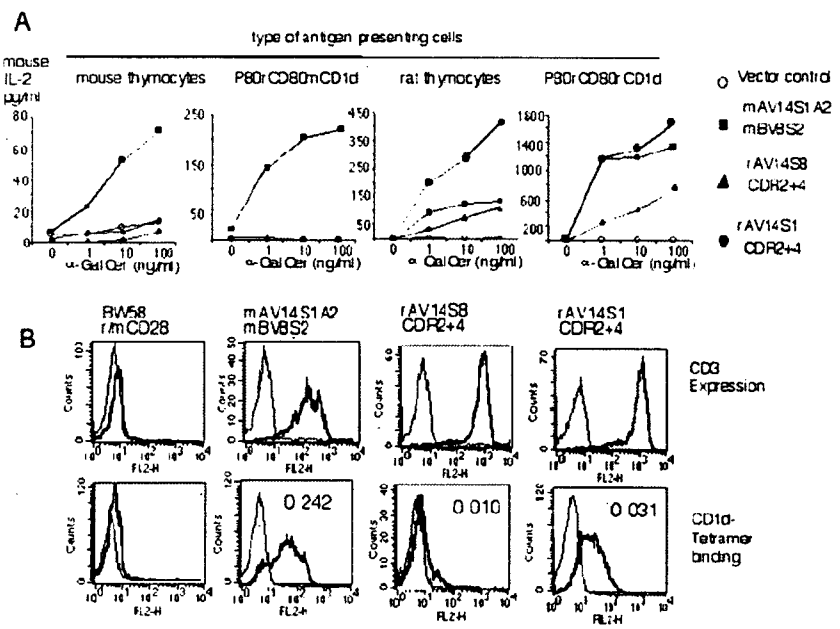

FIGURE 3. *A*, Species specificity of CD1d-restricted α-GalCer recognition by rat iNKT TCR-transduced cells. The graphs in the upper row indicate degree of IL-2 production (please note the different scales of the ordinates) by TCR-transduced BW58r/mCD28 cells after stimulation with α-GalCer presented by different APC-expressing mouse or rat CD1d. Transduced TCR: ○, vector control; ■, mAV14S1A2+mBV8S2 (mouse α-chain + mouse β-chain); ▲, AV14S8 + rat CDR2+4 β-chain (rat AVS8 α-chain + BV8S4-like rat β-chain); ●, AV14S1 + rat CDR2+4 β-chain (rat AVS1 α-chain + BV8S4-like rat β-chain). Amino acid sequences of the TCR chains used by these are given in Fig. 2. The type of α-GalCer-presenting cells and concentrations of α-GalCer used for stimulation are indicated on top of the respective graphs and at the abscissa, respectively. Zero ng/ml indicates the use of vehicle control. *B*, *Upper row*, CD3 expression of TCR-transduced cell lines used in *A*. Binding of isotype control (□) or anti-CD3 (□). *Lower row*, Mouse CD1d tetramer staining. Binding of unloaded control (350 ng/50 μl sample, □) and of α-GalCer-loaded tetramers (350 ng/50-μl sample, □). The type of transduced TCR is given on top of the histograms. The numbers in the histogram give ratios of MFIs for staining with α-GalCer-loaded mouse CD1d tetramers divided by that for staining with anti-CD3.

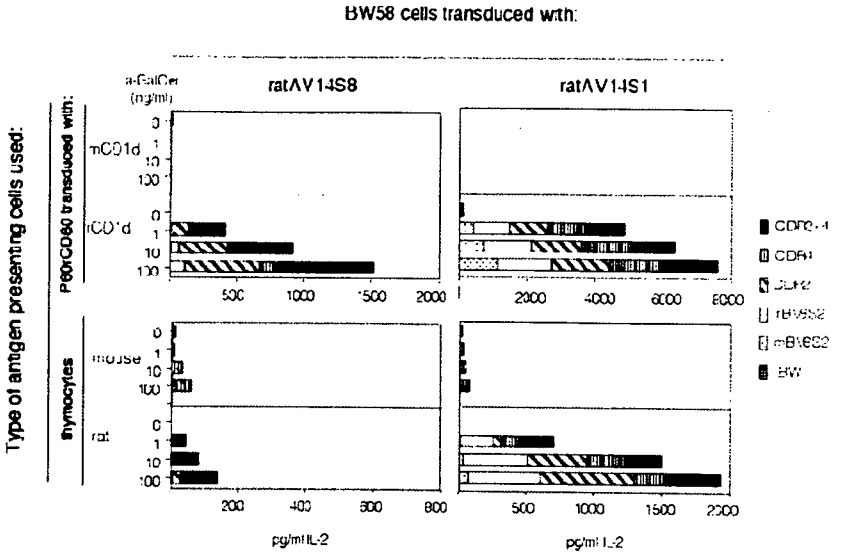

FIGURE 4. CD1d-restricted α-GalCer recognition of rat iNKT TCR. Species specificity of CD1d restriction. Shown is the α-GalCer-induced IL-2 production of BW58r/m CD28 transduced with rat AV14S1 or AV148 α-chains and various β-chains and different types of APC. Each section of the column indicates IL-2 production by cells expressing a certain α-β-chain combination. The α-chain is indicated at the top of the graph, the β-chains are indicated by the symbols in the graph. BW, Cells transduced with vector control. Ordinate, Type of APCs and the origin of CD1d. Note the variation of the scales indicating IL-2 production in the various graphs. α-GalCer concentrations are given in nanograms per milliliter. Vehicle designates culture with solvent (DMSO) only.

Effects of iNKT TCR α- and β-chain differences on CD1d-restricted α-GalCer recognition

We have previously analyzed the effects of CDR2 and/or CDR4 mutations of rat BV8S2 on the recognition of peptide Ags and superantigens (33). To learn whether the known BV encoded (super)Ag recognition sites may also contribute to α-GalCer recognition, AV14 α-chains were coexpressed with the various rat BV8S2 β-chain mutants and a mouse BV8S2 β-chain. These lines were then tested for the response to α-GalCer presented either by rat or mouse CD1d and for binding of α-GalCer-loaded mouse CD1d tetramer.

All lines expressed similar levels of TCR (summarized in Fig. 6) and produced similar amounts of IL-2 after stimulation with anti-CD3 mAb, with the exception of the mouse β-chain-expressing lines, which sometimes showed a rather low level of IL-2 production (data not shown). All lines were tested two to five times; and although the overall degree of stimulation varied between experiments, the patterns of α-GalCer reactivity remained the same. Figs. 4 and 5 show results from a representative experiment comparing all cell lines and Fig. 6 summarizes the results of all experiments.

The iNKT TCR composition affected the α-GalCer reactivity as follows: 1) the α-chain sequence of the transduced TCR largely affected the general degree of α-GalCer reactivity, because all rat AV14S1 α-chain-expressing lines responded considerably better to α-GalCer than the corresponding rat AV14S8 α-chain-expressing lines (Figs. 3 and 4); 2) lines with TCR comprising the two rat α-chains showed no or only a marginal response to α-GalCer which was presented by mouse CD1d, regardless of the type of the pairing β-chain (Fig. 4). These findings confirmed and extended the results on the species specificity of CD1d-restricted α-GalCer recognition by rat iNKT TCR shown in Fig. 3) only lines with TCR comprising the mouse α-chain in combination with mouse β-chain or with suitable rat β-chains responded to α-GalCer presented by mouse CD1d (Fig. 5). Suitable were those rat β-chains, which contained the BV8S4-like CDR2 (CDR2 or CDR2+4 mutant), whereas β-chains with the CDR2 of rat BV8S2 (CDR4 mutant and wild-type BV8S2) showed in the same setting only a marginal or no response. This pattern of reactivity maps the CDR2 of the β-chain as a region contributing to CD1d-restricted α-GalCer recognition in the interspecies comparison.

In contrast to the variation in the response to α-GalCer presented by mouse CD1d, recognition of rat CD1d-α-GalCer complexes was largely unaffected by the β-chain of iNKT TCR. All lines expressing TCR comprising the rat or mouse AV14S1 α-chains (Figs. 4 and 5) showed a very similar response. The

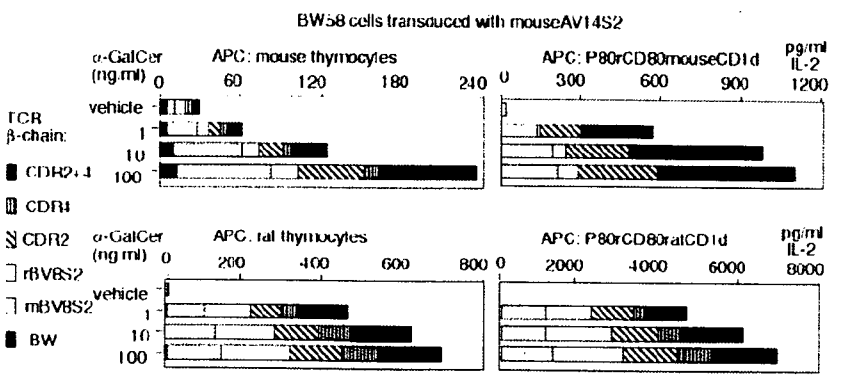

FIGURE 5. Analysis of mouse CD1d restricted α -GalCer recognition by chimeric mouse α- rat-β-chain iNKT TCR reveals contribution of CDR2β to ligand recognition by iNKT TCR. Shown is IL-2 production of BW58r/m CD28 transduced with AV14S1A2 α-chains and various β-chains indicated by the symbols in the graph to α-GalCer presented by indicated APC. BW indicates cells transduced with vector control. Every section of the columns indicates IL-2 production by cells expressing a certain α-β-chain combination. Note the variation of the scales indicating IL-2 production in the various graphs. α-GalCer concentrations are given in ng/ml. Vehicle designates culture with solvent (DMSO) only.

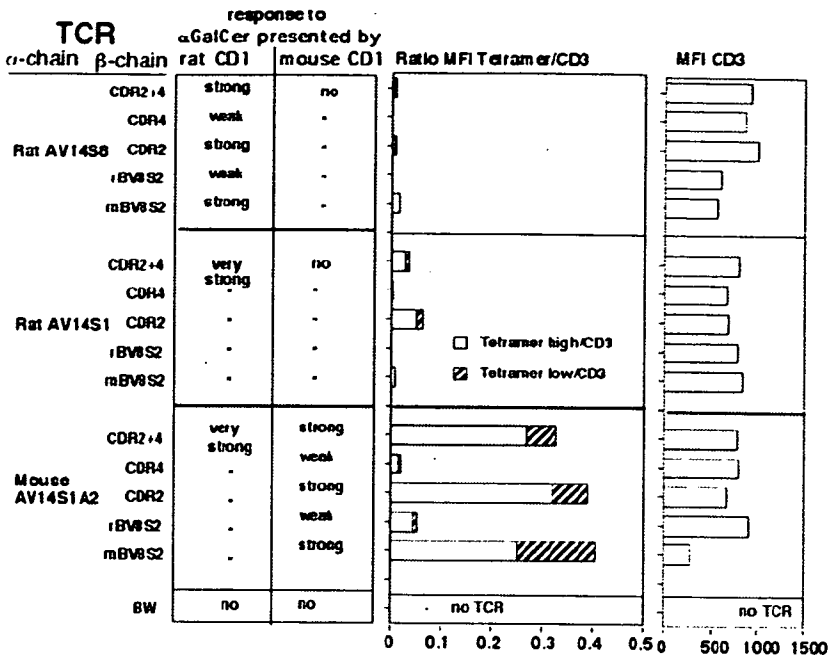

FIGURE 6. Survey of α-GalCer responsiveness of TCR-transduced cell lines, their CD1d tetramer binding, and TCR expression. The left part of the figure summarizes functional data of three to five experiments on the response to α-GalCer presented by rat or mouse CD1d by cell lines expressing the indicated iNKT TCR combinations (see also Figs. 3–5). The central part gives an estimate on CD1d tetramer binding for different cell lines. The stacked bars depict the ratio of MFIs obtained by staining with 350 ng (tetramer high) or 35 ng (tetramer low) of CD1d tetramer divided by the MFI of anti-CD3 staining. Numbers on the abscissa indicate this ratio. Examples for the staining are given in Fig. 3. The right part gives the MFI of CD3 staining of the cell lines used to generate of the data depicted in the central part of the graph.

somewhat lower IL-2 production of the line coexpressing rat AV14S1 α-chain and the CDR4 mutant β-chain probably reflects a generally weaker capacity in TCR-triggered IL-2 production, because anti-CD3 induced IL-2 secretion (not shown) was only about one-half of that found for the other lines. Less clear were the results for cell lines expressing rat AV14S8 α-chain. In two of four experiments, β-chain composition affected the response to rat CD1-α-GalCer complexes of the lines. An example for such a differential response is given in Fig. 4, where the lines expressing the CDR2 or CDR2+4 mutant β-chains reacted far better than those lines expressing the wild-type BV8S2 or the CDR4 mutant.

Finally, effects of the α-chain composition were also seen for the three mouse β-chain-expressing lines. The rat AV14S8 α-chain/mouse β-chain-expressing line completely lacked α-GalCer reactivity (Fig. 4), whereas the rat AV14S1 α-chain/mouse β-chain expressing line responded to α-GalCer if it was presented by rat CD1d-transduced P80rCD80 cells (Fig. 4), implicating rat Vα interactions with CD1d in imparting the observed species specificity. Only the mouse AV14S1A2 α-chain/mouse β-chain expressing line responded irrespective of the types of APC or origin of CD1d used to present the α-GalCer (Fig. 4).

Differential binding of α-GalCer-loaded mouse CD1d tetramers to iNKT-TCR-transduced lines

All cell lines were also tested for TCR expression and binding of α-GalCer-loaded tetramers at two different concentrations. In all cases, binding of unloaded tetramer control was negligible. Fig. 6 summarizes data from such an experiment and gives an overview of the results obtained in the functional assays. The capacity to bind α-GalCer-loaded mouse CD1d tetramers is presented by the ratio of MFI of tetramer binding and MFI of anti-CD3 binding. The best binding was found for the TCR comprising mouse AV14S1 α-chain paired with the CDR2, CDR2+4 mutants of rat BV8S2 β-chains or the mouse BV8S2 β-chain (Figs. 3 and 6), which is consistent with their exclusive capacity to respond to α-GalCer presented by mouse CD1d. At least 8 times weaker was the tetramer binding of lines coexpressing mouse α-chains and rat BV8S2 and CDR4 mutants.

Interestingly, the tetramer binding varied also between the rat α-chain-expressing lines. The poorly responding rat AV14S8 α-chain-expressing lines showed essentially no binding, whereas at least some tetramer binding was found for the more reactive lines coexpressing rat AV14S1 α-chain and the suitable CDR2 or CDR2+4 mutated β-chains. Finally, and again consistent with the functional assays, the rat α-chains paired with mouse β-chain bound no tetramer, whereas the original mouse iNKT TCR bound it very well. Indeed, the efficient binding of this TCR at the lower tetramer concentration suggests a rather high avidity of the original mouse iNKT TCR for α-GalCer-mouse CD1d complexes, consistent with measurements conducted with other mouse iNKT cell hybridomas and T cell populations.

Fig. 6 summarizes our results on the CD1d-restricted α-GalCer response and binding of α-GalCer-loaded mouse CD1d tetramers to iNKT TCR-transduced cell lines. It appears that the lack of reactivity to α-GalCer presented by mouse CD1d results from an impaired binding of the rat iNKT TCR α- rather than β-chain to mouse CD1d. Furthermore, comparison of iNKT TCR sharing the same α-chain but comprising different β-chains revealed that the amino acid composition of CDR2 of the β-chain strongly affects the CD1d-restricted glycolipid reactivity.

Discussion

This study was initiated to characterize the phenotype and the α-GalCer response of rat iNKT cells in a side by side comparison of mouse and rat IHL. As previously described (1, 6, 28), ~30% of mouse IHL coexpressed NK1.1 and TCR and were either $CD4^+$ or $CD4^-CD8^-$, whereas rat IHL comprised rather low numbers of NKR-P1A (rat homolog of NK1.1) and TCR^+ cells, most of them being $CD8\alpha\beta^+$. Our attempts to directly detect rat iNKT cells by staining with α-GalCer-loaded mouse CD1d tetramers failed, although the capacity of rat IHL to produce IFN-γ and IL-4 production after stimulation with α-GalCer suggested that there is indeed a functional iNKT cell population in F344/Crl rats. Analysis of newly generated cell lines expressing CD1d and iNKT TCR of both species allowed us to directly demonstrate the functionality of the rat elements of cognate Ag recognition by iNKT cells. In

addition, this analysis revealed that Ag recognition by rat iNKT TCR required its presentation by syngeneic CD1d, which was unexpected, given that mouse and human CD1d tetramers and dimers (18–20) bind to iNKT cells of the opposite species. Nevertheless, despite this cross-species reactivity, mouse iNKT TCRs bind mouse CD1d better than human CD1d, as was shown with α-GalCer-loaded mouse CD1d dimers (4). In addition, the weakly binding human dimers showed a stronger preference for mouse BV8S2 iNKT TCR than for mouse dimers, a result that underlines the substantial contribution of the β-chain to binding of α-GalCer CD1d complexes (4).

Interestingly, mouse iNKT TCR-transduced lines responded quite well to α-GalCer presented by rat and by mouse CD1d, whereas the rat iNKT TCR-expressing lines responded only when Ag was presented by rat CD1d. What could be the reason for the need of syngenicity between iNKT TCR and CD1d only in one direction? One possibility could be that higher numbers of α-GalCer complexes on rat $CD1d^+$ APCs could have compensated for the generally low avidity of rat iNKT TCR for CD1d, in particular for mouse CD1d. This possibility cannot be formally excluded but seems to be rather unlikely because homologous types of APC were used for presentation. Alternatively, we suggest a higher degree of promiscuity either in Ag recognition by mouse vs rat iNKT TCR or in Ag presentation by rat vs mouse CD1d.

With the help of chimeric and mutated iNKT TCR, we could identify TCR regions, which contribute to binding of α-GalCer and (mouse) CD1d. Cell lines expressing TCR comprising a mouse iNKT TCR α-chain and a suitable β-chain transgressed the threshold for the induction of a response to Ags presented by mouse CD1d, and these cells efficiently bound mouse CD1d tetramers. The differential reactivity of the rat BV8S2 β-chain mutants allowed us for the first time to demonstrate the important role of BV-encoded parts in the α-GalCer response, without a possible interference by CDR3 diversity. In addition, analysis of mutants swapping the CDR2 of BV8S2 with that of BV8S4 provided evidence for an involvement of the CDR2 of the β-chain in recognition of the α-GalCer-CD1d complex. In this context, it is of interest that the CDR2 of rat BV8S4, which in the combination with the mouse iNKT TCR α-chain permitted binding of α-GalCer-mouse CD1d complexes, and the CDR2 of mouse BV8S differ from each other by only one amino acid (Fig. 2). In contrast, the CDR2 of rat BV8S2, which in the interspecies comparison was nonpermissive, differed from that of mouse BV8S2 by 3 aa.

Rat *Tcrb* haplotypes vary in expression of functional BV8S2 and BV8S4 genes. The $Tcrb^u$ haplotype, which is found in F344/Crl and DA rats, expresses BV8S2 and BV8S4, whereas the $Tcrb^l$ haplotype of LEW/Cr, BN, and PVG rats expresses only BV8S2 (24, 31, 41, 42). These rat strains are widely used as models for autoimmune diseases; therefore, it is of special interest to investigate whether differences in reactivity to natural iNKT TCR ligands based on differences in the CDR2s of BV8S2 vs BV8S4 could lead to a rat strain-specific variation in iNKT T cell development or Ag reactivity.

The three α-chains tested contributed not only to restricted recognition of syngeneic CD1d, but also to the overall magnitude of the α-GalCer response. The lines expressing TCR with rat AV14S1 chains and mouse AV14S1A2 α-chains showed a much better response than the rat AV14S8-expressing lines. By analogy to what is known from MHC-restricted recognition of peptide Ags, the differences in α-GalCer reactivity of the two rat α-chains could have been explained by the K50T substitution in the CDR2α and by the A93V difference in the CDR3α (43). Two reasons lead us to assume that the CDR2α difference is of minor importance. In a comprehensive study on a mouse AV14S1 polymorphism, Sim et al. (44) demonstrated that a pronounced CDR2α difference between mouse AV14S1A1 and AV14S1A2 (see also Fig. 2) had little if any effect on TCR-binding to α-GalCer-CD1d complexes (44). Also, our own preliminary results (E. Pyz, I. Müller, and T. Herrmann, unpublished observations) obtained with rat AV14S1 and AV14S8 chain mutants showed little effect of the K50T substitution on the α-GalCer response, whereas a pronounced effect was found for the V93A substitution.

To sum up, we showed that efficient activation of rat iNKT TCR-expressing lines requires presentation of α-GalCer by syngeneic CD1d, and that reactivity to complexes of α-GalCer and mouse CD1d can be obtained by replacing the rat α-chain against that of the mouse and by using a β-chain comprising the CDR2 of rat BV8S4.

This finding thus provides the first description of a germline-encoded CDR involved in ligand recognition by iNKT TCR. The generation and functional analysis of further chimeric rat/mouse iNKT TCR and of chimeric rat/mouse CD1d molecules should strongly facilitate the characterization of the TCR/CD1d/Ag complex. At a certain point of chimerism of TCR or CD1d, cells expressing iNKT TCR comprising rat/mouse α-chain chimeras would be expected to gain specificity for α-GalCer presented by mouse CD1d and mouse/rat CD1d chimeras should gain the capacity to efficiently present Ag to rat iNKT TCR. Finally, combined functional assays with cells expressing such chimeric or mutated receptors and ligands, at best together with binding studies of recombinant molecules, may even allow definition of direct contacts in the ternary complex comprising iNKT TCR/Ag and CD1d.

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Disclosures

The authors have no financial conflict of interest.

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