Cutting Edge: IFN-γ Signaling to Macrophages Is Required for Optimal Vα14i NK T/NK Cell Cross-Talk

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Activated NK T cells are known to rapidly stimulate NK cells and, subsequently, CD8\(^+\) T cells and B cells. In this report, we first demonstrate that the downstream effects induced by \(\alpha\)-galactosylceramide activated NK T cells on NK cells are mainly dependent on IFN-\(\gamma\). We found that NK T cell activation of NK cells requires a functional IFN-\(\gamma\) signaling in macrophages and dendritic cells but not in B cells, NK cells, or NK T cells. NK T cell activation is dendritic cell-dependent whereas NK T cell activation of NK cells is indirect and in part mediated by macrophages. Interestingly, in this context, macrophage participation in the CD1d Ag presentation of \(\alpha\)-galactosylceramide to NK T cells is not necessary. These data indicate that NK T cell-dependent activation of macrophages is required for optimal NK T cell-induced stimulation of NK cells. The Journal of Immunology, 2005, 174: 3864–3868.

Materials and Methods

Abs and reagents

TCRB-FITC, CD5-FITC, CD19-FITC, CD45.1-FITC, CD45.2-FITC, CD4-allophycocyanin, CD8-PE, CD1d-PE, NK1.1-PE, CD11c-FITC, CD4-PE, CD4-allophycocyanin, and isotype controls were purchased from BD Pharmingen. \(\alpha\)-GalCer was synthesized by the Kirin Brewery and kindly provided by Dr. K. Miyayama (Kirin Brewery, Gunma, Japan). \(\alpha\)-GalCer and vehicle-loaded CD1d tetramers conjugated with PE were produced as described previously (7) or obtained from the National Institute of Allergy and Infectious Disease MHC Tetramer Core Facility. The following mAbs were purchased from BD Pharmingen and used for ELISA: IFN-\(\gamma\) mAbs (clone R4-6a2, and clone XMG1.2), IL-4 mAbs (clone 4B11 and BV6D-2G2), and streptavidin-peroxidase. For the in vivo neutralization of IL-12, functional grade purified anti-IL-12p70 (clone C17.8) was purchased from eBioscience.

Mice and in vivo treatment protocols

Inbred C57BL/6 and B6.129P2-Ppkr^+/+Boa/Tac mice were purchased from Taconic Laboratory Animals and Services. C57BL/6 H2-\(\text{K}^d\)-\text{b2m}^−/− and C57BL/6 IL-12p40−/− mice were purchased from The Jackson Laboratory. C57BL/6 CD1d−/− mice (17) were kindly provided by Dr. L. Van Kaer (Vanderbilt University, Nashville, TN) and bred in our facility. STAT1−/− mice on a C57BL/6 background were kindly provided by Drs. C. Biron and J. Durbin (Brown University, Providence, RI). C57BL/6 Jx281−/− mice (4) were kindly provided by Dr. M. Taniguchi (Riken Research Center for Allergy and Immunology, Yokohama, Japan) and bred in our facility. Mice were treated i.p. with either 2 \(\mu\)g of \(\alpha\)-GalCer or 100 \(\mu\)l vehicle (0.5% polysorbate-20). For in vivo neutralization of IL-12, mice were treated i.p. with anti-IL-12p70 12 h before \(\alpha\)-GalCer treatment. All mice were 4–8 wk-old males, except where indicated, and experiments were conducted in accordance with institutional guidelines for animal care.

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3 Abbreviations used in this paper: \(\alpha\)14i: NK T, \(\alpha\)14i18 NK T cell; \(\alpha\)-GalCer, \(\alpha\)-galactosylceramide; DC, dendritic cell; Mø, macrophage; ICS, intracellular staining.
Isolation of lymphocytes and flow cytometric analysis

Hepatic lymphocytes were isolated by mincing tissue and pressing through a 70 μm nylon cell strainer (Falcon). Cell suspensions were layered onto a two-step discontinuous Percoll gradient (Pharmacia Fine Chemicals). Splenic lymphocytes were isolated by mincing and passing through nylon mesh (Tekko). Cell suspensions were layered onto Lympholyte-M (Cedarlane Laboratories). For intracellular staining of IL-12 (BD Pharmingen). For analysis of intracellular IL-12, lymphocytes were first digested with collagenase (Liberase CI; Roche Diagnostics) as described (18, 19). In brief, spleens were injected with 1 ml of Liberase CI in serum-free DMEM, macerated with a needle, and transferred to a 37°C water bath for 45 min. RBC were lysed using ammonium chloride and cells were then washed with PBS containing 3% serum and passed through nylon mesh before separation with Lympholyte-M. Where indicated, lymphocytes were positively selected for CD19+, DX5+, CD11c+ and/or CD11b+ cells before culture using magnetic beads specific for each surface marker and the AutoMACS cell separator (Miltenyi Biotec). Isolated lymphocytes were prepared for flow cytometric analysis, blocked with 2.4G2 anti-Fc mAb, and labeled with indicated mAbs. ICS for IFN-γ protein was performed using the Cytofix/Cytoperm kit (BD Pharmingen). For analysis of intracellular IL-12, lymphocytes were first digested with CD19+, DX5+, CD11c+ and/or CD11b+ cells using the AutoMACS ex vivo and treated with brefeldin A (Sigma-Aldrich) for 3 h before preparation for FACS analysis. All flow cytometric analyses were performed using the FACS Calibur and Cell Quest software (BD Biosciences).

In vitro α-GalCer stimulation assay

Following isolation of lymphocytes and positive selection for specific subsets as described above, 5 × 10⁶ splenocytes from STAT1−/−, IL-12p40−/−, C57BL6/J and C57BL6.SJL were cultured alone or 2.5 × 10⁶ splenocytes from STAT1−/−, IL-12p40−/−, and C57BL6/J were cocultured with equal numbers of C57BL6.SJL splenocytes in the presence of 100 ng/ml α-GalCer or 1 μl/ml DMSO (Sigma-Aldrich). In additional experiments, 5 × 10⁶ splenocytes from IL-12p40−/− or C57BL6/J were cocultured with α-GalCer or DMSO and either 2.5 × 10⁶ CD11c+ or CD11b+CD11c+ cells isolated from CD1d−/− spleens, or 1 × 10⁶ total CD1d−/− splenocytes. All cultures were plated onto 24-well culture plates (Corning) in 1 ml of DMEM with 8% FCS per well for 36–40 h. The supernatant was then collected for ELISA and lymphocytes were prepared for flow cytometric analysis as described above.

Results and Discussion

IFN-γ signaling is required for optimal Vα14i NK T/NK cell cross-talk

Previous studies have suggested that both IFN-γ and IL-12 are essential components of the Vα14i NK T cell-induced activation of NK cells (12, 13). Here, upon immunization of wild-type mice or mice deficient in either IFN-γ signaling or IL-12 with α-GalCer or vehicle, we confirmed the critical role of IFN-γ and IL-12 (Fig. 1A). A clear reduction of the percentage of NK cells producing IFN-γ following α-GalCer immunization can be seen in IL-12, STAT1, and IFN-γR1-deficient animals when compared with wild-type mice. At 6 h postimmunization, 31.26 ± 2.09% of B6 hepatic NK cells synthesize IFN-γ whereas only 9.86 ± 3.44 of IFN-γR1−/− and 5.10 ± 0.96% STAT1−/− liver NK cells synthesize IFN-γ. Interestingly, the absence of IFN-γ signaling results in a more pronounced NK cell IFN-γ defect in the liver than in the spleen. The data also demonstrate that, in the absence of IFN-γ signaling, a significant number of NK cells can still be activated by Vα14i NK T cells whereas only a few NK cells can be activated in the absence of IL-12 (Fig. 1A). Other cytokines such as IL-18 could explain the residual NK activation seen in IL-12-deficient animals (20, 21). Further, we also show that the transcription factor STAT1 is essential in the Vα14i NK T cell/NK cell cross-talk (Fig. 1A). Interestingly, the treatment of STAT1−/− mice, i.p., with anti-IL-12p70 mAb 12 h before α-GalCer injection did not completely abolish the Vα14i NK T cell’s ability to stimulate NK cells (Fig. 1B). In the STAT1−/− mice that received both α-GalCer and the neutralizing mAb, the decrease in NK cell IFN-γ observed in these mice was only slightly greater than those that received only the α-GalCer injection (3.18 ± 0.09% vs 5.82 ± 0.35% in the liver). Taken together, these findings suggest that STAT1 signaling is required for the induction of IL-12 (Fig. 1B), and demonstrate that Vα14i NK T cell activation of NK cells is dependent on IL-12 and IFN-γ signaling.

Vα14i NK T cell number and CD1d expression in IL-12p40−/− and IFN-γR1−/− mice are comparable to wild-type mice

A lower number of Vα14i NK T cells in IL-12p40−/− and IFN-γR1−/− mice could explain the results seen in Fig. 1. Therefore, we examined the Vα14i NK T cell population in the STAT1−/−, IFN-γR1−/−, and IL-12p40−/− mice in comparison to the wild-type C57BL6/J animals. Using CD1d tetramers loaded with α-GalCer, we demonstrate that the percentage and absolute number of the Vα14i NK T cells in IFN-γR1−/−, IL-12p40−/−, and wild-type mice are similar (Fig. 2, A and B). However, although not statistically significant, we consistently observed a lower number of Vα14i NK T cells in the STAT1−/− mice in comparison to the wild-type animals. It is also well known that mice deficient in IFN-γ and STAT1 have a lower level of MHC class I expression and it is conceivable that the effects described above were the result of a lower level of CD1d expression. We found that CD1d cell surface expression on STAT1−/−, IFN-γR1−/−, IL-12p40−/−, and C57BL6/J splenocytes is comparable (Fig. 2C).
We found that both the IL-4 serum level and the percent of NK T cell's ability to respond to Ag-specific stimulation (22) is dependent of both IFN-γ. The efficiency of NK cell-derived IFN-γ can be subsequently detected in the supernatant. Therefore, we measured the serum IL-4 concentration and hepatic intracellular IFN-γ by permeabilization and staining for intracellular IFN-γ. In IL-4, NK cells are functional in the absence of IFN-γ signaling and IL-12. Results shown are from one experiment representative of three and are expressed as mean ± SD of five mice per group.

The lower percentage of NK cells producing IFN-γ following α-GalCer immunization seen in IL-12 and STAT-1-deficient mice could result from a functional defect of the Vα14i NK T and/or NK cells. To investigate these possibilities, we first measured the serum IL-4 concentration and hepatic intracellular IFN-γ of mice at 2 h post-α-GalCer immunization. At this early time point, serum IL-4 is strictly dependent of the Vα14i NK T cells and is therefore an excellent indicator of the Vα14i NK T cell's ability to respond to Ag-specific stimulation (22). We found that both the IL-4 serum level and the percent of IFN-γ is dependent of both IFN-γ signaling and IL-12.

To address whether the NK cell populations isolated from the STAT1−/− and IL-12p40−/− mice were functional, we established a method that would allow us to measure NK cell IFN-γ intracellularly in vitro. It is well known that the addition of α-GalCer to splenocytes activates Vα14i NK T cells and that cytokines can be subsequently detected in the supernatant. However, we found that NK cell-derived IFN-γ is best detected by ICS if spleens are first digested with Liberase CI (18). This enzyme treatment considerably increases the total cell yield, and the percentage of splenic DC and Mφ, presumably enhancing the efficiency of α-GalCer presentation. Initially, time course experiments were performed to determine the optimal time-point at which to measure IFN-γ from NK cells by ICS. In these conditions, NK cell-derived IFN-γ peaks between 36 and 40 h postculture. 
Using this method, cocultures of either STAT1–/– or IL-12p40–/– and congenic C57BL/6 SJL-derived splenocytes were established in the presence of α-GalCer or DMSO. The STAT1–/– and IL-12p40–/– NK cells, defined as CD5+/NK1.1– cells, distinguishable from the wild-type congenic NK cells based on their expression of CD45.2, responded as well as the wild-type cells (CD45.2-negative) within the same well (Fig. 3C). In contrast when STAT1–/– or IL-12p40–/– splenocytes are cultured in the presence of α-GalCer but in the absence of congenic B6 splenocytes, only a weak stimulation of the STAT1–/– NK cells and no stimulation of the IL-12p40–/– NK cells could be observed (Fig. 3C, bottom left panel). These data confirm that STAT1–/– and IL-12p40–/– NK cells are functional and, additionally, provide evidence that the Vα14i NK T/NK cell cross-talk is indirect.

Taken together these results demonstrate that the inability of NK cells from IL-12p40–/– and STAT1–/– mice to respond fully to α-GalCer-stimulated Vα14i NK T cells is not due to an intrinsic functional defect in either the Vα14i NK T or NK cells.

**IL-12-producing CD11b+CD11c− cells are critical for optimal NK T cell-induced activation of NK cells**

As shown in Fig. 3, our experiments using the congenic wild-type B6-derived splenocytes mixed with either splenocytes from STAT1–/– or IL-12p40–/– mice indicate that their NK cell-IFN-γ response could be rescued to, or near, wild-type levels. A plausible explanation for these results was that a critical subset of cells possessing both a functional IFN-γ signaling pathway and the ability to secrete IL-12 was added to the culture. To define this population, we established coculture experiments from IL-12p40–/– splenocytes and purified cellular subsets from CD1d–/– mice. The use of CD1d–/– mice allows a direct examination of the effects of the addition of different cell populations without concerns that any observed recovery was merely the result of the addition of CD1d+ APC or Vα14i NK T cells. Also, CD1d can serve as a marker to distinguish between the different sources of cells.

We found that addition of CD19+/CD1d–/– or DX5+/CD1d–/– cells to IL-12p40–/– splenocytes had no effect on the NK cell-derived IFN-γ response 36 h postculture (not shown). The addition of CD11c+/CD1d–/– cells (DC) had a relatively weak effect on this response (Fig. 4A) presumably due to the lack of CD1d expression of these cells. In contrast, addition of the CD11b+/CD11c+/CD1d–/– subset (Mφ) was sufficient to render NK cells responsive to α-GalCer-activated Vα14i NK T cells. These data demonstrate an unanticipated and critical role for macrophages during the Vα14i NK T cell activation of NK cells in vitro. To further confirm that the identified intermediate was a macrophage, additional experiments were conducted in which the CD11c–CD11b+CD1d–/– subset was separated into two separate populations based on their expression of the Mφ-specific marker F4/80 using FACS. Total splenocytes isolated from IL-12p40–/– animals were cocultured with either CD11b+F4/80+ or CD11b–F4/80– subsets in the presence of α-GalCer for 36–40 h. The addition of the CD11b–F4/80– subset failed to recover the IL-12p40–/– NK cell-derived IFN-γ response in vitro whereas the coculture of the CD11b+F4/80+ subset and IL-12p40–/– splenocytes significantly enhanced this response (not shown). Taken together, these data provide clear evidence that, in vitro, a Mφ intermediate is critical for optimal Vα14i NK T cell-induced activation of NK cells. In addition, the fact that the CD11b+/CD11c– cells were derived from CD1d–/– mice demonstrates that the participation of the macrophages is independent of Ag presentation.

**FIGURE 4.** CD11c−CD11b+ cells are required for optimal Vα14i NK T/NK cell cross-talk. A, A total of 5 × 106 total splenic lymphocytes from IL-12p40–/– or B6 mice were cocultured with either 2.5 × 106 CD11c+ or CD11b+CD11c− cells from CD1d–/– mice at 37°C in the presence of either 100 ng/ml α-GalCer or DMSO. At 40 h postculture, cells were harvested and stained for CD5, NK1.1, CD1d, and intracellular IFN-γ. IFN-γ production in NK cells is shown. The percentage of IFN-γ+ NK cells from IL-12p40–/– splenocytes cocultured with CD1d+CD11b+CD11c− is statistically significantly different (p ≤ 0.0007) in all five experiments when compared with NK cell IFN-γ from IL-12p40–/– splenocytes. B, Splenic leukocytes from B6 and IL-12p40–/– mice were isolated from α-GalCer or vehicle-immunized mice. CD19, CD5, and DX5 depleted cells were stained for intracellular IL-12. One experiment representative of three independent experiments is shown. C, Splenic leukocytes from B6 and IL-12p40–/– mice were isolated from α-GalCer or vehicle-immunized mice at 2, 4, and 6 h postinjection. Results were analyzed as in Fig. 4B. One experiment representative of three independent experiments is shown.
in agreement with the in vitro results described above, we found that in addition to DC (Fig. 4B and Ref. 23), CD11c−CD11b−F4/80− cells produce IL-12 following α-GalCer immunization (Fig. 4B). Interestingly, Mø-derived IL-12 peaks at 4 h (Fig. 4C) and precedes the peak of NK cell IFN-γ (not shown and Ref. 10) highlighting the critical role of the macrophages.

Previous reports have suggested that optimal activation of Vα14i NK T cells is dependent of CD40-CD40L interaction and release of IL-12 from DC (24, 25). Our findings demonstrated that IL-12 is not critical for the initial Vα14i NK T cell stimulation. Others have shown that activation of Vα14i NK T cells by α-GalCer induces a full maturation of DCs in vivo (26–28). In addition to DC, which are essential for the initial NK T cell activation, we also identified Mφ as a critical intermediate of the Vα14i NK T cell activation of NK cells. These experiments further confirm that IFN-γ does not directly induce the activation of NK cells to produce IFN-γ. Rather, this cytokine acts indirectly by inducing the production of IL-12, which activates the NK cell population. These findings may have broader consequences for understanding the role of Vα14i NK T cells in bridging innate and adaptive immunity. For example, we speculate that a targeted activation of Vα14i NK T cells leading to NK cell and Mφ activation could significantly contribute to the generation of an optimal immune response to intracellular pathogens.

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Disclosures

The authors have no financial conflict of interest.

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