CD1d-Restricted IFN-γ–Secreting NKT Cells Promote Immune Complex-Induced Acute Lung Injury by Regulating Macrophage-Inflammatory Protein-1 α Production and Activation of Macrophages and Dendritic Cells

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CD1d-Restricted IFN-γ–Secreting NKT Cells Promote Immune Complex-Induced Acute Lung Injury by Regulating Macrophage-Inflammatory Protein-1α Production and Activation of Macrophages and Dendritic Cells

Ji Hyung Kim and Doo Hyun Chung

Immune complex-induced acute lung injury (IC-ALI) has been implicated in various pulmonary disease states. However, the role of NKT cells in IC-ALI remains unknown. Therefore, we explored NKT cell functions in IC-ALI using chicken egg albumin and anti-chicken egg albumin IgG. The bronchoalveolar lavage fluid of CD1d−/− and Jα18−/− mice contained few Ly6G+CD11b+ granulocytes, whereas levels in B6 mice were greater and were increased further by α-galactosyl ceramide. IFN-γ and MIP-1α production in the lungs was greater in B6 than CD1d−/− mice. Adaptive transfer of wild type (WT) but not IFN-γ−/−, MIP-1α−/−, or FcγR-deficient NKT cells into CD1d−/− mice caused recruitment of inflammatory cells to the lungs. Moreover, adoptive transfer of IFN-γR-deficient NKT cells enhanced MIP-1α production and cell recruitment in the lungs of CD1d−/− or CD1d−/− IFN-γ−/− mice, but to a lesser extent than WT NKT cells. This suggests that IFN-γ–producing NKT cells enhance MIP-1α production in both an autocrine and a paracrine manner. IFN-γ-deficient NKT cells induced less IL-1β and TNF-α production by alveolar macrophages and dendritic cells in CD1d−/− mice than did WT NKT cells. Taken together, these data suggest that CD1d-restricted IFN-γ–producing NKT cells promote IC-ALI by producing MIP-1α and enhancing proinflammatory cytokine production by alveolar macrophages and dendritic cells.

1α in both an autocrine and paracrine manner, and enhancing cyto-
kine production by alveolar macrophages and dendritic cells (DCs).

Materials and Methods

Mice
C57BL/6 (B6) mice were purchased from Orient Company Ltd (Seoul, Korea) at 8–10 wk of age. CD1d−/− and Jo18 (C57BL/6 background) mice were generously provided by Dr. L. Van Kaer (Vanderbilt University, Nashville, TN) and M. Taniguchi (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan), respectively. IFN-γ−/−, IFN-γR−/−, and MIP-1α−/− mice in a B6 background were purchased from The Jackson Laboratory. The FcerIγ−/− (FcγR−/−) mice in a B6 background were purchased from the Taconic Company. CD1d−/− IFN-γ−/− mice were generated by crossing CD1d−/− and IFN-γ−/− mice. Mice aged 8 to 12 wk were used in all experiments. Mice were bred and maintained under spe-
cific pathogen-free conditions in the Clinical Research Institute of Seoul National University Hospital (Seoul, Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute of Seoul National University Hospital.

Experimental model of IC-ALI
Mice were anesthetized with 2,2,2-trichloromethanol, and 150 μg rabbit anti-CEA IgG Abs (Sigma-Aldrich, Munich, Germany) was injected via cannulated tracheas. Then 5 to 10 min later, CEA (20 mg/kg; Sigma-Aldrich, St. Louis, MO) was administered i.v. For histological examina-
tion, 225 μg α-galactosyl ceramide (α-GalCer; 1 μg), anti–MIP-1α mAbs (250 μg), or isotype control mAbs (250 μg) were injected i.v. 4 h or 1 d prior to injection of CEA. α-GalCer was provided by Dr. Sanghiee Kim (College of Pharmacy, Seoul National University, Seoul, Korea).

Flow cytometry
Mice were sacrificed at appropriate time points (4, 8, 12, 24, 36, and 48 h) after induction of IC-ALI. Bronchoalveolar lavage was performed five times with 700 μl PBS at 4°C. Mononuclear cells were isolated from whole-lung homogenates using Lymphocytes M (CEDALANE, Hornby, Ontario, Canada). Total bronchoalveolar lavage fluid (BALF) and mononuclear cell counts in whole lungs were determined using a hemocytometer, and the cells were incubated on ice with anti-FcγRIII/II mAb (BD Bioscience, San Jose, CA) for 15 min. After washing, the cells were stained with a combina-
tion of the following mAbs (1 μg/200 μl PBS): anti–TCR-β, anti-NK1.1, anti-Gr1, anti-CD11b, anti-F4/80, anti-MHC class II (I-A β), anti-CD11c, and c-kit mAbs (BD Bioscience or eBioscience, San Diego, CA). For in-
tracellular cytokine staining, BALF cells were cultured for 4 h in 24-well plates (105 cells/well) with RPMI 1640 (1 ml; WelGENE, Seoul, Korea) containing concanavalin A (Sigma-Aldrich, St. Louis, MO) and Golgi inhibitor (BD Bioscience). Cells were then spun down, incubated with mAb for surface labeling, and stained for intracellular cytokines using the Cytotox/Cytoperm kit (BD Bioscience) according to the manufacturer’s instructions. Flow cytometric analysis was performed using a FACSCalibur instrument (Becton Dickinson, San Jose, CA), and the data were analyzed using the Cell-
Quest software (Becton Dickinson).

ELISA
IFN-γ, IL-4, IL-1β, TNF-α, MIP-1α, IL-12, and IL-6 levels in BALF were measured using ELISA cytosets (BD Bioscience or R&D Systems [Min-
neapolis, MN] for MIP-1α). To measure C5a and IL-13, we used purified anti-C5a (BD Bioscience) and IL-13 (R&D Systems) mAbs for capture, and applied biotinylated anti-C5a (BD Bioscience) or IL-13 (R&D Sys-
tems) mAbs for detection. Standard curves were generated using known amounts of purified murine recombinant C5a and IL-13 (R&D Systems). The reaction was stopped with HCl (3N), and absorbance at 450 nm and 570 nm were determined using a spectrophotometer.

Real-time PCR
For quantitative real-time PCR, total RNA was isolated from lung homo-
genates using an RNasey kit (Qiagen, Valencia, CA) according to the man-
ufacturer’s instructions. RNA was reverse transcribed using Moloney murine leukemia virus-RT Taq polymerase (Koschem, Seoul, Korea) prior to PCR. All reagents including primers and probes used in our experiments were purchased from Applied Biosystems (Foster City, CA). cDNA was amplified using TaqMan universal master mix, gene-specific probes/primer, and water. PCR product concentrations were determined using an Applied Bio-
systems 7500 Sequence Detection System, and results were normalized to GAPDH expression.

Measurement of myeloperoxidase levels
Homogenized lung tissues were suspended in potassium phosphate buffer (50 mM; pH 6.0) and hexadecyltrimethyl ammonium bromide (0.5%); Sigma-Aldrich, St. Louis, MO), subjected to three freeze/thaw cycles, and sonicated, to measure myeloperoxidase (MPO) levels. α-Diadosidase dihyd-
rochloride (0.167 mg/ml; Sigma-Aldrich, St. Louis, MO) and hydrogen peroxide (0.005%); Sigma-Aldrich) were then added to the supernatant, and the change in OD at λ = 450 nm was determined. MPO from human polymorphonuclear neutrophils (Calbiochem, Bad Soden, Germany) was used as a standard.

Adoptive transfer
Mouse livers were homogenized, resuspended in DMEM (20 ml; 2.5% PBS, 1% Alsever’s solution; Sigma), and then mixed with 10 ml Percoll (GE Healthcare, Uppsala, Sweden). After centrifugation for 20 min at 2000 rpm and 25°C, mononuclear cells were isolated from pellets, washed in PBS, and incubated in RBC lysis solution (4 ml; Qiagen, Valencia, CA) for 10 min at room temperature. To sort NKT cells, we stained hepatic mononuclear cells with PE-conjugated anti-NK1.1 (BD Bioscience) and Cy-
Chrom-conjugated anti-TCR-β mAbs (BD Bioscience, San Jose, CA). The stained cells were then sorted using a BD FACSaria (Franklin Lakes, NJ) to a purity of >98%. In all experiments, sorted NKT cells (3 × 105) were adoptively transferred via i.v. injection 1 d prior to CEA injection.

Migration assay
Sorted T or NKT cells (2 × 105/well) were incubated with aggregated IgGs (10 μg/ml), anti-CD3 mAbs (100 ng/ml; BD Bioscience), or both. After 48 h, supernatants were placed in the bottom chamber of a Costar plate (5-μm pore size membrane; Corning Incorporated Costar Transwell). BALF cells were removed from B6 mice 24 h after CEA injection and placed in the upper chamber (1 × 105/well). The anti–MIP-1α-neutralizing mAbs (1 μg/ml) or isotype control mAbs were added as appropriate. After 12-h incubation, the cells in both chambers were removed, spun down, counted, and stained with PE-conjugated anti-CD11b and FITC-conjugated anti-Ly6G mAbs. Migration was calculated by determining the proportion of Ly6G−CD11b+ cells in the bottom chamber.

Histological examination
Mice were euthanized and perfused with buffered (1 ml; pH 7.2) formalin (10%) via the right vena cava, and fixed for 72 h. Whole lungs were paraffin embedded using standard histological techniques, sectioned, and stained with H&E.

Statistical analysis
Statistical significance was analyzed using the Prism 3.0 program (Graph-
Pad Software). Student t test was used to determine p values when comparing two groups. The p values <0.05 were considered significant. A one-
way ANOVA test was performed, and the post test was used if overall p < 0.05. Data are expressed as mean ± SEM.

Results

CD1d-restricted NKT cells promote IC-ALI
To investigate whether NKT cells play a critical role in IC-
ALI, we introduced anti-CEA IgG intratracheally into B6 and CD1d−/− mice, which were injected i.v. 10 min later with CEA.

To evaluate IC-ALI, we estimated the numbers of total cells and Ly6G−CD11b+ granulocytes in BALF 0, 4, 8, 12, 24, 36, and 48 h after injection of CEA. In B6 mice, the numbers of total cells and Ly6G−CD11b+ granulocytes in BALF increased minimal-
ly from 0 to 12 h after CEA injection but increased abruptly at 24 h, followed by a decrease after 36 h (Fig. 1A). In contrast, CD1d−/− mice showed minimal infiltration of cells, including Ly6G−CD11b+ granulocytes, in BALF from 0 to 48 h after CEA injection. Subset analysis performed 24 h after CEA injection revealed that the numbers of macrophages, NK cells, Gr-1+ granulocytes, MHC class II−CD11c− DCs, and T cells in BALF of B6 were greater than those of CD1d−/− mice during IC-ALI (Fig. 1A). Consistent with cell infiltration in the lungs during IC-ALI, microscopic examination revealed that pathological
changes (congestion, alveolar hemorrhage, and inflammation) in the lungs were lower in CD1d
$^{-/-}$ mice, although pathological alteration was not even in the lungs (Fig. 1B). Moreover, total cell and Ly6G$^{+}$CD11b$^{+}$ granulocyte counts, and MPO levels in the lungs of CD1d
$^{-/-}$ mice were similar to those of Jx18
$^{-/-}$ mice (Fig. 1C). These findings indicate that IC-ALI is attenuated in NKT cell-deficient mice compared with B6 mice, and suggest that NKT cells promote IC-ALI. To address this suggestion, we analyzed the kinetics of NKT cell infiltration into the lung and adoptively transferred the sorted NKT cells into CD1d
$^{-/-}$ mice in the IC-ALI model. Vx14 TCR transcript levels in the lungs of B6 mice increased gradually beginning 4 h after CEA injection, peaked at 12 h, and then gradually decreased, whereas no Vx14 TCR transcript was detected in the lungs of CD1d
$^{-/-}$ mice during IC-ALI (Fig. 1D). Adoptive transfer of NKT cells increased total cell and Ly6G$^{+}$CD11b$^{+}$ granulocyte counts in BALF, and pulmonary histological change of CD1d
$^{-/-}$ mice, to levels similar to those in B6 mice (Fig. 1B, 1E). These data indicate that CD1d-restricted NKT cells promote IC-ALI.
IFN-γ and MIP-1α production by CD1d-restricted NKT cells contributes to IC-ALI

To evaluate the mechanism by which NKT cells promote IC-ALI, we assessed the IL-4, IFN-γ, MIP-1α, MCP-1, and RANTES transcriptional levels in the lungs of B6 and CD1d<sup>-/-</sup> mice (Fig. 1A). Transcription of IFN-γ, MIP-1α, MCP-1, and RANTES increased gradually and peaked 24 h after CEA injection. IFN-γ and MIP-1α transcript levels were greater in B6 mice than in CD1d<sup>-/-</sup> mice, consistent with the amounts of proteins detected (Fig. 2A, 2B). In contrast, the levels of MCP-1 and RANTES transcripts in B6 and CD1d<sup>-/-</sup> mice were similar. Unlike IFN-γ, IL-4 in the lungs of B6 and CD1d<sup>-/-</sup> mice was detected at low levels. Adoptive transfer of NKT cells restored IFN-γ and MIP-1α production in the lungs of CD1d<sup>-/-</sup> mice to levels similar to those of B6 mice, whereas no change in IL-4, TGF-β, MCP-1, and RANTES levels was detected (Fig. 2C). These findings suggest that IFN-γ and MIP-1α may contribute to promoting IC-ALI by CD1d-restricted NKT cells.

TCR and FcγR signaling on CD1d-restricted NKT cells enhance IFN-γ and MIP-1α production during IC-ALI

To explore the mechanism responsible for the promotion of IC-ALI by NKT cells, we measured BALF cell numbers, and the transcriptional levels of cytokines and chemokines in the lungs of B6 mice given α-GalCer. On α-GalCer injection, both BALF total cell count and IFN-γ and MIP-1α production in the lungs increased in B6 but not in CD1d<sup>-/-</sup> mice (Fig. 3A, 3B). In contrast, no change in IL-4 production in the lungs of B6 mice was detected. These findings suggest that signals through TCR on CD1d-restricted NKT cells are involved in IC-ALI.

Previously, we reported that NKT cells constitutively express FcγRIII but not FcγRI, FcγRII, and FcγRIV on cell surface, and engagement of FcγRIII by IgG in joint tissues in Ab-induced arthritis provides activating signals to NKT cells (12). Therefore, it is possible that FcγRIII may also activate NKT cells, promoting IC-ALI. NKT cells of B6 or FcγR<sup>-/-</sup> mice were adoptively transferred to CD1d<sup>-/-</sup> mice during IC-ALI to address this. Adoptive transfer of wild type (WT) NKT but not FcγR-deficient NKT cells increased Ly6G<sup>+</sup>CD11b<sup>+</sup> cell numbers and IFN-γ and MIP-1α production in BALF (Fig. 3C). These data suggest that engagement of FcγR by IC plays a critical role in the activation of NKT cells during IC-ALI. Taken together, these findings suggest that signals via both TCR and FcγRIII on CD1d-restricted NKT cells might be involved in promoting IC-ALI by enhancing production of IFN-γ and MIP-1α.

IFN-γ produced by CD1d-restricted NKT cells enhances MIP-1α production in both an autocrine and a paracrine manner during IC-ALI

To explore whether IFN-γ produced by NKT cells plays a critical role in IC-ALI, we adoptively transferred NKT cells of WT or IFN-γ<sup>-/-</sup> mice into CD1d<sup>-/-</sup> mice. Adoptive transfer of WT but not IFN-γ-deficient NKT cells increased BALF cell numbers and IFN-γ and MIP-1α production in the lungs of CD1d<sup>-/-</sup> mice to levels similar to those of B6 mice (Fig. 4A). These findings suggest that IFN-γ-producing NKT cells promote IC-ALI by producing MIP-1α. Sorted NKT cells of B6 or IFN-γ<sup>-/-</sup> mice were stimulated with aggregated IgGs with or without anti–IFN-γ mAb to address this (Fig. 4B). WT NKT cells produced MIP-1α, which was inhibited by anti–IFN-γ mAb. In contrast, IFN-γ-deficient NKT cells did not produce MIP-1α in the presence of aggregated IgG stimulation; production was restored by adding recombinant IFN-γ. These findings suggest that IFN-γ-producing NKT cells enhance their own MIP-1α production during IC-ALI.

We postulate that IFN-γ produced by NKT cells may affect MIP-1α production of either NKT cells (autocrine) or other cells (paracrine), or both. To explore this, we adoptively transferred NKT cells from either WT or IFN-γR<sup>-/-</sup> mice into CD1d<sup>-/-</sup> mice and CD1d<sup>-/-</sup> IFN-γ<sup>-/-</sup> double knockout mice (Fig. 4C). IFN-γ production was similar in B6 and CD1d<sup>-/-</sup> mice adoptively transferred with WT or IFN-γR-deficient NKT cells, whereas CD1d<sup>-/-</sup> mice produced only minimal amounts of IFN-γ. Moreover, adoptive transfer of IFN-γR-deficient NKT cells increased MIP-1α production in the lungs of CD1d<sup>-/-</sup> mice, but to a lesser extent than WT NKT cells. The production of IFN-γ by CD1d<sup>-/-</sup> IFN-γ<sup>-/-</sup> mice transferred with WT or IFN-γR-deficient NKT cells was similar to that in CD1d<sup>-/-</sup> mice given WT or IFN-γR-deficient NKT cells, respectively (Fig. 4D). In contrast, WT NKT cells, but not IFN-γR-deficient NKT cells, enhanced MIP-1α production in the lungs of CD1d<sup>-/-</sup>IFN-γ<sup>-/-</sup> mice. These results suggest that IFN-γ is produced in the murine lung primarily by CD1d-restricted NKT cells. This, in turn, causes pulmonary CD1d-restricted NKT cells, together with other IFN-γ-expressing cells, to increase MIP-1α expression during IC-ALI.

CD1d-restricted NKT cells promote recruitment of inflammatory cells by producing MIP-1α during IC-ALI

Sorted NKT cells were incubated with aggregated IgGs and MIP-1α production was assayed to explore whether NKT cells induce migration of inflammatory cells by producing MIP-1α by stimulation with anti-CD3 mAb and/or aggregated IgGs. On stimulation with aggregated IgGs, NKT cells produced MIP-1α in a dose-dependent manner (Fig. 5A). Furthermore, stimulation with both anti-CD3 mAb and aggregated IgGs showed an additive effect on MIP-1α production. In migration assays, the supernatant of activated NKT cells increased migration of Ly6G<sup>+</sup>CD11b<sup>+</sup> BALF cells (Fig. 5B). This effect was inhibited by anti–MIP-1α mAb. In contrast, the T cell culture supernatant did not induce migration of Ly6G<sup>+</sup>CD11b<sup>+</sup> cells from BALF. Moreover, administration of anti–MIP-1α mAb caused a reduction in Ly6G<sup>+</sup>CD11b<sup>+</sup> cells in B6 but not in CD1d<sup>-/-</sup> mice (Fig. 5C). These findings suggest that MIP-1α, produced by NKT cells stimulated with CD3 and FcγR, induces migration of inflammatory cells into the lung during IC-ALI.

To determine whether MIP-1α produced by NKT cells promotes inflammatory cell recruitment, we adoptively transferred NKT cells from WT or MIP-1α<sup>-/-</sup> mice into CD1d<sup>-/-</sup> mice and then measured BALF cell numbers and IFN-γ and MIP-1α levels in the lungs (Fig. 5D). Adoptive transfer of WT but not MIP-1α-deficient NKT cells restored BALF cell numbers and MIP-1α production in CD1d<sup>-/-</sup> mice to levels similar to those in B6 mice. Moreover, flow cytometric analysis demonstrated that adoptive transfer of WT but not MIP-1α-deficient NKT cells promoted recruitment of Ly6G<sup>+</sup>CD11b<sup>+</sup> granulocytes in the lungs of CD1d<sup>-/-</sup> mice (Fig. 5E). However, IFN-γ production in the lungs of CD1d<sup>-/-</sup> mice adoptively transferred with WT NKT cells was similar to that of CD1d<sup>-/-</sup> mice given MIP-1α-deficient NKT cells. These findings suggest that MIP-1α, produced by CD1d-restricted NKT cells, promotes the recruitment of Ly6G<sup>+</sup>CD11b<sup>+</sup> granulocytes into the lungs during IC-ALI but does not affect IFN-γ production.

CD1d-restricted IFN-γ-producing NKT cells enhance cytokine production by alveolar macrophages and DCs during IC-ALI

Alveolar macrophages play a critical role in the development of IC-ALI (3). IC activates alveolar macrophages, resulting in synthesis and release of various cytokines, such as TNF-α, IL-1β, IL-6, IL-12, and IL-13 (3). Moreover, C5a, cleaved from C5 by
proteases from activated alveolar macrophages, contributes to the induction of neutrophil infiltration and regulates the expression of cytokines, chemokines, and adhesion molecules during IC-ALI (13). Therefore, to determine whether NKT cells cross talk with alveolar macrophages, the expression of various cytokines and C5a in BALF from B6 and CD1d<sup>−/−</sup> mice was measured. TNF-α and IL-1β production increased in BALF of B6 mice compared with that of CD1d<sup>−/−</sup> mice (Fig. 6A). However, IL-12, IL-13, IL-18, TGF-β, and C5a levels in BALF of B6 mice were similar to those of CD1d<sup>−/−</sup> mice (Fig. 6A, 6B). These findings suggest that NKT cells enhance cytokine production by alveolar macrophages during IC-ALI. To address this, we measured cytoplasmic TNF-α and IL-1β levels in B6 and CD1d<sup>−/−</sup> BALF macrophages and DCs. The percentages of TNF-α<sup>+</sup> or IL-1β<sup>+</sup> F4/80<sup>+</sup> alveolar macrophages and CD11c<sup>+</sup> DCs of B6 mice were greater than those of CD1d<sup>−/−</sup> mice (Fig. 6C, 6E). Moreover, NKT cells activate alveolar macrophages by producing IFN-γ during clearance of Pseudomonas aeruginosa from the lung (14), suggesting that IFN-γ produced by NKT cells indeed enhances cytokine production by alveolar macrophages during IC-ALI. WT or IFN-γ-deficient NKT cells were adoptively transferred into CD1d<sup>−/−</sup> mice, and IL-1β and TNF-α levels in alveolar macrophages, CD11c<sup>+</sup> DCs, and BALF were assayed to further elucidate the effect of IFN-γ. WT, but not IFN-γ-deficient, NKT cells enhanced IL-1β and TNF-α production in alveolar macrophages, CD11c<sup>+</sup> DCs, and BALF of CD1d<sup>−/−</sup> mice to levels similar to those in B6 mice (Fig. 6E).

**FIGURE 2.** IFN-γ and MIP-1α production by NKT cells contributes to IC-ALI. Anti-CEA IgG was administered intratracheally into B6 and CD1d<sup>−/−</sup> mice, and 5 min later, mice were injected i.v. with CEA. A, IFN-γ, IL-4, MIP-1α, MCP-1, and RANTES transcript levels in the lungs of B6 and CD1d<sup>−/−</sup> mice were estimated using real-time PCR 0, 4, 8, 12, 24, 36, and 48 h after CEA injection. B, IFN-γ and MIP-1α levels in BALF of B6 and CD1d<sup>−/−</sup> mice were determined by ELISA 0, 4, 8, 12, 24, 36, and 48 h after CEA injection. A and B, *p < 0.05, **p < 0.01, B6 versus CD1d<sup>−/−</sup> mice. C, Sorted NKT cells from B6 mice were adoptively transferred into CD1d<sup>−/−</sup> mice 1 d prior to CEA injection. IFN-γ, IL-4, TGF-β, MIP-1α, MCP-1, and RANTES transcript levels in the lungs of B6, CD1d<sup>−/−</sup>, and CD1d<sup>−/−</sup> mice adoptively transferred with WT NKT cells were evaluated using real-time PCR 24 h after CEA injection. *p < 0.05, **p < 0.01, B6 versus CD1d<sup>−/−</sup> mice or CD1d<sup>−/−</sup> versus WT NKT → CD1d<sup>−/−</sup> mice. Data from a representative of three repeated independent experiments are shown (n = 3 per group).
Therefore, these data suggest that production of IL-1β and TNF-α by alveolar macrophages and DCs depends on IFN-γ-producing CD1d-restricted NKT cells during IC-ALI.

**Discussion**

The data presented in this study demonstrated that CD1d-restricted NKT cells infiltrated into the lungs, underwent activation, and induced inflammation and subsequent pulmonary injury during IC-ALI. To understand the mechanisms by which CD1d-restricted NKT cells aggravate lung injury, it is necessary to first elucidate the signals that induce their activation. Administration of α-GalCer into B6 mice increased BALF cell numbers and IFN-γ and MIP-1α production in the lungs. Furthermore, adoptive transfer of FcγR-deficient NKT cells into CD1d−/− mice did not induce pulmonary tissue damage, suggesting that FcγRIII signaling is critical to pathogenesis. Consistent with these findings, Chouchakova et al. (15) demonstrated that FcγRIII signaling is critical in the lungs by stimulating the production of TNF-α and CXC chemokines. These findings suggest that signaling via TCR and/or FcγRIII contributes to the activation of NKT cells in IC-ALI, similar to that which occurs during IC-mediated joint inflammation (10, 12). CD1d molecules activate NKT cells by presenting cellular glycolipid (16). Moreover, Fas/Fas ligand-mediated apoptosis of respiratory epithelial, inflammatory, and endothelial cells contributes to the development of ALI (17). Therefore, it is feasible that during IC-ALI, cellular glycolipid from apoptotic cells presented by CD1d, and circulating or deposited IC provide activating signals to CD1d-restricted NKT cells by engaging TCR and FcγRIII, respectively.

NKT cells have been implicated in the prevention of autoimmune diseases, transplantation, and metastasis of tumors. In contrast with the protective role of NKT cells in the aforementioned diseases, NKT cells promote other immune conditions, such as rheumatoid arthritis (10) and atherosclerosis (18). Furthermore, in the respiratory system, NKT cells attenuate pulmonary fibrosis (19) and hypersensitivity pneumonitis (20), but promote asthma (21). Thus, NKT cells can exert either beneficial or harmful effects, depending on the target tissues’ microenvironment. NKT cells aggravate IC-mediated joint inflammation by suppressing TGF-β (10). Unlike the IC-mediated arthritis model, TGF-β production in the lungs of B6 mice with IC-ALI was similar to that of CD1d−/− mice; thus, it is unlikely that NKT cells regulate TGF-β production in IC-ALI. Therefore, NKT cells differentially regulate IC-induced inflammation via different pathways in the...
FIGURE 4. IFN-γ produced by NKT cells enhances MIP-1α production in both an autocrine and a paracrine manner. A. Sorted NKT cells from B6 or IFN-γ−/− mice were adoptively transferred into CD1d−/− mice 24 h prior to CEA injection. Total cell counts and IFN-γ and MIP-1α levels in BALF of B6, CD1d−/−, and CD1d−/− mice adoptively transferred with WT or IFN-γ−/−deficient NKT cells were measured by ELISA. *p < 0.05, **p < 0.01, ***p < 0.001, B6 versus CD1d−/− mice, CD1d−/− versus WT NKT → CD1d−/− mice, or WT NKT → CD1d−/− versus IFN-γ−/− NKT → CD1d−/− mice. B. Sorted NKT cells of B6 or IFN-γ−/− mice were incubated with aggregated IgGs (algG; 50 μg/ml) with or without anti–IFN-γ mAb (1 μg/ml) or recombinant IFN-γ (10 ng/ml). Then, 24 h later, MIP-1α concentrations in culture supernatant were determined. *p < 0.05, **p < 0.01, no treatment versus algG or algG versus algG + anti–IFN-γ mAbs. C and D, Sorted NKT cells from B6 or IFN-γR−/− mice were adoptively transferred into CD1d−/− IFN-γ−/− or CD1d−/− mice 1 d prior to CEA injection. IFN-γ and MIP-1α levels in BALF of B6, CD1d−/−, CD1d−/− IFN-γ−/−, and CD1d−/−IFN-γ−/− mice adoptively transferred with WT or IFN-γR−/−deficient NKT cells were measured by ELISA. C and D, *p < 0.05, **p < 0.01, IFN-γ−/−CD1d−/− versus WT NKT → IFN-γ−/−CD1d−/− mice, or WT NKT → IFN-γ−/−CD1d−/− mice versus IFN-γR−/− NKT → IFN-γ−/−CD1d−/− mice). Data from a representative of three repeated independent experiments are shown (n = 3 per group).

in vitro studies revealed that engagement of the TCR and/or FcγRIII caused NKT cells to produce MIP-1α. Moreover, studies in vivo demonstrated that IFN-γ produced by NKT cells enhanced either their own MIP-1α expression (autocrine) or that of other immune cells (paracrine), resulting in greater inflammatory cell infiltration. Indeed, MIP-1α has been shown to enhance recruitment of neutrophils into the lungs during IC-ALI (24). Furthermore, MIP-1α mobilizes human Vx24 Vβ11+NKT cells by engaging CCR1 (25), suggesting that MIP-1α produced by NKT cells may recruit both more NKT cells and granulocytes into the lungs during IC-ALI. In contrast, Ota et al. (26) demonstrated that α-GalCer–induced IFN-γ upregulated Qa-1, which, in turn, suppressed secondary invariant NKT cell activation via CD94/NKG2. These findings suggest that IFN-γ produced by NKT cells might also negatively regulate NKT cell activation. Therefore, it is feasible that NKT cells might regulate migration and

lungs and joints in terms of TGF-β, although the mechanisms of activation and provocative effects of NKT cells in IC-mediated inflammation in these tissues are similar. Furthermore, IFN-γ produced by CD1d-restricted NKT cells enhanced MIP-1α production in the lungs during IC-ALI. Several studies have demonstrated a functional link between IFN-γ and MIP-1α in the regulation of immune responses (22, 23). Dorner et al. (22) demonstrated that various chemokines, such as MIP-1α, MIP-1β, and RANTES, are secreted together with IFN-γ by NK, CD8+, and Th1 cells, suggesting that IFN-γ and MIP-1α act together as a functional unit. It was also reported that IFN-γ increases MIP-1α release by alveolar macrophages with or without IgE/anti-IgE (23). Thus, IFN-γ potentiates the production of MIP-1α by immune cells during immune responses. However, to the best of our knowledge, no previous study has reported that NKT cells stimulate MIP-1α production by releasing IFN-γ. Our
activation of NKT cells, as well as other immune cells, by producing IFN-\(\gamma\). Taken together, IFN-\(\gamma\) produced by CD1d-restricted NKT cells enhances production of MIP-1\(\alpha\) by inflammatory and NKT cells in the lungs, resulting in increased recruitment of NKT and inflammatory cells and promoting tissue damage.

Alveolar macrophages promote IC-ALI by producing various inflammatory mediators (3). Therefore, it is interesting to speculate whether NKT cells cross talk with alveolar macrophages and CD11c\(^+\) DCs. TNF-\(\alpha\) and IL-1\(\beta\) enhance neutrophil infiltration during ALI (27, 28). Intracellular TNF-\(\alpha\) and IL-1\(\beta\) levels in alveolar macrophages and CD11c\(^+\) DCs were greater in B6 than in NKT cell-deficient mice, and IFN-\(\gamma\)-producing NKT cells enhanced production of TNF-\(\alpha\) and IL-1\(\beta\) in alveolar macrophages and CD11c\(^+\) DCs. These findings suggest that CD1d-restricted NKT cells recruit and activate alveolar macrophages and CD11c\(^+\) DCs in the lungs by producing IFN-\(\gamma\), resulting in enhanced cytokine production.

Considering the central role of NKT cells in IC-induced inflammation, inhibition of NKT cell function may be therapeutically useful. Recently, we demonstrated that FTY720 suppresses cytokine production in NKT cells via S1P1, but does not inhibit NKT cell migration (29). Thus, FTY720 may be therapeutically useful for controlling acute pulmonary inflammation. An alternative approach would be development of therapeutic approaches that specifically block TCR or Fc\(\gamma\)RIII signaling.

In conclusion, CD1d-restricted IFN-\(\gamma\)-producing NKT cells promoted IC-ALI by stimulating production of MIP-1\(\alpha\) in both an
FIGURE 6. NKT cells cross talk with alveolar macrophages and DCs during IC-ALI. Anti-CEA IgG was administered intratracheally into B6 and CD1d$^{-/-}$ mice, followed 5 min later by i.v. injection of CEA. A and B, IL-1β, IL-6, IL-12, IL-13, TNF-α, and C5a levels in B6 and CD1d$^{-/-}$ mouse BALF were determined by ELISA, and IL-18 and TGF-β transcript levels were determined in the lung homogenates by real-time PCR, 24 h after CEA injection. The percentages of TNF-α$^+$ or IL-1β$^+$ F4/80$^+$ macrophages (C) and CD11c$^+$ DCs (E) were analyzed by intracellular staining and are presented as a flow cytometry diagram (C) and graphs (E). Cytokine levels in F4/80$^+$ cells (D) and total BALF (F) were determined by measuring mean fluorescence intensity (MFI) in flow cytometric analysis and using ELISA, respectively. A–F, *p < 0.05, **p < 0.01, ***p < 0.001, B6 versus CD1d$^{-/-}$ mice, CD1d$^{-/-}$ versus WT NKT→CD1d$^{-/-}$ mice, or WT NKT→CD1d$^{-/-}$ versus IFN-γ$^{-/-}$ NKT→CD1d$^{-/-}$ mice. Data from a representative of four repeated independent experiments are shown (n = 3 per group).
autocrine and a paracrine manner, and enhancing cytokine production by alveolar macrophages and CD11c+ DCs. Therefore, therapeutic modalities that inhibit NKT cell function may be useful for treatment of IC-ALI.

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