Activation of Invariant NKT Cells Ameliorates Experimental Ocular Autoimmunity by A Mechanism Involving Innate IFN-γ Production and Dampening of the Adaptive Th1 and Th17 Responses

Rafael S. Grajewski, Anna M. Hansen, Rajeev K. Agarwal, Mitchell Kronenberg, Stephane Sidobre, Shao Bo Su, Phyllis B. Silver, Moriya Tsuji, Richard W. Franck, Anne P. Lawton, Chi-Chao Chan and Rachel R. Caspi

J Immunol 2008; 181:4791-4797; doi: 10.4049/jimmunol.181.7.4791
http://www.jimmunol.org/content/181/7/4791

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References This article cites 36 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/181/7/4791.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Activation of Invariant NKT Cells Ameliorates Experimental Ocular Autoimmunity by A Mechanism Involving Innate IFN-γ Production and Dampening of the Adaptive Th1 and Th17 Responses

Rafael S. Grajewski, Anna M. Hansen, Rajeev K. Agarwal, Mitchell Kronenberg, Stephane Sidobre, Shao Bo Su, Phyllis B. Silver, Moriya Tsuji, Richard W. Franck, Anne P. Lawton, Chi-Chao Chan, and Rachel R. Caspi

Invariant NKT cells (iNKT) have been reported to play a role not only in innate immunity but also to regulate several models of autoimmunity. Furthermore, iNKT cells are necessary for the generation of the prototypic eye-related immune regulatory phenomenon, anterior chamber associated immune deviation (ACAI). In this study, we explore the role of iNKT cells in regulation of autoimmunity to retina, using a model of experimental autoimmune uveitis (EAU) in mice immunized with a uveitogenic regimen of the retinal Ag, interphotoreceptor retinoid-binding protein. Natural strain-specific variation in iNKT number or induced genetic deficiencies in iNKT did not alter baseline susceptibility to EAU. However, iNKT function seemed to correlate with susceptibility and its pharmacological enhancement in vivo by treatment with iNKT TCR ligands at the time of uveitogenic immunization reproducibly ameliorated disease scores. Use of different iNKT TCR ligands revealed dependence on the elicited cytokine profile. Surprisingly, superior protection against EAU was achieved with α-C-GalCer, which induces a strong IFN-γ but only a weak IL-4 production by iNKT cells, in contrast to the ligands α-GalCer (both IFN-γ and IL-4) and OCH (primarily IL-4). The protective effect of α-C-GalCer was associated with a reduction of adaptive Ag-specific IFN-γ and IL-17 production and was negated by systemic neutralization of IFN-γ. These data suggest that pharmacological activation of iNKT cells protects from EAU at least in part by a mechanism involving innate production of IFN-γ and a consequent dampening of the Th1 as well as the Th17 effector responses. The Journal of Immunology, 2008, 181: 4791–4797.

 invariant NKT cells (iNKT) are considered to represent an innate subset of T cells. iNKT cells have a semi-invariant Vα14-Jα18 TCR repertoire specific for lipid Ags that are presented on the MHC class I-like CD1d molecule. α-Galactosylceramide (α-GalCer), a synthetic glycolipid derived from marine sponges, is a well known iNKT TCR ligand (1). Recently, natural ligands of iNKT TCR have been described, such as α-glucuronosylceramide and glycosphingolipids from Sphingomonadaceae (2–4), that resemble cell wall constituents of some Gram-negative bacteria, supporting an innate role of iNKT cells in some infectious diseases. Other studies also revealed regulatory and protective properties in various models of autoimmunity such as experimental autoimmune encephalomyelitis (EAE) and type-1 diabetes (reviewed in Ref. 1). Most of these studies used synthetic ligands for iNKT cell activation and therapeutic regimen.

Upon ligation of their invariant TCR with α-GalCer, iNKT cells rapidly produce large amounts of cytokines such as IFN-γ and IL-4 (5). Analogues of α-GalCer prepared by total synthesis led to the identification of ligands that induce a cytokine pattern that is more biased toward either a Th1-type (IFN-γ) or a Th2-type (IL-4) response. An example of the former is α-C-GalCer, whereas the latter includes the ligand (2s,3s,4r)-1-O-α-D-galactopyranosyl-N-tetrasaccharide-2-amino-1,3,4-nonenetriol) (OCH) (6, 7). Some studies demonstrated altered biological effects of these α-GalCer analogues on autoimmunity and cancer that could be ascribed to the cytokine profiles they elicit (6, 7).

Experimental autoimmune uveitis (EAU) induced in animals by immunization with retinal Ags in CFA is a model for human autoimmune uveitis, a disease that accounts for ~10–15% of severe visual handicap in the US. EAU is induced by immunization with...
the same retinal Ags that are recognized by uveitis patients and is dependent on CD4+ Th1 and Th17 effector cells (8, 9). EAU in mice is induced with the interphotoreceptor retinoid-binding protein (IRBP) or with its pathogenic fragments emulsified in CFA (8).

The relationship between NKT cells and eye-related immune responses is not well understood. Although iNKT cells have been shown to play an important role in the eye-related regulatory phenomenon known as anterior chamber-associated immune deviation (ACAI) (10, 11), the possible role of iNKT cells in regulation of EAU has not been established. In the present study, we examine the role of iNKT cells and the effects of the iNKT cell ligands on EAU. Our data show that although natural strain-specific variations or genetically induced lack of iNKT cells do not seem to affect the threshold of susceptibility to EAU, iNKT function seemed to correlate with susceptibility. Importantly, a pharmacological enhancement of these cells using glycopolipid iNKT cell ligands was able to inhibit induction of disease. This appeared to be due at least in part to iNKT-produced IFN-γ and a consequent dampening of the adaptive Th1 and Th17 pathogenic effector responses.

Materials and Methods

Animals

B10.RIII, B10.A, C57BL/6, DBA/2, AKR, and BALB/c mice (wild-type (WT) and C1d-knockout (KO)) were purchased from The Jackson Laboratory. All experiments were approved by the National Eye Institute Animal Care and Use Committee. Animal care and use conformed to Institutional guidelines and to the Association for Research in Vision and Ophthalmology guidelines on the use of animals in ophthalmic and vision research.

Ags and reagents

Bovine IRBP was purified as described (12, 13). CFA was purchased from Difco and was supplemented with additional Mycobacterium tuberculosis H37RA to 2.5 mg/ml. Purified derivative of tuberculin (PPD) was purchased from the Statens Seruminstitut. α-GalCer (KRN7000) was provided by the Kirin Brewery (14). α-C-GalCer was synthesized as described previously (6). OCH was synthesized by Drs. Chi-Huey Wong and Douglas Wu of the Scripps Research Institute, La Jolla, CA. Neutralizing anti-IFN-γ Abs (clone R4-6A2) were obtained from the Biological Resources Branch, National Cancer Institute.

Isolation of lymphoid cells from liver

Livers were perfused in situ through the hepatic portal vein with PBS and minced into small pieces in PBS with 2% FCS and 0.02% sodium azide (PBS/FCS/Az). The tissue was then pressed through a 200-gauge mesh and minced into small pieces in PBS with 2% FCS and 0.02% sodium azide. The tissue was then pressed through a 200-gauge mesh and minced into small pieces in PBS with 2% FCS and 0.02% sodium azide.

Immunization, EAU induction, and EAU scoring

C57BL/6 and BALB/c mice were immunized with 150 μg of bovine IRBP emulsified in CFA supplemented with Mycobacterium tuberculosis, strain H37RA from Difco to 2.5 mg/ml. Clinical disease was evaluated by fundus examination in a masked fashion and was scored on a scale from 0 (no inflammation) to 4 (complete destruction of the retina) in half-point increments, as described previously (8). Eyes were harvested for histopathology at 21 days after immunization. Disease was scored by an ophthalmic pathologist (C.-C. Chan) in a masked fashion as described previously (8).

α-GalCer and other treatments

Unless otherwise noted, 5 μg of either α-GalCer, α-C-GalCer, or OCH were added to and emulsified with the uveitogenic Ag preparation (IRBP/CF A 1:1 v/v). The emulsion was injected s.c., divided into three doses (both thighs and base of tail). Systemic IFN-γ neutralization was achieved by treatment with monoclonal anti-IFN-γ Ab, 150 μg/mouse injected i.p. on days −2, 0, and 2.

Determination of immunological responses

Cytokine production to α-GalCer analogues in culture was examined on splenocytes obtained from naive mice. Cell suspensions of 2.5 × 10⁶ cells/ml were incubated with 100 ng/ml of the stimulant and supernatants were collected after 48 h. Cytokine production in vivo to α-GalCer analogues was measured in sera collected at the indicated time points after i.p. injection of 5 μg of the analog. For determination of Ag-specific cytokine production and proliferation, spleens and lymph nodes draining the site of immunization (inguinal and iliac) were collected on day 21 and were pooled within each group. Proliferation to the indicated doses of Ag was assayed by [³H]thymidine uptake during the last 16 h of a 72-h culture on triplicate cultures of 0.2 ml, as described (15). Cytokine responses were determined using the Pierce Chemical multiplex bead technology (Ref. 16 and http://www.endogen.com/services).

Flow cytometry

iNKT cells were enumerated by flow cytometry after exclusion of dead cells by DNA staining with 7-amino-actinomycin D. Cells were reacted with PE-labeled CD1d/α-GalCer tetramers, allophycocyanin-labeled β-TCR, and FITC-labeled anti-CD4 Abs. β-TCR-positive cells were gated, and the percentage of α-GalCer-positive cells (either CD4+ or CD8+) was determined by counting of 1 × 10⁶ viable β-TCR-positive cells. Multiplication of percentages by absolute numbers of lymphocytes that were isolated from each organ (β counter) resulted in the total iNKT numbers shown in Fig. 1.

Statistical analysis and data presentation

All experiments were performed at least twice and results were highly reproducible. Figures show data from representative or from pooled experiments, as specified. EAU severity is represented by fundoscopy scores determined on days 19–21. All fundoscopy scores were confirmed by histopathology. Where appropriate, statistical analysis of EAU severity was performed using the Snedecor and Cochran z test for linear trend in proportions (17). This is a nonparametric, frequency-based test that takes into account both disease severity and incidence. Probability values of <0.05 were considered to be significant. Values determined to be significantly different from controls are marked with an asterisk in the figures.

Results

iNKT cytokine profile, but not their number, may correlate with susceptibility to EAU

EAU is a disease model where susceptibility varies considerably among different mouse strains. The strain with the highest known susceptibility is B10.RIII. B10.A is another susceptible strain that can develop high disease scores, but, unlike the B10.RIII strain, it requires administration of pertussis toxin at the time of immunization to develop EAU, as do all other susceptible strains. C57BL/6 and DBA/2 mice have mild to moderate susceptibility, and AKR as well as BALB/c mice are resistant to disease. We asked the question whether susceptibility to disease in a series of EAU-characterized mouse strains correlated with their numbers of iNKT cells. Fig. 1a shows a schematic representation of typical EAU scores for six mouse strains, summarizing previously reported findings (18–20). Representative pictures of severe, mild and no disease are shown in the inset.

Most of the iNKT cells in the body are concentrated in the thymus, liver and spleen. We enumerated invariant TCR-bearing NKT cells in these three organs in the different mouse strains using flow cytometry, by binding of PE-labeled CD1d/α-GalCer-tetramers. Although there were strain-specific differences in iNKT cell numbers and variations in their content in the different organs, there was no correlation with strain-specific differences in disease susceptibility (Fig. 1, b and c). In addition, a low iNKT number in one organ (e.g., thymus B10.A
and spleen AKR) tended to be counterbalanced by a higher number in another organ (e.g., liver of B10.A and AKR) in some strains. Consequently, the total number of iNKT cells was often similar between strains with different EAU susceptibilities (Fig. 1).

We next examined the percent of CD4\(^+\) iNKT cells of the total iNKT cells, as this subset plays a role in the eye-specific regulatory phenomenon known as ACAID (11, 21). CD4\(^+\) iNKT cells were enumerated by double staining for CD4 and for the invariant TCR using CD1d/\(\alpha\)-GalCer tetramers, anti-CD4, and anti-\(\beta\)-TCR Abs as described in Materials and Methods. The data revealed that the differences in CD4\(^+\) iNKT cells between the strains were even less pronounced than total iNKT numbers and did not correlate with disease susceptibility (Fig. 1).

Lastly, we selected three strains that were either resistant, moderately susceptible, or highly susceptible to EAU (BALB/c, C57BL/6, and B10RIII, respectively) and tested their iNKT cells in vitro using two different ligands, \(\alpha\)-GalCer and OCH. The data showed that OCH protected no better than \(\alpha\)-GalCer, whereas \(\alpha\)-GalCer was the most effective (Fig. 4).

**Genetic lack of iNKT cells does not enhance susceptibility to EAU**

If iNKT cells had a role in raising the threshold of susceptibility to EAU, we would expect that iNKT deficiency would result in more severe disease. However, mice deficient in CD1d (lacking CD1d-dependent NKT cells) (24) did not show enhanced EAU susceptibility compared with their WT counterparts (Fig. 3). The time of onset as well as the course of disease as determined by periodic fundus examinations were also not affected (data not shown). Mice deficient in CD1d on the resistant BALB/c background remained resistant (Fig. 3). These data are consistent with observations made by others in the EAE model (25–27).

**Activation of iNKT cells ameliorates EAU but analogues of \(\alpha\)-GalCer differ in their efficacy**

We next examined whether functional triggering of iNKT cells using invariant TCR ligands can affect EAU. Five micrograms of \(\alpha\)-GalCer incorporated into the IRBP/CFA emulsion ameliorated EAU severity in C57BL/6 mice (Fig. 4a). To examine the effect of \(\alpha\)-GalCer analogues, mice were similarly treated with \(\alpha\)-C-GalCer and OCH. The data showed that OCH protected no better than \(\alpha\)-GalCer, whereas \(\alpha\)-C-GalCer was the most effective (Fig. 4b).

**FIGURE 1.** Total iNKT cell numbers and percentages of CD4\(^+\) NKT cells seem unrelated to susceptibility to EAU. Six different mouse strains (six mice per strain, 8 wk of age) were immunized with 150 \(\mu\)g IRBP in CFA and an additional i.p. injection of 0.3 \(\mu\)g pertussis toxin. B10.RIII mice were immunized with 10 \(\mu\)g IRBP without additional pertussis toxin. a, Schematic representation of typical strain-specific disease scores and representative histopathology (inset), based on established data. Lymphocytes from naive livers, thymuses and spleens were isolated on a density gradient, and iNKT cell numbers were determined by flow cytometry using labeled CD1d/\(\alpha\)-GalCer tetramers, anti-CD4, and anti-\(\beta\)-TCR Abs as described in Materials and Methods. b, Average of isolated iNKT cell numbers in thymus, lymph node, and spleen, expressed as a total cell number collected from each mouse. c, Average of isolated NKT cell numbers in thymus, lymph node and spleen, expressed as a percentage of the total cell number collected from each mouse. d, Proportion of CD4\(^+\) iNKT cells expressed as a percentage of total iNKT cells. The data are pooled from two identical experiments of three mice each (total six individual animals per point).
This pattern of protection was unexpected because OCH deviates the iNKT response to TCR ligation toward IL-4 production (whereas α-C-GalCer skews toward IFN-γ) and has moreover been shown in experimental models of arthritis, diabetes in the NOD mouse, and encephalomyelitis (EAE) to be more protective than α-GalCer in its original form through an IL-4 dependent mechanism (reviewed in Refs. 7, 22). We therefore examined whether our α-GalCer, α-C-GalCer, and OCH preparations had the expected effect on iNKT cytokine production. Data obtained by measuring IL-4 and IFN-γ in serum of mice injected with the three α-GalCer analogues confirmed that indeed the three analogues elicited cytokine profiles that were in keeping with what has been reported by others (Fig. 5). These data suggested that

FIGURE 2. Functional differences are present in iNKT cells from susceptible, compared with resistant, strains. Splenocytes from BALB/c, C57BL/6, and B10RIII mice were isolated and cultured in HL-1 media containing 1% normal mouse serum and were left either unstimulated or were treated with 100 ng/ml of either α-GalCer or OCH, as indicated in the figure. Cell supernatants were collected after 48 h and cytokines were analyzed by Pierce Searchlight Technology. Data are representative of two experiments with three individual mice in each group. Supernatants were pooled before analysis.

FIGURE 3. Lack of NKT cells does not seem to alter the disease course or the susceptibility to EAU. An EAU-susceptible (C57BL/6) and resistant strain (BALB/c) was immunized as described in Fig. 1a. Likewise NKT-deficient strains on these genetic backgrounds were immunized, and EAU scores were compared with the WT mice. EAU scores of C57BL/6 WT and Jα18-KO mice. Representative experiment of two showing the same pattern (data were not pooled due to inter-experiment variation in disease severity). Positive mice of total are indicated within each bar.

FIGURE 4. Effect of iNKT cell activation on EAU. a. Activation of NKT cells ameliorates EAU. C57BL/6 WT mice were immunized with IRBP as described in Fig. 1a with (black column) or without (white column) 5 μg of the synthetic iNKT cell ligand α-GalCer. EAU scores on day 19 after immunization are shown (p < 0.005). b. Analogues of α-GalCer differ in their ability to ameliorate EAU. C57BL/6 WT mice were immunized as described in Fig. 1a without (white column) or with 5 μg of the synthetic iNKT cell ligand α-GalCer (black column, p vs control <0.09) or α-C-GalCer (gray column, p vs control <0.02) or OCH (dark gray column, p vs control <0.22). Data combined from three experiments.

4794 NKT CELLS PROTECT FROM EAU by guest on November 13, 2017 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/ Downloaded from
an IFN-γ-dominated cytokine profile elicited by α-C-GalCer is more efficient in protecting from EAU than a deviation toward an IL-4-dominated profile in this model.

**Discussion**

In the present study, we demonstrate that iNKT cells can have a role in EAU regulation. Their role appears to be not in setting the threshold of susceptibility to EAU, as do the natural CD4+CD25+ regulatory cells whose function in deterring development of ocular autoimmunity we have characterized in recent studies (30, 31). Rather, they can inhibit developing disease following a pharmacological enhancement of their activity at or around the time of priming. In chronic autoimmunity, priming of new effector T cells is believed to be occurring on a continuous basis. Since endogenous ligands for iNKT cells exist in the body and can trigger iNKT activity (4, 5), it is conceivable that iNKT cells can participate in modulating the course of ocular autoimmune disease. Thus, there appears to be a “division of labor” between the natural CD4+CD25+ regulatory T cells and iNKT cells, with the former setting the threshold of susceptibility and the latter possibly regulating the autoimmune response after that threshold has been passed.

The group of Stein-Streilein (10, 11, 21) demonstrated that iNKT cells have a central role in ACAID, a prototypic regulatory phenomenon elicited by injection of Ag into the anterior chamber of the eye and its transport by eye-derived APC to the spleen.
NKT CELLS PROTECT FROM EAU

iNKT cells are recruited into the spleen via a mechanism involving MIP-2 and participate in priming the adaptive T regulatory cells typically associated with ACAID. Although prior elicitation of ACAID to IRBP can inhibit a subsequent episode of EAU (32), it is unlikely that the protection from EAU by iNKT that we observe here bears a relationship to their role in ACAID. In ACAID, the eliciting Ag originates from the eye, which has to be perturbed (injected with Ag) in order for this phenomenon to be observed, and iNKT activation, if any, occurs without additional manipulation. In contrast, in our study, pharmacological activation of iNKT cells is needed and is applied when the eye is still intact. Thus, it is conceivable that iNKT cells may regulate ocular immune responses at more than one level. Studies in the models of experimental arthritis, NOD diabetes, and EAE (reviewed in Ref. 22) had indicated that activation of iNKT cells by OCH is more effective than by α-GalCer, which was attributed to its induction of IL-4 and Th2 skewing. We were therefore surprised to find that OCH was not more effective than α-GalCer in protecting from EAU, and that the most efficient protection followed administration of α-C-GalCer, which induces an IFN-γ dominated iNKT cytokine response. Thus, effectiveness of protection paralleled the innate IFN-γ inducing ability of the invariant TCR ligand. The protection was accompanied by reduction in the IRBP-specific adaptive Th1 and Th17 pathogenic effector responses, as judged by production of their respective hallmark cytokines IFN-γ and IL-17 to in vitro recall with IRBP. The functional role of IFN-γ in the protective and regulatory effects of iNKT cells is strongly supported by direct evidence showing that neutralization of innate IFN-γ reverses the protective effect of α-C-GalCer and restored the subsequent proinflammatory cytokine production of the adaptive response. This is not to say that the mechanism of protection is the same for all the three analogues. Our data do not negate the possibility that protection from EAU by OCH and by α-GalCer could involve IL-4, as was previously demonstrated in several other autoimmune disease models (22).

Our data are in line with some previous reports, which revealed that protection from autoimmune disease by iNKT may not always involve IL-4 and Th2 skewing. Studies by Lehuen and her colleagues (33, 34) demonstrated that even in the absence of IL-4, iNKT cells can control EAE and experimental type 1 diabetes. This was associated with a decrease in Th1-associated pathogenic autoimmune responses without inducing Th2 responses and was due at least in part to induction of anergy in the autoreactive T cells (35). Such a mechanism could also be involved in the prevention of EAU observed here. Although these studies did not directly implicate IFN-γ in these effects, participation of IFN-γ (rather than IL-4) in protection from EAE was suggested by Furlan et al. (27).

It should be noted that high systemic levels of IFN-γ early or late in the disease can be protective, but likely by different mechanisms. Initial production of IFN-γ would be mostly from NKT and NK cells, whereas later in disease Ag-specific Th1 cells are a major source of IFN-γ. We previously showed that early up-regulation of IFN-γ by injections of IL-12 inhibits development of EAU and associated immunological responses by aborting priming, through a process that involves induction of NO and apoptosis (29). The innate IFN-γ produced by α-C-GalCer-triggered NKT cells may well work in a similar fashion. In contrast, protective effects of IFN-γ later in the disease appear to be due to its role in elimination of spent effector cells by activation-induced cell death (36, 37). Thus, neutralization of systemic IFN-γ at that stage also enhances disease (19), although at that point it is not possible to distinguish between effects of IFN-γ produced by Ag-specific T cells and iNKT cells.

In summary, we have demonstrated that iNKT cells can actively participate in regulating the autoimmune response to immunologically privileged retinal Ags. This apparently occurs at a different level than their role in induction of ACAID. The mechanism involves the induction of innate IFN-γ production through ligation of the invariant TCR and results in inhibited development of adaptive Th1 and Th17 responses that represent pathogenic effector mechanisms in uveitis.

Acknowledgments

We thank Dr. S. Yamano of the Kirin Brewery, Tokyo, Japan for providing α-GalCer (KRN7000) and Drs. Chi-Huey Wong and Douglas Wu of the Scripps Research Institute, La Jolla, CA for synthesizing the OCH used in this study.

Disclosures

The authors have no financial conflict of interest.

References

CD4+ T cells, are required to generate efferent CD8+ T regulatory cells following antigen inoculation in an immune-privileged site. *J. Immunol.* 171: 1266–1271.


