The Journal of Immunology

Plasticity of Invariant NKT Cell Regulation of Allergic Airway Disease Is Dependent on IFN-γ Production

Hiroyuki Matsuda, Katsuyuki Takeda, Toshiyuki Koya, Masakazu Okamoto, Yoshiki Shiraishi, Nobuaki Miyahara, Azzeddine Dakhama, Jennifer L. Matsuda, Laurent Gapin and Erwin W. Gelfand

J Immunol 2010; 185:253-262; Prepublished online 4 June 2010;
doi: 10.4049/jimmunol.0902301
http://www.jimmunol.org/content/185/1/253

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Plasticity of Invariant NKT Cell Regulation of Allergic Airway Disease Is Dependent on IFN-γ Production

Hiroyuki Matsuda,*[,1] Katsuyuki Takeda,*[,1] Toshiyuki Koya,* Masakazu Okamoto,* Yoshiki Shiraishi,* Nobuaki Miyahara,* Azzeddine Dakhama,* Jennifer L. Matsuda,[†] Laurent Gapin,[†] and Erwin W. Gelfand*

Invariant NKT cells (iNKT cells) play a pivotal role in the development of allergen-induced airway hyperresponsiveness (AHR) and inflammation. However, it is unclear what role they play in the initiation (sensitization) phase as opposed to the effector (challenge) phase. The role of iNKT cells during sensitization was examined by determining the response of mice to intratracheal transfer of OVA-pulsed or OVA–α-galactosylceramide (OVA/αGalCer)-pulsed bone marrow-derived dendritic cells (BMDCs) prior to allergen challenge. Wild-type (WT) recipients of OVA-BMDCs developed AHR, increased airway eosinophilia, and increased levels of Th2 cytokines in bronchoalveolar lavage fluid, whereas recipients of OVA/αGalCer BMDCs failed to do so. In contrast, transfer of these same OVA/αGalCer BMDCs into IFN-γ–deficient (IFN-$\gamma^{-/-}$) mice enhanced the development of these lung allergic responses, which was reversed by exogenous IFN-γ treatment following OVA-BMDC transfer. Further, Jα18-deficient recipients, which lack iNKT cells, developed the full spectrum of lung allergic responses following reconstitution with highly purified WT liver or spleen iNKT cells and transfer of OVA-BMDCs, whereas reconstituted recipients of OVA/αGalCer BMDCs failed to do so. Transfer of iNKT cells from IFN-$\gamma^{-/-}$ mice restored the development of these responses in Jα18-deficient recipients following OVA-BMDC transfer; the responses were enhanced following OVA/αGalCer BMDC transfer. iNKT cells from these IFN-$\gamma^{-/-}$ mice produced higher levels of IL-13 in vitro compared with WT iNKT cells. These data identify IFN-γ as playing a critical role in dictating the consequences of iNKT cell activation in the initiation phase of the development of AHR and airway inflammation. ***The Journal of Immunology*****, 2010, 185: 253–262.**

The characteristic features of bronchial asthma include variable airflow obstruction, airway hyperresponsiveness (AHR), mucus hypersecretion, and airway inflammation (1). Many types of cells are involved in the development of airway inflammation in the asthmatic lung, including lymphocytes, mast cells, eosinophils (Eos), and dendritic cells (DCs) (1–3). Much of the supporting data identify Th2 cells that produce Th2 cytokines, such as IL-4, -5, and -13, as being essential in the development of allergic airway inflammation and AHR in humans (4) and mice (5, 6).

Differentiation of Th2 cells from naive T cells is an essential component of the allergic response. Naive T cells require interaction with mature APCs, such as DCs, to initiate the expansion and acquisition of Th2 effector cell functions in response to Ag exposure (7, 8). As a result, DCs play a pivotal role in asthma development, regulating downstream responses to allergen exposure (7). In the lung, DCs may represent the most important APCs and play an essential role in the induction of allergic airway inflammation and AHR (7, 9). Following intratracheal transfer of OVA-pulsed bone marrow-derived dendritic cells (BMDCs), mice develop AHR and eosinophilic airway inflammation after OVA challenge alone (10, 11). In such studies, the Ag-pulsed BMDCs replace the active sensitization phase, priming the airways to subsequent allergen challenge.

Invariant NKT cells (iNKT cells) represent a distinct lymphocyte subpopulation that has important immunoregulatory functions (12, 13). iNKT cells express a semi-invariant TCR that recognizes glycolipid Ags presented by the nonpolymorphic MHC class I-like molecule CD1d (14, 15). α-galactosylceramide (αGalCer), a specific ligand for iNKT cells isolated from a marine sponge (12), rapidly induces the production of Th1 and Th2 cytokines, including IFN-γ and IL-4, by iNKT cells. Through the release of these cytokines, iNKT cells modulate a variety of immune responses, such as tumor immunity, autoimmune disease, and infection (12–14).

The role of iNKT cells in the initiation of asthma has been intensively studied but remains controversial (14, 15). In humans, Akbari et al. (16) reported that the percentages of iNKT cells strikingly increase in the airways of asthmatics. Although other investigators found that the number of iNKT cells was not increased or increased only marginally in the airway lumens or airways of patients with asthma (17–19), recent studies indicated

*Division of Cell Biology, Department of Pediatrics, National Jewish Health, Denver, CO 80206; and [†]Integrated Department of Immunology, University of Colorado Health Sciences Center, Denver, CO 80217

[1]H.M. and K.T. contributed equally to this work.

Received for publication July 17, 2009. Accepted for publication April 19, 2010.

This work was supported by National Institutes of Health Grants HL-36577, HL-61005, and AI-77609 (to E.W.G.).

The content of this publication is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

Address correspondence and reprint requests to Dr. Erwin W. Gelfand, National Jewish Health, 1400 Jackson Street, Denver, CO 80206. E-mail address: gelfande@njhealth.org

Abbreviations used in this paper: αGalCer, α-galactosylceramide; αGC, BMDCs cultured with αGalCer; AHR, airway hyperresponsiveness; AM, alveolar macrophage; BAL, bronchoalveolar lavage; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; Eos, eosinophils; IFN-$\gamma^{-/-}$, IFN-γ deficient; IFN-γKO, IFN-γ^{-} mice; IFN-γ+OVA/αGC, BMDCs cultured with OVA and αGalCer prior to exogenous IFN-γ administration; iNKT cells, invariant NKT cells; Jα18$^{-/-}$, Jα18 deficient; Jα18KO, Jα18$^{-/-}$ mice; Lym, lymphocytes; MCh, methacholine; Medium, BMDCs cultured without OVA or αGalCer; mIFN-γ, mouse IFN-γ; MNC, mononuclear cell; Neut, neutrophils; OVA, BMDCs cultured with OVA; OVA/αGC, BMDCs cultured with OVA and αGalCer; PAS, periodic acid-Schiff; PBLN, peribronchial lymph node; RL, lung resistance; TC, total cell; WT, wild-type.

that the numbers of iNKT cells in the airways of severe asthmatics tend to be increased (20, 21). However, their role in the initiation or amplification of asthma pathogenesis is not fully defined. In the mouse, two reports showed that iNKT cells play an essential role in the development of allergic airway inflammation and AHR (22, 23), whereas other groups did not find these effects (24–26). The reasons for such discrepancies are unclear. They might suggest that iNKT cell regulatory activities have a certain plasticity that might be subject to a number of regulatory factors under different experimental conditions.

We and other investigators showed that a single i.p. administration of αGalCer prior to Ag challenge of sensitized mice inhibits allergic airway inflammation and AHR through iNKT cells and in an IFN-γ–dependent manner (25, 27, 28). It is unclear whether such effects are restricted to the challenge phase or whether activation of iNKT cells during the sensitization (initiation) phase also regulates development of allergic inflammation and AHR, because they were shown to be a potent producers of Th1- and Th2-type proinflammatory cytokines (29). Intratracheal transfer of Ag-pulsed BMDCs leads to the full development of lung allergic responses on allergen challenge alone, 10 d later (10, 11). In this study, we show that transfer of BMDCs treated with αGalCer, a specific ligand of NKT cells, prevented the development of lung allergic responses, and this was dependent on IFN-γ production by recipient iNKT cells. In the absence of IFN-γ in recipients, the OVA-pulsed BMDCs retained the ability to induce allergic airway inflammation and AHR, and these responses were further enhanced following transfer of OVA/αGalCer BMDCs.

Materials and Methods

Animals

Eight- to 12 wk-old female C57BL/6 wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used throughout the study. IFN-γ–deficient (IFN-$\gamma^{-/-}$) and Jα18-deficient (Jα18$^{-/-}$) mice on a C57BL/6 background were bred in the animal facility at National Jewish Health. The animals were maintained on an OVA-free diet. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Reagents

Recombinant murine GM-CSF and murine IL-4 were purchased from R&D Systems (Minneapolis, MN). αGalCer was obtained from Axxora (San Diego, CA), and recombinant mouse IFN-γ (mIFN-γ) was obtained from eBioscience (San Diego, CA). FITC-conjugated anti-mouse CD3ε mAb (145-2C11), PE-PerCP–conjugated anti-mouse CD4 mAb (RM4-5), allophycocyanin-conjugated anti-mouse IFN-γ mAb (XMG1.2), allophycocyanin-conjugated anti-mouse IL-4 mAb (11B11), and streptavidin-allophycocyanin conjugate were purchased from BD Biosciences (San Jose, CA). Biotinylated anti-mouse IL-13 Ab was obtained from R&D Systems. PBS57-loaded CD1d tetramer was provided by the National Institute of Allergy and Infectious Disease MHC Tetramer Core Facility (Atlanta, GA).

Generation of BMDCs

BMDCs were generated from bone marrow cells of naive C57BL/6 WT mice, according to the procedure described by Inaba et al. (30), with some modification. In brief, bone marrow cells obtained from femurs and tibias of mice were placed in 75-ml flasks at 37°C in culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, penicillin [100 U/ml], streptomycin [100 μg/ml; Invitrogen, Carlsbad, CA]), recombinant murine GM-CSF (10 ng/ml), and recombinant murine IL-4 (10 ng/ml). Nonadherent cells were collected by aspirating the medium and transferring them into fresh flasks. On day 8, cells were pulsed with OVA (grade V, 150 μg/ml; Sigma-Aldrich, St. Louis, MO) and αGalCer (150 ng/ml) or OVA alone for 24 h and washed three times with PBS.

In vitro assay of BMDCs

BMDCs (1×10^6 cells) were incubated with or without OVA and/or αGalCer for 24 h at 37°C. After harvesting BMDCs, cytokine levels in culture supernatants were measured by ELISA, and surface Ags of BMDCs were analyzed by flow cytometry. The surface phenotype of BMDCs was analyzed using FITC-conjugated anti–I-A^b (AF6-120.1), FITC-conjugated anti-CD11b (M1/70), allophycocyanin-conjugated anti-CD11c (HL3), PE-conjugated anti-CD80 (16-10A1), and PE-conjugated anti-CD86 (GL1) (all obtained from BD Pharmingen, San Diego, CA). For control staining, similarly labeled, isotype-matched control Abs were used.

Transfer of allergen-pulsed BMDCs into naive mice

OVA-, αGalCer-, or OVA- and αGalCer-pulsed BMDCs (1×10^6) were instilled intratracheally into naive WT or IFN-$\gamma^{-/-}$ mice on day 1; mice that received nonpulsed BMDCs served as controls. Ten days after transfer of BMDCs, animals were challenged with nebulized OVA (1% in saline) for 20 min on days 11–13. Forty-eight hours after the last OVA challenge (day 15), AHR was assessed, and bronchoalveolar lavage (BAL) fluid, serum, and tissues were obtained for further analyses. A group of IFN-$\gamma^{-/-}$ mice received 1 μg mIFN-γ in 25 μl PBS, intratracheally, 1 d after OVA/αGalCer BMDC transfer, followed by OVA challenge via the airways.

Determination of airway responsiveness

Airway function was assessed, as previously described, measuring changes in lung resistance (RL) in response to increasing doses of inhaled methacholine (MCh) (31). Data are expressed as the percentage of change from baseline RL values obtained after inhalation of saline. There were no significant differences in baseline RL values among the groups.

Bronchoalveolar lavage

Immediately after assessment of airway function, lungs were lavaged via the tracheal tube with 1 ml HBSS at room temperature. Total leukocyte numbers were measured using a Coulter Counter (Coulter, Hialeah, FL). Cytospin slides were stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA) and differentiated by standard hematological procedures in a blinded fashion.

Lung histology

Lungs were fixed in 10% formalin and processed into paraffin. Mucus-containing goblet cells were detected by staining of paraffin sections (5-μm thick) with periodic acid-Schiff (PAS). Sections were also stained with H&E to analyze inflammatory cell infiltration. Histological analyses were performed in a blinded manner under a light microscope linked to an image-capture system. The numbers of PAS^+ goblet cells were determined in cross-sectional areas of the airway wall. Eight to 10 sections were evaluated per animal. The measurements were averaged for each animal, and the mean value ± SE was determined for each group.

Measurement of cytokines

Levels of cytokines in BAL fluid and cell culture supernatants were determined using commercially available ELISAs, following the manufacturers' instructions. ELISA kits for the detection of IL-4, -5, -10, and -12 (p70) and IFN-γ were obtained from BD Pharmingen. The IL-13 ELISA kit was purchased from R&D Systems. ELISA kits for mouse IL-18 were obtained from Bender Medbioscience (Burlingame, CA), and the IL-6 kit was from eBioscience. The limits of detection for each assay were as follows: 4 pg/ml for IL-4, -5, and -6; 10 pg/ml for IL-10, -12, and -18 and IFN-γ; and 1.5 pg/ml for IL-13.

Lung leukocyte isolation

Lung leukocytes were isolated, as previously described (32), using collagenase digestion, followed by centrifugation on 35% Percoll density gradients (Sigma-Aldrich).

Intracellular cytokine staining

Intracellular cytokine staining was performed as previously described (33). Briefly, lung mononuclear cells (MNCs) were stimulated for 3 h with PMA and ionomycin (10 and 500 ng/ml, respectively) in the presence of brefeldin A (10 μg/ml). After washing, cells were stained for cell surface markers with mAbs against CD3, CD4, and CD1d tetramer. After fixation and permeabilization, cells were stained with allophycocyanin-conjugated anti–IFN-γ or anti–IL-4 mAb or biotin-conjugated anti–IL-13. In parallel, cells were similarly labeled with isotype-matched control Ab. After washing, staining was analyzed by flow cytometry on a FACSCalibur using CellQuest software (BD Biosciences).

In vitro cytokine production in peribronchial lymph nodes

Peribronchial lymph nodes (PBLNs) were removed and subsequently passed through a stainless steel sieve. Single-cell preparations were suspended in complete RPMI 1640 with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. PBLN MNCs (4×10^5 cells) were cultured for 24 h and 5 d in 96-well round-bottom plates in the presence of OVA (100 μg/ml). Levels of IL-4, -5, and -13 and IFN-γ in culture supernatants were measured by ELISA.

In vitro activation of iNKT cells

Livers from WT or IFN-$\gamma^{-/-}$ mice were harvested and subsequently passed through a stainless steel sieve. After washing with PBS, MNCs were isolated by 35% Percoll gradient centrifugation (Sigma-Aldrich). Liver MNCs were cocultured with αGalCer WT BMDCs. Liver MNCs were adjusted to 1×10^6 cells/ml of iNKT cells following tetramer staining and mixed with 0.33×10^6 BMDCs/ml. After 24 h, culture supernatants were collected, and the levels of IL-4 and -13 were measured by ELISA. Cells were also analyzed by intracellular cytokine staining.

Adoptive transfer of iNKT cells into Jα18$^{-/-}$ mice

Liver cells from WT, IFN-$\gamma^{-/-}$, or Jα18$^{-/-}$ mice were harvested and subsequently passed through a stainless steel sieve. After washing with PBS, MNCs were isolated by 35% Percoll density gradient centrifugation (Sigma-Aldrich). Enrichment of iNKT cells was carried out by negative selection using the CD4 isolation kit (CD4 Cellect Immunocolumn Kit Mouse, Cedarlane Laboratories, Burlington, Ontario, Canada), in accordance with the manufacturer's instructions. Purity of iNKT cells from WT and IFN-$\gamma^{-/-}$ mice after isolation was 35–40%, as assessed by flow cytometry. iNKT cell-enriched liver MNCs (0.8×10^6 cells) were transferred into Jα18$^{-/-}$ mice via the tail vein 1 d before the intratracheal instillation of allergen-pulsed cell isolation, as described above. Isolated CD4$^+$ cells were stained with PE-conjugated PBS57-loaded CD1d tetramer and purified with anti-PE MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). To further purify iNKT cells, PE-positive cells were sorted on MoFlo (DakoCytomation, Fort Collins, CO) following MicroBeads separation. Purified spleen iNKT cells (>95% were CD1d tetramer$^+$ and CD3$^+$) (0.3×10^6 cells) were transferred into Jα18$^{-/-}$ mice via the tail vein 1 d before the intratracheal instillation of OVA BMDCs. Control mice received PBS prior to OVA BMDCs. Both groups of recipient mice were challenged with OVA for 3 consecutive days and assayed 48 h after the last challenge, 10 d after injection of BMDCs.

Statistical analysis

The *t* test was used to compare differences between two groups, whereas ANOVA and the Tukey–Kramer multiple-means comparison tests were used for comparisons among three or more groups. Statistical analyses using nonparametric analysis (Mann–Whitney *U* test or Kruskal–Wallis test) were also performed. The *p* values for significance were set to 0.05 for all tests with statistical software (JMP, SAS Institute, Cary, NC). The data were pooled from three independent experiments with four mice/group in each experiment ($n = 12$). Values for all measurements are expressed as mean ± SEM.

Results

Effect of αGalCer on BMDCs in vitro

To determine whether incubation with αGalCer alters the function of BMDCs in vitro, we measured cytokine levels in culture supernatants and analyzed the expression of several surface Ags by flow cytometry. As shown in Fig. 1*A*, OVA, but not αGalCer, induced IL-6 release from BMDCs. Other cytokines (i.e., IL-10, -12, -13, and -18 were not detected (data not shown).

There were few differences in the levels of expression of CD80, CD86, CD40, and MHC class II on αGalCer BMDCs and nonpulsed BMDCs (Fig. 1*B*). OVA BMDCs expressed higher levels of these surface Ags than did BMDCs cultured without OVA. OVA/αGalCer BMDCs expressed the same levels of these Ags as did OVA BMDCs. Collectively, αGalCer added to BMDCs did not seem to alter the phenotype (cytokine profile, surface Ag expression) of the BMDCs in vitro.

Transfer of allergen-pulsed BMDCs in vivo into WT mice

To determine whether incubating BMDCs with αGalCer prior to transfer into allergen- challenged recipients could alter the allergic phenotype, we transferred OVA BMDCs, αGalCer BMDCs, or OVA/αGalCer BMDCs intratracheally into naive WT mice prior to challenge with OVA on three consecutive days. As shown in Fig. 2*A*, mice administered OVA BMDCs and challenged with OVA developed significant increases in RL in response to increasing doses of inhaled MCh. However, mice receiving αGalCer BMDCs or OVA/αGalCer BMDCs failed to develop AHR to MCh; RL levels were the same as in mice that received nonpulsed BMDCs. Cell-composition analysis of BAL fluid demonstrated that airway eosinophilia developed in WT mice that received OVA BMDCs. In contrast, WT mice that received αGalCer BMDCs or OVA/αGalCer BMDCs had decreased numbers of Eos in the BAL fluid (Fig. 2*B*).

Examination of cytokine levels in the BAL fluid showed that IL-4, -5, and -13 were elevated in recipients of OVA BMDCs, whereas these cytokine levels were significantly lower in recipients of OVA/αGalCer BMDCs. IFN-γ levels in the BAL fluid of OVA/αGalCer BMDC recipients were significantly increased compared with recipients of OVA BMDCs (Fig. 2*C*).

Lung histology (Fig. 2*D*) revealed that recipients of OVA BMDCs developed a marked infiltration of inflammatory cells, including Eos, around the airways and vessels. However, these lung inflammatory responses were not observed following transfer of OVA/αGalCer BMDCs.

Animal models of allergic airway inflammation are accompanied by goblet cell metaplasia and mucus hypersecretion in the airways (34), which is a prominent feature of asthma. As shown in Fig. 2*E* and 2*F*, challenge with OVA in recipients of OVA BMDCs resulted in marked increases in the numbers of PAS$^+$ cells. In the mice that received OVA/αGalCer BMDCs, few PAS$^+$ goblet cells could be detected.

Intracellular cytokine staining of lung iNKT cells

In previously sensitized mice, IFN-γ was shown to be critical to the inhibition of allergic airway inflammation and AHR induced by αGalCer (25, 27, 28). To determine whether transfer of OVA BMDCs exposed to αGalCer modulated the numbers of iNKT cells in the lung and their capacity for IFN-γ production, we quantified the number of iNKT cells in the lungs and the numbers of IFN-γ–producing iNKT cells by intracellular cytokine staining. In mice that received OVA/αGalCer BMDCs, a significant increase in the number of CD3$^+$CD1d-tetramer$^+$ cells (Fig. 3*A*, 3*B*) and CD3$^+$CD1d-tetramer$^+$IFN-γ$^+$ cells was observed in the lung compared with mice that received OVA BMDCs (Fig. 3*C*).

Transfer of allergen-pulsed BMDCs in IFN-$\gamma^{-/-}$ mice

Together, these data suggested that activation of iNKT cells by αGalCer during the initiation phase (i.e., before allergen challenge) attenuates the development of allergic airway inflammation and AHR through increasing numbers of recipient IFN-γ–producing iNKT cells in the lung. To directly determine the role of IFN-γ in this inhibition, we examined the effects of administering αGalCer BMDCs or exogenous mIFN-γ to IFN-$\gamma^{-/-}$ recipients.

In contrast to WT recipients, in which the development of AHR was inhibited following administration of OVA/αGalCer BMDCs (Fig. 2*A*), IFN-$\gamma^{-/-}$ recipients of OVA/αGalCer BMDCs showed a striking increase in AHR compared with IFN-$\gamma^{-/-}$ mice that received OVA BMDCs or αGalCer (non–OVA-pulsed) BMDCs (Fig. 4*A*). Analysis of the cell composition of BAL fluid demonstrated that airway eosinophilia was also significantly enhanced in

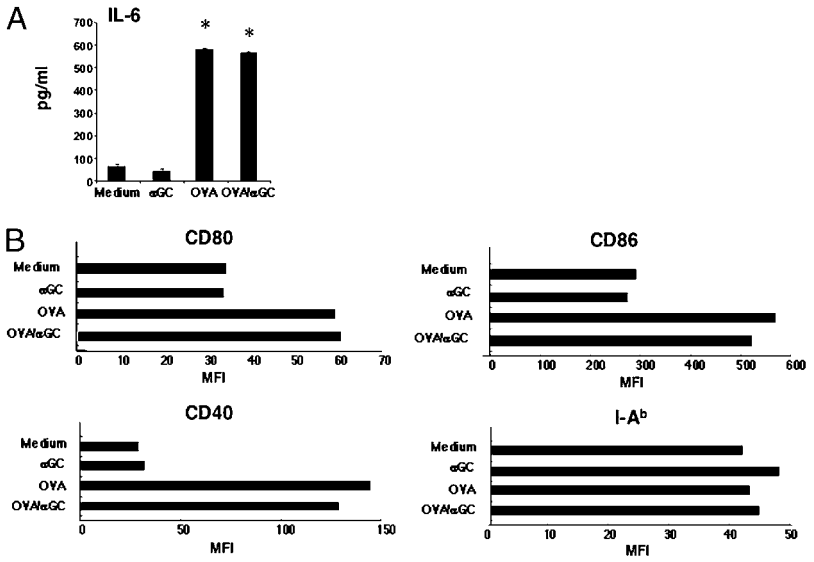

FIGURE 1. αGalCer does not alter the phenotype of BMDCs in vitro. BMDCs (1×10^6 cells) were incubated with or without OVA and/or αGalCer for 24 h at 37°C. Cytokine levels in culture supernatants were measured by ELISA, and surface Ag analyses were done by flow cytometry. IL-6 levels (*A*) and expression of surface Ags (*B*) in BMDCs in vitro. Data are representative of three independent experiments ($n = 12$). $*p < 0.05$, comparing OVA-pulsed BMDCs and OVA/αGalCer-pulsed BMDCs versus medium only and αGalCer-pulsed BMDCs. αGC, BMDCs cultured with αGalCer; Medium, BMDCs cultured without OVA or αGalCer; OVA, BMDCs cultured with OVA; OVA/αGC, BMDCs cultured with OVA and αGalCer.

IFN-$\gamma^{-/-}$ recipients of OVA/αGalCer BMDCs compared with recipients of OVA BMDCs (Fig. 4*B*). The development of AHR in IFN-$\gamma^{-/-}$ recipients of OVA/αGalCer BMDCs was prevented by exogenous IFN-γ administration (Fig. 4*A*, 4*B*). Examination of cytokines in the BAL fluid demonstrated that levels of IL-4 and -13 were also significantly higher in IFN-$\gamma^{-/-}$ recipients of OVA/αGalCer BMDCs; these cytokines, as well as IL-5, were decreased by mIFN-γ administration (Fig. 4*C*).

On histological analysis, IFN-$\gamma^{-/-}$ recipients of OVA/αGalCer BMDCs showed a greater inflammatory cell accumulation compared with IFN-$\gamma^{-/-}$ recipients of OVA BMDCs (Fig. 4*D*), and the number of PAS$^+$ cells was also increased in IFN-$\gamma^{-/-}$ recipients of OVA/αGalCer BMDCs (Fig. 4*E*).

These changes in IFN-$\gamma^{-/-}$ recipients of OVA/αGalCer BMDCs were accompanied by similar increases in the numbers of CD3$^+$CD1d-tetramer$^+$ cells in their lungs, as observed in WT recipients (Fig. 3*B*). However, unlike WT recipients, the numbers of lung CD3$^+$CD1d-tetramer$^+$IL-4$^+$ and CD3$^+$CD1d-tetramer$^+$ IL-13$^+$ cells were markedly increased compared with IFN-$\gamma^{-/-}$ mice that received OVA BMDCs (Fig. 5*A–C*). These findings associated with transfer of OVA/αGalCer BMDCs into IFN-$\gamma^{-/-}$ mice identified a conversion of the responses with enhancement

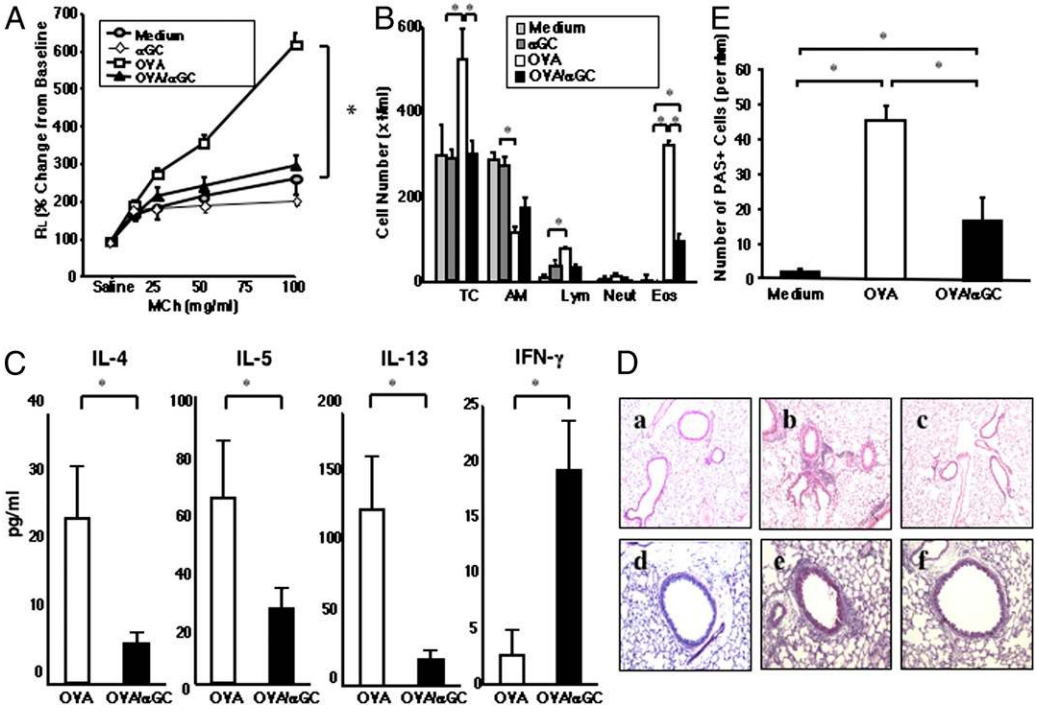

FIGURE 2. Transfer of OVA/αGalCer-pulsed BMDCs to WT mice prior to OVA challenge prevents development of allergic airway inflammation and AHR. *A*, Airway resistance. *B*, BAL cell composition. *C*, Cytokine levels in BAL fluid. *D*, Representative photomicrographs (original magnificaion ×200). The tissues were obtained 48 h after the last challenge and stained with H&E (*a–c*) or PAS (*d–f*). Shown are photomicrographs of WT recipients of nonpulsed DCs (*a*, *d*), OVA-pulsed BMDCs (*b*, *e*), and OVA and αGalCer-pulsed BMDCs (*c*, *f*). *E*, Quantitative analysis of PAS$^+$ cells in the lung tissue. Data represent mean ± SEM ($n = 12$). $*p < 0.05$, comparing WT recipients of OVA-pulsed BMDCs versus medium, αGalCer-pulsed BMDCs, or OVA/αGalCer-pulsed BMDCs. αGC, BMDCs cultured with αGalCer; AM, alveolar macrophages; Eos, eosinophils; Lym, lymphocytes; Medium, BMDCs cultured without OVA or αGalCer stimulation; Neut, neutrophils; OVA, BMDCs cultured with OVA; OVA/αGC, BMDCs cultured with OVA and αGalCer; TC, total cell.

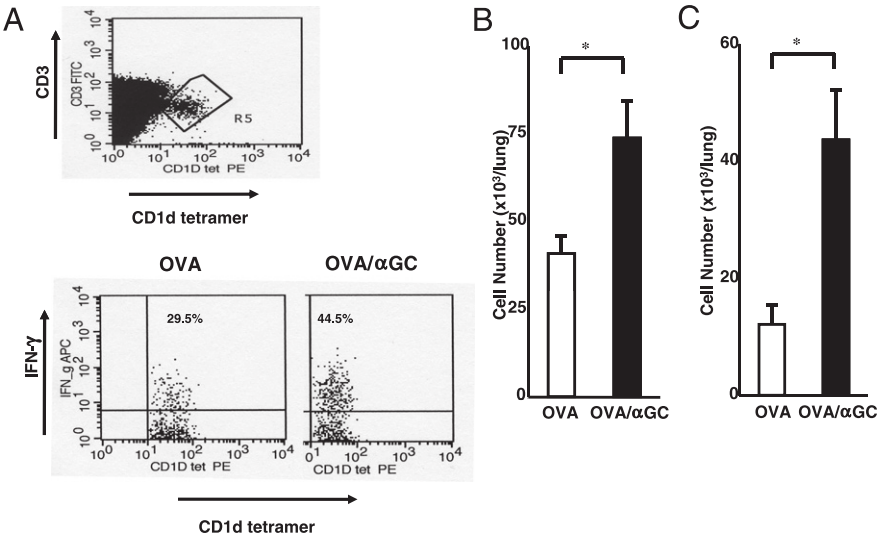

FIGURE 3. WT recipients of OVA- and αGalCer-pulsed BMDCs have increased numbers of iNKT cells and production of IFN-γ in the lung. Lung MNCs were isolated and stimulated with phorbol/ionomycin, fixed, permeabilized, and stained with anti-mouse CD3, CD1d tetramer, and IFN-γ Ab and quantified as described in *Materials and Methods*. $CD3^+CD1d^+$ $tetramer^+$ T cells were gated on and analyzed for intracellular IFN-γ (*A*), number of $CD3^+CD1d^+$ T cells (*B*), and number of $CD3^+CD1d$ $tetramer^+IFN\text{-}\gamma^+$ T cells (*C*). Means ± SEM from three independent experiments are shown ($n = 12$). $*p < 0.05$. OVA, BMDCs pulsed with OVA; OVA/αGC, BMDCs pulsed with OVA and αGalCer.

of AHR, airway eosinophilia, and Th2 cytokine production in association with changes in the numbers and pattern of cytokine-producing iNKT cells in the lung.

Further, to identify a conversion of the cytokine profile of T cells in regional lymph nodes, PBLNs were recovered from WT or IFN-$\gamma^{-/-}$ mice following OVA or OVA/αGalCer BMDC transfer and allergen challenge, and in vitro cytokine production was analyzed. As shown in Fig. 5*D*, the levels of IL-4, -5, and -13 were increased and IFN-γ was decreased in WT recipients of OVA BMDCs compared with recipients of OVA/αGalCer BMDCs. Conversely, in IFN-$\gamma^{-/-}$ recipient mice, the levels of IL-4, -5, and -13 were increased in recipients of OVA/αGalCer BMDCs.

IFN-γ plays a pivotal role in the phenotype of iNKT cells and development of allergic airway inflammation and AHR

The data suggested that IFN-γ production by recipient iNKT cells was pivotal in dictating the outcome of OVA/αGalCer BMDC transfer on the development of lung allergic responses.

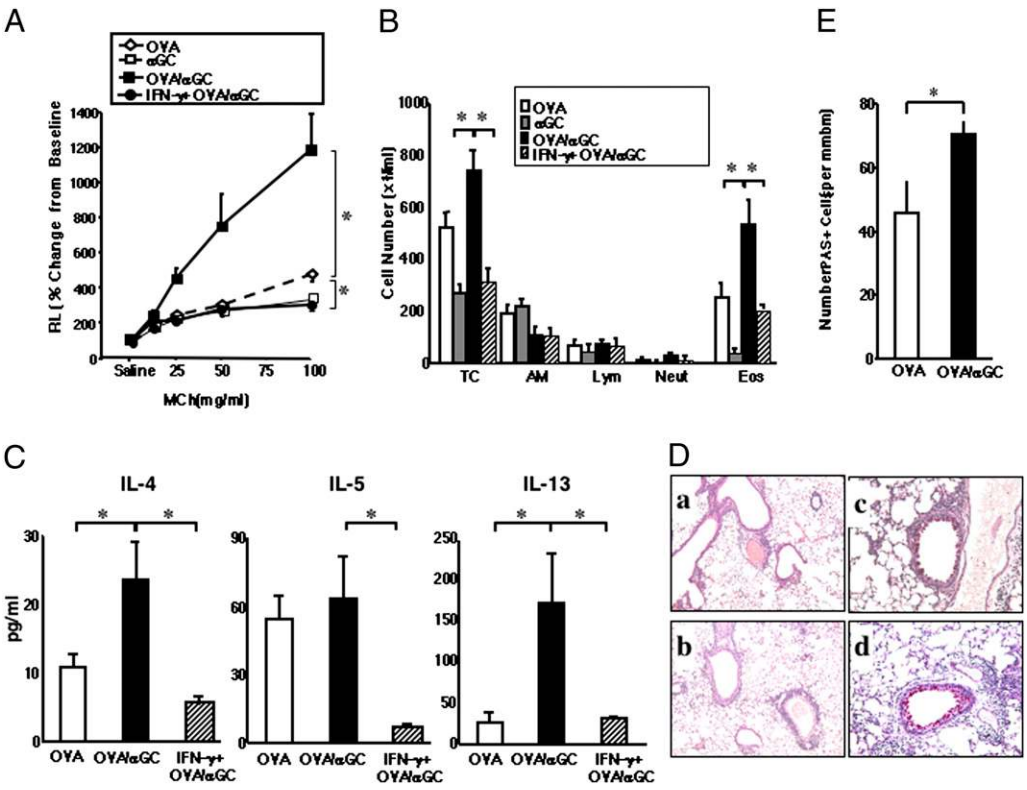

FIGURE 4. Transfer of OVA/αGalCer-pulsed BMDCs prior to OVA challenge enhances allergic airway inflammation and AHR in IFN-$\gamma^{-/-}$ recipients; exogenous IFN-γ inhibited this enhancement. *A*, Airway resistance. *B*, BAL cell composition. *C*, Cytokine levels in BAL fluid. *D*, Representative photomicrographs from IFN-$\gamma^{-/-}$ recipients of OVA-pulsed BMDCs (*a*, *c*) or OVA/αGalCer-pulsed BMDCs (*b*, *d*) (H&E, *a*, *b*; PAS, *c*, *d*). *E*, Quantitative analysis of PAS^+ cell number. Data represent mean ± SEM from three independent experiments. ($n = 12$). $*p < 0.05$, comparing IFN-$\gamma^{-/-}$ recipients of OVA/αGalCer-pulsed BMDCs versus recipients of OVA-pulsed BMDCs and OVA-pulsed BMDCs in IFN-γ recipients. αGC, BMDCs cultured with αGalCer; AM, alveolar macrophages; Eos, eosinophils; Lym, lymphocytes; OVA, BMDCs cultured with OVA; OVA/αGC, BMDCs cultured with OVA and αGalCer; IFN-γ+OVA/αGC, BMDCs cultured with OVA and αGalCer prior to exogenous IFN-γ administration; Neut, neutrophils; TC, total cell.

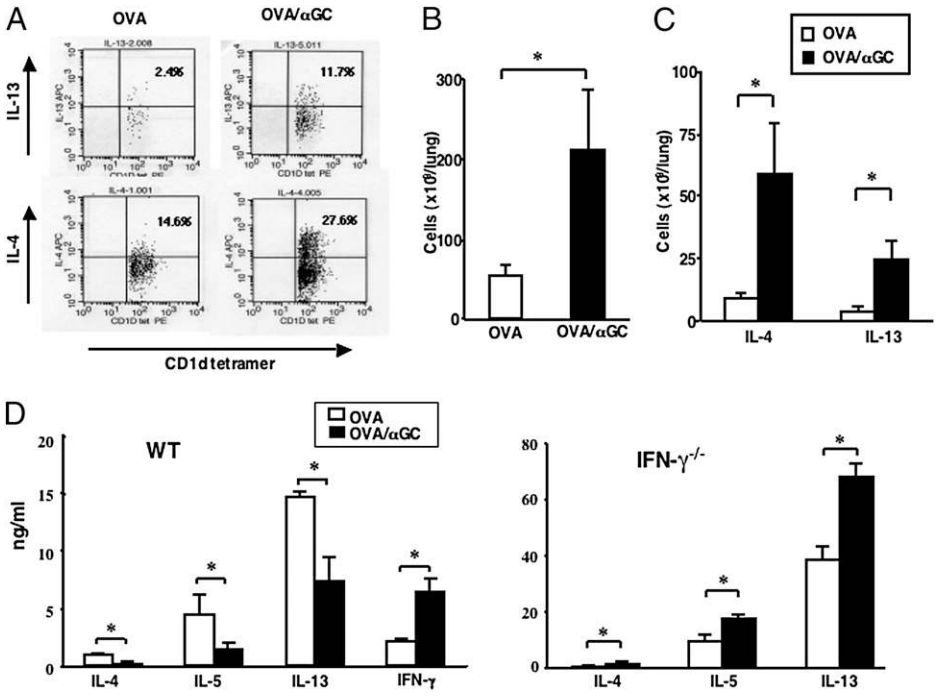

FIGURE 5. IFN-$\gamma^{-/-}$ recipients of OVA/αGalCer-pulsed BMDCs have increased numbers of iNKT cells and IL-4– and -13–producing cells in the lung. Lung MNCs were isolated and stimulated with phorbol/ionomycin, fixed, permeabilized, and stained with anti-mouse CD3, CD1d tetramer, and IL-4 or -13 Ab and quantified as described in *Materials and Methods*. *A*, $CD3^+CD1d$ tetramer$^+$ T cells were gated on and analyzed for intracellular IL-4 and -13. *B*, Numbers of $CD3^+CD1d^+$ tetramer$^+$ T cells. *C*, Numbers of $CD3^+$ CD1d tetramer$^+$ IL-4^+ or -13^+ T cells. OVA, BMDCs cultured with OVA; OVA/αGC, BMDCs cultured with OVA and αGalCer. *D*, PBLN cells from WT or IFN-$\gamma^{-/-}$ mice, which received OVA or OVA/αGC BMDC followed by allergen challenge were cultured with OVA (100 ng/ml) and supernate cytokine levels were determined. Means ± SEM from three independent experiments are shown ($n = 12$). $*p < 0.05$.

To determine whether iNKT cells represented the primary source of IFN-γ production in dictating the outcome, $CD4^+$ T cells were enriched from the liver of WT, IFN-$\gamma^{-/-}$, or J$\alpha18^{-/-}$ mice and adoptively transferred into J$\alpha18^{-/-}$ recipients before OVA or OVA/αGalCer BMDC transfer prior to OVA challenge. J$\alpha18^{-/-}$ recipients of cells purified from J$\alpha18^{-/-}$ mice did not develop AHR, and the numbers of Eos in BAL fluid were reduced after transfer of OVA BMDCs (Fig. 6). J$\alpha18^{-/-}$ mice that received WT cells followed by OVA BMDCs exhibited significantly increased AHR and airway eosinophilia. However, if these mice received OVA/αGalCer BMDCs, AHR and airway eosinophilia were markedly reduced. In contrast, J$\alpha18^{-/-}$ recipients of cells from IFN-$\gamma^{-/-}$ mice and OVA BMDCs developed levels of AHR and airway eosinophilia comparable to the WT recipients. J$\alpha18^{-/-}$ recipients of iNKT-enriched cells from IFN-$\gamma^{-/-}$ mice and OVA/αGalCer BMDCs demonstrated the highest level of AHR and the greatest number of Eos in the BAL fluid (Fig. 6). These data indicate that IFN-γ production from iNKT cells plays a pivotal role in determining the outcome of BMDC transfer in naive mice exposed to allergen challenge.

To determine the capacity for Th2 cytokine production in IFN-$\gamma^{-/-}$ iNKT cells, liver MNCs from naive IFN-$\gamma^{-/-}$ or WT mice were cultured with αGalCer BMDCs and cytokine levels were examined. As shown in Fig. 7, IFN-$\gamma^{-/-}$ iNKT cells were more capable of producing IL-13 compared with WT iNKT cells. There were no significant differences in IL-4 production levels between WT and IFN-$\gamma^{-/-}$ iNKT cells (data not shown).

Adoptive transfer of iNKT cells purified from spleen triggers allergic airway inflammation and AHR

The functions of iNKT cells may differ when obtained from different tissues with distinct effects in a tumor model (35). To determine whether iNKT cells from different tissues are capable of initiating allergic airway inflammation and AHR, we examined the activity of iNKT cells from the spleens of J$\alpha18^{-/-}$ mice. As shown in Fig. 8*A*, iNKT cells were purified to >95% and transferred into J$\alpha18^{-/-}$ mice prior to OVA BMDC transfer and OVA challenge. Mice that received spleen iNKT cells developed AHR and eosinophilic airway inflammation (Fig. 8*B*, 8*C*).

Discussion

Systemic administration of αGalCer, a specific ligand for iNKT cells, was shown to prevent the development of allergic airway inflammation and AHR under certain conditions (25, 27, 28). However, these findings could not distinguish whether the effects were manifested during the initiation phase of the response or specifically altered the subsequent airway response to allergen challenge. Because DCs are important APCs in the lung and play a critical role in the induction or the initiation phase of allergic airway inflammation and AHR (7, 8), we sought to define whether this ligand for iNKT cells could modify DC function and, in turn, iNKT cell function. To focus on the initiation phase, we showed that transfer of OVA BMDCs intratracheally could initiate the development of AHR and airway inflammation in response to OVA challenge in the absence of prior sensitization with adjuvant (10, 11). In this way, allergen-pulsed BMDCs that are exposed to a ligand for iNKT cells may be used to determine the potential role for iNKT cells in the initiation phase, prior to allergen challenge.

First, we examined whether incubation of BMDCs with αGalCer or allergen altered some of the characteristics of these cells. Although pulsing of BMDCs with OVA increased IL-6 production and levels of certain surface markers (CD80, CD86, CD40, and I-A^b), no significant differences were found in vitro when comparing the responses with the addition of OVA/αGalCer. IL-6 production from OVA BMDCs was likely induced through the small amounts of LPS contaminating the OVA preparation (36). Because IL-6 was shown to induce a polarization toward Th2 differentiation and suppression of T regulatory cell function (37), OVA BMDCs may be potent inducers of allergic airway responses.

However, when using these two populations of allergen-pulsed BMDCs, we found important differences in vivo. Intratracheal instillation of allergen-pulsed BMDCs incubated with αGalCer prevented the development of allergen-specific airway inflammation and AHR in response to allergen challenge in WT recipients. The decreases in airway responsiveness to inhaled MCh and airway eosinophilia were associated with decreases in the levels of Th2 cytokines, including IL-4, -5, and -13, in BAL fluid and goblet cell metaplasia and increases in IFN-γ levels. Recipients of OVA/

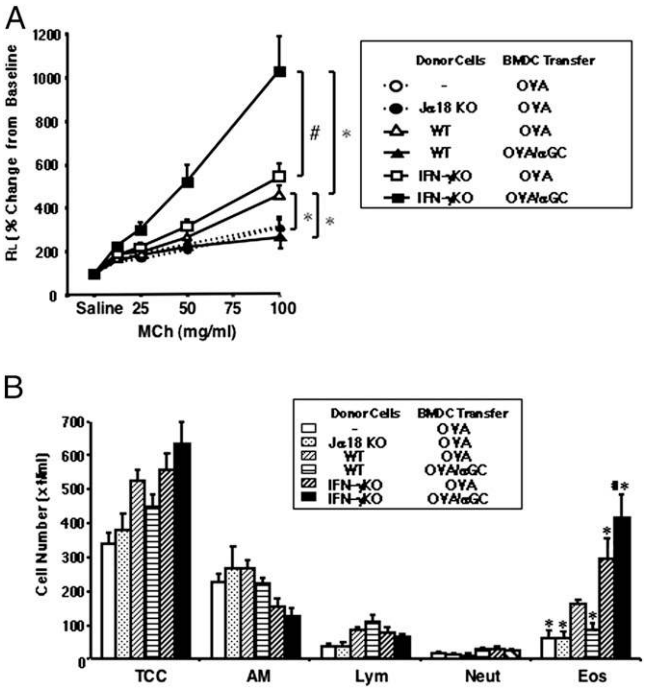

FIGURE 6. Levels of AHR and allergic airway inflammation in J$\alpha18^{-/-}$ mice are decreased after receiving iNKT cells from the livers of WT mice but are enhanced following reconstitution with iNKT cells from the livers of IFN-$\gamma^{-/-}$ mice prior to transfer of OVA/αGalCer BMDCs and OVA challenge. J$\alpha18^{-/-}$ mice received iNKT cells from J$\alpha18^{-/-}$, WT, or IFN-$\gamma^{-/-}$ mice prior to OVA or OVA/αGC BMDC transfer followed by aerosolized OVA challenge. *A*, Airway resistance. *B*, BAL cell composition. Mean ± SEM from three independent experiments are shown (n = 12). $*p < 0.05$, comparing J$\alpha18^{-/-}$ recipients of iNKT cells from WT mice and transfer of OVA BMDCs; $^{\#}p < 0.05$, comparing J$\alpha18^{-/-}$ recipients reconstituted with iNKT cells from IFN-$\gamma^{-/-}$ mice and transfer of OVA/αGalCer BMDCs and OVA BMDCs. AM, alveolar macrophages; Eos, eosinophils; IFN-γKO, IFN-γ^{-} mice; Jα18KO, J$\alpha18^{-/-}$ mice; Lym, lymphocytes; Neut, neutrophils; OVA, BMDCs pulsed with OVA; OVA/αGC, BMDCs pulsed with OVA and αGalCer; TC, total cell.

αGalCer BMDCs also demonstrated significant increases in levels of IFN-γ in PBLNs, where allergen-captured DCs migrate and present Ag to recirculating naive $CD4^+$ and $CD8^+$ cells (7, 10, 11). In the lungs of recipients of OVA/αGalCer BMDCs, the number of iNKT cells that produced IFN-γ was significantly increased compared with the numbers in recipients of OVA BMDCs.

However, in contrast to WT recipients, the transfer of OVA/αGalCer BMDCs into IFN-γ–deficient recipients prior to allergen challenge markedly augmented development of airway inflammation and AHR accompanied by increases in the levels of BAL Th2 cytokines and goblet cell metaplasia. Transfer of OVA/αGalCer BMDCs also resulted in increases in the number of lung iNKT cells that produced IL-4 and -13, as demonstrated by intracellular cytokine staining in $tetramer^+$ cells. These data indicated that activation of iNKT cells by DCs treated with αGalCer in the initiation phase played a pivotal role in the regulation of the host response to allergen challenge, and central to this outcome was whether host cells produced IFN-γ.

To further address the role of IFN-γ and iNKT cells, J$\alpha18^{-/-}$ mice, which were deficient in iNKT cells, received iNKT cells enriched from the liver of WT, IFN-$\gamma^{-/-}$, or J$\alpha18^{-/-}$ mice prior to BMDC transfer and allergen challenge. Notably, J$\alpha18^{-/-}$ mice did not develop AHR and airway inflammation following OVA-BMDC transfer and allergen challenge unless they received WT iNKT cells. Similar to WT recipients, J$\alpha18^{-/-}$ recipients showed decreased airway responses to allergen challenge following transfer of iNKT cells from WT mice prior to OVA/αGalCer BMDC transfer. However, in the J$\alpha18^{-/-}$ mice that received iNKT cells from IFN-$\gamma^{-/-}$ mice, where only the iNKT (or donor) cells were incapable of producing IFN-γ in the recipient mice, transfer of OVA/αGalCer BMDCs significantly enhanced AHR and increased the number of Eos in BAL fluid. Although the transferred cells were only enriched for iNKT cells, these findings suggest that IFN-γ production by iNKT cells can act as a "brake" on an otherwise Th2-biased response. It is unclear whether the IFN-γ produced by iNKT cells directly antagonizes the Th2 response or whether IFN-γ produced by iNKT cells acts on some undefined host cells that then block the development of a Th2 response. Fujita et al. (38) suggested that IL-27 together with IFN-γ secreted by iNKT cells played a role in the suppression of allergen-induced airway inflammation and Th2-type cytokine production. Future experiments are needed to resolve this issue.

The present study demonstrated that activation of iNKT cells prior to allergen challenge can prevent or enhance the development of allergic airway inflammation and AHR, depending on whether iNKT cells can produce IFN-γ. These results share some features with previous studies indicating that activation of iNKT cells in the initial phase was critical to the development of allergic airway inflammation and AHR (39, 40). Kim et al. (39) demonstrated that αGalCer, coadministered intranasally with OVA on three consecutive days, led to the development of AHR and airway inflammation, whereas OVA priming alone did not result in airway inflammation and AHR. Bilenki et al. (40) showed that in vivo stimulation of NKT cells by systemic administration of αGalCer in the initial phase enhanced ragweed-induced airway eosinophilia. Because they administered αGalCer i.v., iNKT cells were likely activated systemically, whereas in the current study, activation was likely restricted to lung iNKT cells as a result of the intratracheal administration of αGalCer-treated DCs. Unlike the report of Meyer et al. (41), which showed that intranasal instillation of αGalCer enhanced AHR and airway eosinophilia, we were unable to alter these responses in WT or IFN-$\gamma^{-/-}$ recipients of αGalCer-treated DCs.

Some of the inconsistencies among the various studies may be related to the number of treatments with αGalCer and/or the mode of delivery (systemic versus local). Recent experiments demonstrated that iNKT cells with different cytokine-secretion capacity seemed to segregate in a tissue-specific manner. In a tumor model, Crowe et al. (35) compared NKT cells from liver, spleen, and thymus for their ability to mediate rejection of a sarcoma cell line in vivo and showed that only liver-derived NKT cells could prevent tumor growth. They concluded that iNKT cells exist in functionally distinct subpopulations among different tissues. In our model, we demonstrated that iNKT cells from at least two organs showed similar function; iNKT purified from spleen played a role in the initiation phase of the development of AHR and allergic inflammation similar to that of iNKT cells isolated from liver.

αGalCer-primed mice re-exposed to the same Ag in vivo retained the ability to produce systemic IL-4 rapidly, whereas IFN-γ could not be detected in the serum (29). Earlier priming with αGalCer enhanced systemic cytokine secretion, especially serum levels of IL-4 by 17-fold, 4 h after injection compared with naive mice (42). A number of reports demonstrated that repeated administration of αGalCer favors Th2 activation, skewing responses to IL-4 production rather than IFN-γ (29, 43–45). However, it is unclear how this Th2 polarization is achieved; it was reported that a single injection of αGalCer while first stimulating iNKT cells led to a state of unresponsiveness upon

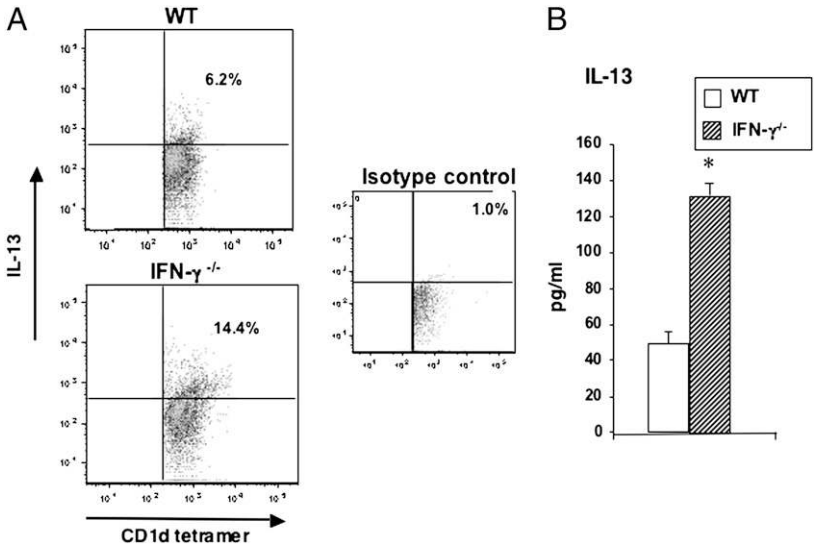

FIGURE 7. iNKT cells from the livers of IFN-$\gamma^{-/-}$ mice produce higher levels of IL-13 compared with WT iNKT cells. Liver MNCs from naive IFN-$\gamma^{-/-}$ or WT mice were isolated and stimulated with αGalCer-pulsed BMDCs for 24 h. IL-13 production in the cytoplasm of iNKT cells or culture supernatants was determined. *A*, Representative scattergram with IL-13 cytoplasmic staining in $CD3^+CD1d$ tetramer$^+$ cells. *B*, IL-13 levels in supernatants from culture of naive liver MNCs with αGalCer-pulsed BMDCs. $*p <$ 0.05, comparing IL-13 production from IFN-$\gamma^{-/-}$ and WT iNKT cells.

rechallenge with this Ag (45). In the current study, iNKT cells were stimulated in vivo by αGalCer-pulsed DCs, and these DCs are known to induce prolonged IFN-γ–producing NKT cell responses (46). As shown in this study, upregulation of IFN-γ, but not IL-4, was associated with inhibition of eosinophilic airway inflammation, AHR, and Th2 responses.

It is of interest that OVA/αGalCer BMDCs inhibited the development of lung allergic responses in WT recipients, but these same cells augmented allergic inflammation and AHR in IFN-$\gamma^{-/-}$ recipients. This suggested that the effect of αGalCer in IFN-$\gamma^{-/-}$ mice may be the result of activation of a default pathway of cytokine production by activated (recipient) iNKT cells. In WT

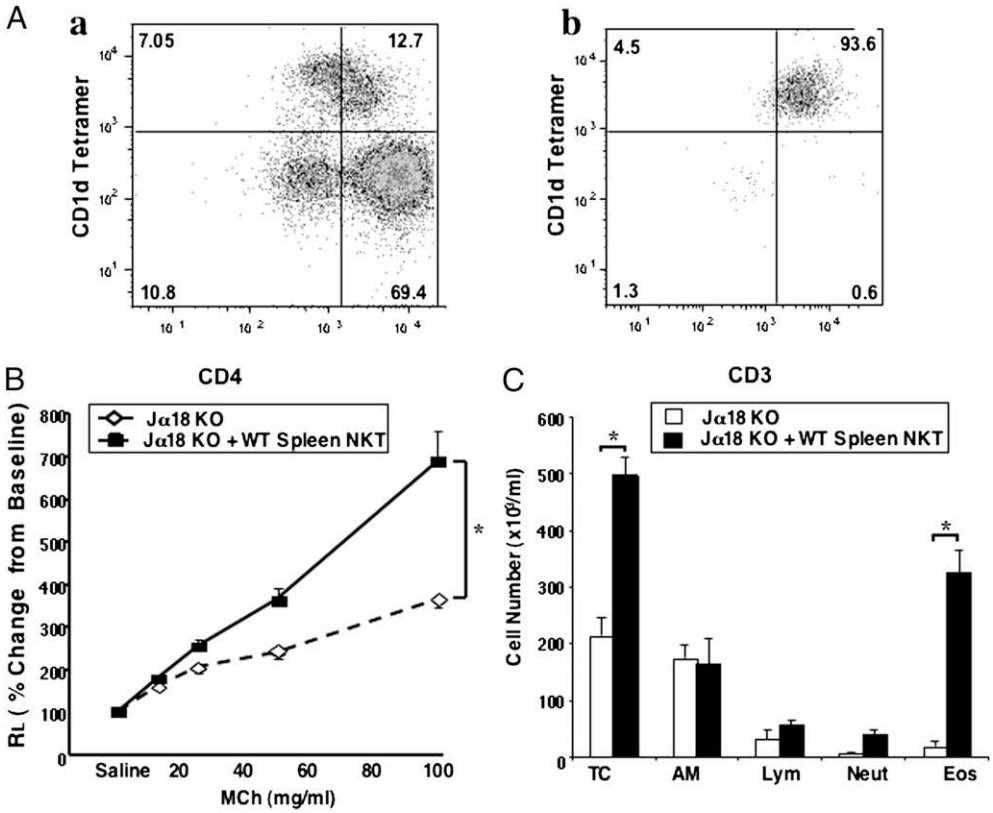

FIGURE 8. Jα18$^{-/-}$ recipients of spleen iNKT cells from WT mice prior to OVA-pulsed BMDC transfer and OVA challenge developed allergic airway inflammation and AHR. *A*, WT spleen iNKT cells were purified using three steps: initially, $CD4^+$ T cells were enriched following negative selection. *Aa*, These cells were stained with CD1d-PE tetramer and isolated by magnetic bead sorting. *Ab*, The $CD1d^+$ cells were further purified by cell sorting. Purity of the cell suspensions was analyzed using the Accuri (C6, Ann Arbor, MI) flow cytometer. Purified iNKT cells were transferred into Jα18$^{-/-}$ mice followed by OVA-pulsed BMDCs and allergen challenge. *B*, Airway resistance. *C*, BAL cell composition. Data represent mean ± SEM (n = 12). $*p < 0.05$, comparing Jα18$^{-/-}$ mice treated with PBS prior to OVA-pulsed BMDCs and OVA challenge versus Jα18$^{-/-}$ recipients of purified spleen iNKT cells prior to OVA-pulsed BMDCs and OVA. AM, alveolar macrophages; Eos, eosinophils; Jα18KO, Jα18$^{-/-}$ mice receiving PBS prior to OVA-pulsed BMDCs; Jα18KO+WT spleen iNKT, Jα18$^{-/-}$ mice receiving spleen iNKT cells prior to OVA-pulsed BMDCs; Lym, lymphocytes; Neut, neutrophils; TC, total cell.

mice, iNKT cells activated by αGalCer-pulsed DCs preferentially produced IFN-γ rather than IL-4. In IFN-γ–deficient mice, these same DCs stimulated Th2 cytokine production in the iNKT cells. The effects of iNKT cells on the allergic phenotype may be direct (22) but more likely are indirect, modulating the activity of other cells. IFN-γ production from iNKT cells can affect bystander cells, such as NK cells, $CD4^+$ T cells, and $CD8^+$ T cells (29, 47–49), and inhibit the development of Th2 responses, AHR, and eosinophilic airway inflammation (28). iNKT cells from IFN-$\gamma^{-/-}$ mice, while failing to produce IFN-γ did produce IL-4 and -13. IL-4 from iNKT cells can prime several cell types (50–52), resulting in an upregulation of Th2 responses, IL-13 production, and the enhancement of AHR and airway inflammation.

The hygiene hypothesis suggests that early-life environmental exposure to microbes or other pathogens and their products promotes innate immune responses that protect against the development of atopy and asthma (53). Many microorganisms have the ability to indirectly activate iNKT cells during infection (14, 54), and some microbial glycolipid Ags were shown to directly activate iNKT cells (55–60). Thus, IFN-γ plays a critical role in determining the consequences of activated iNKT cells in the development of airway inflammation and AHR. As a result, early exposure of IFN-γ–sufficient hosts to microorganisms or pathogen- or microbe-associated products activates iNKT cells and preferentially induces IFN-γ production, protecting against the development of atopy and asthma. However, in individuals who have a lower capacity for IFN-γ production, potentially on a genetic basis or under certain conditions, the activation of iNKT cells might induce the production of IL-4 and -13 and enhance the development of atopy and allergic responses. This concept gains support from findings in infants at genetic risk for developing atopy who have weaker neonatal IFN-γ responses compared with low-risk infants (61), perhaps because of differential patterns of methylation of the IFN-γ promoter (62). It is under such conditions that iNKT cell activation may play a significant role in directing T cell differentiation and Th2 polarization, increasing the risk for developing atopy and asthma.

Acknowledgments

We thank Diana Nabighian for expert help in preparing the manuscript and Lynn Cunningham for performing the immunolabeling studies.

Disclosures

The authors have no financial conflicts of interest.

References

1. Handoyo, S., and L. J. Rosenwasser. 2009. Asthma phenotypes. *Curr. Allergy Asthma Rep.* 9: 439–445.
2. Long, A. A. 2009. Immunomodulators in the treatment of asthma. *Allergy Asthma Proc.* 30: 109–119.
3. Pouliot, P., and M. Olivier. 2009. Opposing forces in asthma: regulation of signaling pathways by kinases and phosphatases. *Crit. Rev. Immunol.* 29: 419–442.
4. O'Byrne, P. M. 2006. Cytokines or their antagonists for the treatment of asthma. *Chest* 130: 244–250.
5. Gelfand, E. W., and A. Dakhama. 2006. CD8+ T lymphocytes and leukotriene B4: novel interactions in the persistence and progression of asthma. *J. Allergy Clin. Immunol.* 117: 577–582.
6. Ngoc, P. L., L. P. Ngoc, D. R. Gold, A. O. Tzianabos, S. T. Weiss, and J. C. Celedón. 2005. Cytokines, allergy, and asthma. *Curr. Opin. Allergy Clin. Immunol.* 5: 161–166.
7. Lambrecht, B. N., and H. Hammad. 2009. Biology of lung dendritic cells at the origin of asthma. *Immunity* 31: 412–424.
8. Grunig, G., A. Banz, and R. de Waal Malefyt. 2005. Molecular regulation of Th2 immunity by dendritic cells. *Pharmacol. Ther.* 106: 75–96.
9. Robays, L. J., T. Maes, G. F. Joos, and K. Y. Vermaelen. 2009. Between a cough and a wheeze: dendritic cells at the nexus of tobacco smoke-induced allergic airway sensitization. *Mucosal Immunol.* 2: 206–219.
10. Miyahara, N., H. Ohnishi, H. Matsuda, S. Miyahara, K. Takeda, T. Koya, S. Matsubara, M. Okamoto, A. Dakhama, B. Haribabu, and E. W. Gelfand. 2008. Leukotriene B4 receptor 1 expression on dendritic cells is required for the development of Th2 responses and allergen-induced airway hyperresponsiveness. *J. Immunol.* 181: 1170–1178.
11. Koya, T., H. Matsuda, S. Matsubara, N. Miyahara, A. Dakhama, K. Takeda, and E. W. Gelfand. 2009. Differential effects of dendritic cell transfer on airway hyperresponsiveness and inflammation. *Am. J. Respir. Cell Mol. Biol.* 41: 271–280.
12. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu. Rev. Immunol.* 25: 297–336.
13. Cerundolo, V., J. D. Silk, S. H. Masri, and M. Salio. 2009. Harnessing invariant NKT cells in vaccination strategies. *Nat. Rev. Immunol.* 9: 28–38.
14. Boyton, R. 2008. The role of natural killer T cells in lung inflammation. *J. Pathol.* 214: 276–282.
15. Matangkasombut, P., M. Pichavant, R. H. Dekruyff, and D. T. Umetsu. 2009. Natural killer T cells and the regulation of asthma. *Mucosal Immunol.* 2: 383–392.
16. Akbari, O., J. L. Faul, E. G. Hoyte, G. J. Berry, J. Wahlström, M. Kronenberg, R. H. DeKruyff, and D. T. Umetsu. 2006. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N. Engl. J. Med.* 354: 1117–1129.
17. Vijayanand, P., G. Seumois, C. Pickard, R. M. Powell, G. Angco, D. Sammut, S. D. Gadola, P. S. Friedmann, and R. Djukanovic. 2007. Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. *N. Engl. J. Med.* 356: 1410–1422.
18. Thomas, S. Y., C. M. Lilly, and A. D. Luster. 2006. Invariant natural killer T cells in bronchial asthma. *N. Engl. J. Med.* 354: 2613–2616, author reply 2613–2616.
19. Pham-Thi, N., J. de Blic, M. Le Bourgeois, M. Dy, P. Scheinmann, and M. C. Leite-de-Moraes. 2006. Enhanced frequency of immunoregulatory invariant natural killer T cells in the airways of children with asthma. *J. Allergy Clin. Immunol.* 117: 217–218.
20. Matangkasombut, P., G. Marigowda, A. Ervine, L. Idris, M. Pichavant, H. Y. Kim, T. Yasumi, S. B. Wilson, R. H. DeKruyff, J. L. Faul, et al. 2009. Natural killer T cells in the lungs of patients with asthma. *J. Allergy Clin. Immunol.* 123: 1181–1185.
21. Reynolds, C., J. Barkans, P. Clark, H. Kariyawasam, D. Altmann, B. Kay, and R. Boyton. 2009. Natural killer T cells in bronchial biopsies from human allergen challenge model of allergic asthma. *J. Allergy Clin. Immunol.* 124: 860–862, author reply 862.
22. Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, T. Nakayama, M. Taniguchi, M. J. Grusby, R. H. DeKruyff, and D. T. Umetsu. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* 9: 582–588.
23. Lisbonne, M., S. Diem, A. de Castro Keller, J. Lefort, L. M. Araujo, P. Hachem, J. M. Fourneau, S. Sidobre, M. Kronenberg, M. Taniguchi, et al. 2003. Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *J. Immunol.* 171: 1637–1641.
24. Korsgren, M., C. G. Persson, F. Sundler, T. Bjerke, T. Hansson, B. J. Chambers, S. Hong, L. Van Kaer, H. G. Ljunggren, and O. Korsgren. 1999. Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice. *J. Exp. Med.* 189: 553–562.
25. Morishima, Y., Y. Ishii, T. Kimura, A. Shibuya, K. Shibuya, A. E. Hegab, T. Iizuka, T. Kiwamoto, Y. Matsuno, T. Sakamoto, et al. 2005. Suppression of eosinophilic airway inflammation by treatment with alpha-galactosylceramide. *Eur. J. Immunol.* 35: 2803–2814.
26. Das, J., P. Eynott, R. Jupp, A. Bothwell, L. Van Kaer, Y. Shi, and G. Das. 2006. Natural killer T cells and CD8+ T cells are dispensable for T cell-dependent allergic airway inflammation. *Nat. Med.* 12: 1345–1346, author reply 1347.
27. Matsuda, H., T. Suda, J. Sato, T. Nagata, Y. Koide, K. Chida, and H. Nakamura. 2005. alpha-Galactosylceramide, a ligand of natural killer T cells, inhibits allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 33: 22–31.
28. Hachem, P., M. Lisbonne, M. L. Michel, S. Diem, S. Roongapinun, J. Lefort, G. Marchal, A. Herbelin, P. W. Askenase, M. Dy, and M. C. Leite-de-Moraes. 2005. Alpha-galactosylceramide-induced iNKT cells suppress experimental allergic asthma in sensitized mice: role of IFN-gamma. *Eur. J. Immunol.* 35: 2793–2802.
29. Matsuda, J. L., L. Gapin, J. L. Baron, S. Sidobre, D. B. Stetson, M. Mohrs, R. M. Locksley, and M. Kronenberg. 2003. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc. Natl. Acad. Sci. USA* 100: 8395–8400.
30. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693–1702.
31. Takeda, K., E. Hamelmann, A. Joetham, L. D. Shultz, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J. Exp. Med.* 186: 449–454.
32. Koya, T., T. Kodama, K. Takeda, N. Miyahara, E. S. Yang, C. Taube, A. Joetham, J. W. Park, A. Dakhama, and E. W. Gelfand. 2006. Importance of myeloid dendritic cells in persistent airway disease after repeated allergen exposure. *Am. J. Respir. Crit. Care Med.* 173: 42–55.
33. Miyahara, N., K. Takeda, T. Kodama, A. Joetham, C. Taube, J. W. Park, S. Miyahara, A. Balhorn, A. Dakhama, and E. W. Gelfand. 2004. Contribution of antigen-primed CD8+ T cells to the development of airway hyperresponsiveness and inflammation is associated with IL-13. *J. Immunol.* 172: 2549–2558.
34. Taube, C., C. Duez, Z. H. Cui, K. Takeda, Y. H. Rha, J. W. Park, A. Balhorn, D. D. Donaldson, A. Dakhama, and E. W. Gelfand. 2002. The role of IL-13 in established allergic airway disease. *J. Immunol.* 169: 6482–6489.
35. Crowe, N. Y., J. M. Coquet, S. P. Berzins, K. Kyparissoudis, R. Keating, D. G. Pellicci, Y. Hayakawa, D. I. Godfrey, and M. J. Smyth. 2005. Differential

antitumor immunity mediated by NKT cell subsets in vivo. *J. Exp. Med.* 202: 1279–1288.
36. Watanabe, J., Y. Miyazaki, G. A. Zimmerman, K. H. Albertine, and T. M. McIntyre. 2003. Endotoxin contamination of ovalbumin suppresses murine immunologic responses and development of airway hyper-reactivity. *J. Biol. Chem.* 278: 42361–42368.
37. Doganci, A., K. Sauer, R. Karwot, and S. Finotto. 2005. Pathological role of IL-6 in the experimental allergic bronchial asthma in mice. *Clin. Rev. Allergy Immunol.* 28: 257–270.
38. Fujita, H., A. Teng, R. Nozawa, Y. Takamoto-Matsui, H. Katagiri-Matsumura, Z. Ikezawa, and Y. Ishii. 2009. Production of both IL-27 and IFN-gamma after the treatment with a ligand for invariant NK T cells is responsible for the suppression of Th2 response and allergic inflammation in a mouse experimental asthma model. *J. Immunol.* 183: 254–260.
39. Kim, J. O., D. H. Kim, W. S. Chang, C. Hong, S. H. Park, S. Kim, and C. Y. Kang. 2004. Asthma is induced by intranasal coadministration of allergen and natural killer T-cell ligand in a mouse model. *J. Allergy Clin. Immunol.* 114: 1332–1338.
40. Bilenki, L., J. Yang, Y. Fan, S. Wang, and X. Yang. 2004. Natural killer T cells contribute to airway eosinophilic inflammation induced by ragweed through enhanced IL-4 and eotaxin production. *Eur. J. Immunol.* 34: 345–354.
41. Meyer, E. H., S. Goya, O. Akbari, G. J. Berry, P. B. Savage, M. Kronenberg, T. Nakayama, R. H. DeKruyff, and D. T. Umetsu. 2006. Glycolipid activation of invariant T cell receptor+ NK T cells is sufficient to induce airway hyperreactivity independent of conventional CD4+ T cells. *Proc. Natl. Acad. Sci. USA* 103: 2782–2787.
42. Ikarashi, Y., A. Iizuka, Y. Koshidaka, Y. Heike, Y. Takaue, M. Yoshida, M. Kronenberg, and H. Wakasugi. 2005. Phenotypical and functional alterations during the expansion phase of invariant Valpha14 natural killer T (Valpha14i NKT) cells in mice primed with alpha-galactosylceramide. *Immunology* 116: 30–37.
43. Singh, N., S. Hong, D. C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 1999. Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J. Immunol.* 163: 2373–2377.
44. Burdin, N., L. Brossay, and M. Kronenberg. 1999. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur. J. Immunol.* 29: 2014–2025.
45. Parekh, V. V., M. T. Wilson, D. Olivares-Villagómez, A. K. Singh, L. Wu, C.-R. Wang, S. Joyce, and L. Van Kaer. 2005. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J. Clin. Invest.* 115: 2572–2583.
46. Fujii, S., K. Shimizu, M. Kronenberg, and R. M. Steinman. 2002. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat. Immunol.* 3: 867–874.
47. Fujii, S., K. Shimizu, C. Smith, L. Bonifaz, and R. M. Steinman. 2003. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J. Exp. Med.* 198: 267–279.
48. Hermans, I. F., J. D. Silk, U. Gileadi, M. Salio, B. Mathew, G. Ritter, R. Schmidt, A. L. Harris, L. Old, and V. Cerundolo. 2003. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J. Immunol.* 171: 5140–5147.
49. Smyth, M. J., M. E. Wallace, S. L. Nutt, H. Yagita, D. I. Godfrey, and Y. Hayakawa. 2005. Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer. *J. Exp. Med.* 201: 1973–1985.
50. Singh, A. K., J. Q. Yang, V. V. Parekh, J. Wei, C. R. Wang, S. Joyce, R. R. Singh, and L. Van Kaer. 2005. The natural killer T cell ligand alpha-galactosylceramide prevents or promotes pristane-induced lupus in mice. *Eur. J. Immunol.* 35: 1143–1154.
51. Hashimoto, D., S. Asakura, S. Miyake, T. Yamamura, L. Van Kaer, C. Liu, M. Tanimoto, and T. Teshima. 2005. Stimulation of host NKT cells by synthetic glycolipid regulates acute graft-versus-host disease by inducing Th2 polarization of donor T cells. *J. Immunol.* 174: 551–556.
52. Miyamoto, K., S. Miyake, and T. Yamamura. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* 413: 531–534.
53. Racila, D. M., and J. N. Kline. 2005. Perspectives in asthma: molecular use of microbial products in asthma prevention and treatment. *J. Allergy Clin. Immunol.* 116: 1202–1205.
54. Brigl, M., L. Bry, S. C. Kent, J. E. Gumperz, and M. B. Brenner. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat. Immunol.* 4: 1230–1237.
55. Amprey, J. L., J. S. Im, S. J. Turco, H. W. Murray, P. A. Illarionov, G. S. Besra, S. A. Porcelli, and G. F. Späth. 2004. A subset of liver NK T cells is activated during *Leishmania donovani* infection by CD1d-bound lipophosphoglycan. *J. Exp. Med.* 200: 895–904.
56. Fischer, K., E. Scotet, M. Niemeyer, H. Koebernick, J. Zerrahn, S. Maillet, R. Hurwitz, M. Kursar, M. Bonneville, S. H. Kaufmann, and U. E. Schaible. 2004. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc. Natl. Acad. Sci. USA* 101: 10685–10690.
57. Mattner, J., K. L. Debord, N. Ismail, R. D. Goff, C. Cantu, 3rd, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, et al. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434: 525–529.
58. Kinjo, Y., D. Wu, G. Kim, G. W. Xing, M. A. Poles, D. D. Ho, M. Tsuji, K. Kawahara, C. H. Wong, and M. Kronenberg. 2005. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 434: 520–525.
59. Sriram, V., W. Du, J. Gervay-Hague, and R. R. Brutkiewicz. 2005. Cell wall glycosphingolipids of *Sphingomonas paucimobilis* are CD1d-specific ligands for NKT cells. *Eur. J. Immunol.* 35: 1692–1701.
60. Kinjo, Y., E. Tupin, D. Wu, M. Fujio, R. Garcia-Navarro, M. R. Benhnia, D. M. Zajonc, G. Ben-Menachem, G. D. Ainge, G. F. Painter, et al. 2006. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat. Immunol.* 7: 978–986.
61. Prescott, S. L., B. King, T. L. Strong, and P. G. Holt. 2003. The value of perinatal immune responses in predicting allergic disease at 6 years of age. *Allergy* 58: 1187–1194.
62. White, G. P., P. M. Watt, B. J. Holt, and P. G. Holt. 2002. Differential patterns of methylation of the IFN-γ promoter at CpG and non-CpG sites underlie differences in IFN-γ gene expression between human neonatal and adult CD45RO-T cells. *J. Immunol.* 168: 2820–2827.