Requirement for IFN-γ in IL-12 production induced by collaboration between Vα14⁺ NKT cells and antigen-presenting cells

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Abstract

Two cytokines IL-4 and IL-12 are known to determine the balance between Th1 and Th2 development. In addition to IL-4 production of Vα14/c9059 NKT cells, they have recently been demonstrated to have the capacity to stimulate IL-12 production by antigen-presenting cells (APC). This study demonstrates that IFN-γ is absolutely required for the NKT cell-stimulated IL-12 production. Culture of B cell-depleted spleen cells from C57BL/6 mice with α-galactosylceramide (α-GalCer) capable of selectively stimulating Vα14/Jα281⁺ NKT cells resulted in the production of IL-12 together with IL-4. Whereas IL-4 production occurred in culture of IFN-γ–/– C57BL/6 splenocytes, the same culture failed to generate IL-12 production. While IL-12 production induced during culture of Vα14/c9059 NKT cells and APC depended on the interaction between CD40 ligand on NKT cells and CD40 on APC, the expression levels of these key molecules were comparable in cells from wild-type and IFN-γ–/– mice. Addition of rIFN-γ to α-GalCer stimulated IFN-γ–/– splenocyte culture, and administration of rIFN-γ to α-GalCer-injected IFN-γ–/– mice resulted in the restoration of IL-12 production in vitro and in vivo. These results illustrate a mandatory role for IFN-γ in Vα14⁺ NKT cell-stimulated IL-12 production by APC.

Introduction

The balance between Th1 and Th2 development is determined by IL-4 and IL-12 (1–6). Therefore, the identification of the sources of these regulatory cytokines and the investigation of regulatory mechanisms underlying the production of these cytokines would be important for understanding the two Th differentiation processes. It has been speculated that effector cells involved in innate immunity play a pivotal role in shaping initial T cell activation (7–10). In this context, a population of T cells expressing NK1.1 and an almost invariant TCRαβ (Vα14/Jα281) specific for CD1 (11–13) was shown to be capable of producing substantial amounts of IL-4 upon the stimulation of their TCR (14–16). In initial studies (14,15), this TCR stimulation leading to IL-4 production was performed using anti-CD3 mAb. However, a recent study identified α-galactosylceramide (α-GalCer) as the ligand that stimulates the Vα14/Jα281 TCR in association with CD1, and demonstrated CD1-restricted activation of Vα14⁺ NKT cells and production of IL-4 following stimulation with α-GalCer (16). More recently, Kitamura et al. (17) and we have also found that this subset of NKT cells, particularly of CD4⁺ NKT cells, has the capacity to stimulate IL-12 production by APC which they interact with (18). Thus, it appears that CD4⁺ NKT cells are bipotential cells that may initiate both Th2- and Th1-type immune responses.

IL-12 production is generally enhanced in the presence of IFN-γ (19–21). Reciprocally, our previous study showed that IFN-γ is secreted by α-GalCer-stimulated CD4⁺ NKT cells largely depending on IL-12 produced by interacting APC (18). Considering this positive feedback loop of IL-12 and IFN-γ production, this study investigated the role of IFN-γ in IL-12 production stimulated by α-GalCer-activated NKT cells.

The results show that APC from IFN-γ–/– mice failed to produce IL-12 during interaction with α-GalCer-stimulated Vα14⁺ NKT cells. α-GalCer-activated NKT cells from wild-type and IFN-γ–/– mice induced comparable levels of IL-4 production, indicating that IL-4 production is predominant over IL-12 production in the absence of IFN-γ. These results suggest that the initiation of Th1 and Th2 immune responses that
occurs in association with the activation of V\(\alpha\)14\(\alpha\) NKT cells is influenced depending on whether IFN-\(\gamma\) is provided by other effector cells co-existing in the microenvironment of the innate immunity.

**Methods**

**Mice**

C57BL/6(B6) mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). IFN-\(\gamma\)-deficient (IFN-\(\gamma\)-\(–\)) B6 mice (B6-lifg\(^{m1Ts}\)) (22) were obtained from Jackson Laboratories (Bar Harbor, MA). These mice were used at 6–9 weeks of age.

**Reagents**

KRN7000, a representative of synthetic \(\alpha\)-GalCer, was prepared in the Pharmaceutical Research Laboratory (Kirin Brewery, Takasaki, Japan). In this paper, KRN7000 is described as \(\alpha\)-GalCer. Mouse rIL-12 was provided by Genetics Institute (Cambridge, MA).

**mAb and polyclonal antibodies**

The following mAb were used: anti-CD3 (145-2C11) (23) and anti-CD16 FcyRIII/II (2.4G2) (24) were each purified from the culture supernatant (SN). FITC-conjugated anti-TCR\(\alpha\) (HS7-157), FITC-conjugated anti-CD8\(\alpha\) (53-6.7), phycoerythrin (PE)-conjugated anti-CD40L (MR1), biotinylated anti-CD11c (HL3), biotinylated anti-NK1.1 (PK136) and allophycocyanin-conjugated anti-CD4 (RM4-5) mAb were obtained from PharMingen (San Diego, CA); PE-conjugated anti-CD40 mAb (3/23) was from Serotec (Kidlington, Oxford, UK); Red670-conjugated streptavidin (Life Technologies, Gaithersburg, MD) and allophycocyanin-conjugated streptavidin (Becton Dickinson, San Jose, CA) were also purchased.

**In vivo administration of \(\alpha\)-GalCer**

\(\alpha\)-GalCer (2 \(\mu\)g/mouse) in PBS containing 0.025% Polysolvate 20 or vehicle (PBS + Polysolvate 20) was injected i.p. into B6 mice.

**Preparation of various lymphoid populations**

**B cell-depleted fraction.** Spleen cells were depleted of B cells by immunomagnetic negative selection as described (25,26). Briefly, spleen cells were incubated with magnetic particles bound to goat anti-mouse Ig (Advanced Magnetic, Cambridge, MA). Surface Ig\(\gamma\)\(\gamma\)\(\gamma\) cells (B cell-depleted fraction) were obtained by removing cells bound to the magnetic particles with a rare earth magnet (Advanced Magnetic).

**DC-enriched population.** Spleen cells were labeled with superparamagnetic microbeads conjugated to rat anti-mouse CD11c mAb (Milenyi Biotec, Sunnyvale, CA). Labeled cells were separated from unlabeled cells by magnetic cell sorting using the MiniMACS (Milenyi Biotec) according to the procedure described in detail (27). The magnetically labeled cells were retained in a MiniMACS column inserted into a MiniMACS magnet while the unlabeled cells passed through. Labeled cells were eluted after the column was removed from the magnet and used as a DC-enriched population.

**Stimulation of spleen cells with \(\alpha\)-GalCer or CD40 ligand (CD40L)-transfected CHO cells (CD40L-CHO)**

B cell-depleted spleen cells were stimulated with \(\alpha\)-GalCer (100 ng/ml in 0.1% DMSO) or cultured with CD40L-expressing CHO cells (kindly provided by Dr H. Yagita, Juntendo University, Tokyo, Japan) in 24-well culture plates (Corning 25820; Corning Glass Works, Corning, NY) in 1 ml of RPMI 1640 medium supplemented with 10% FBS and 2-mercaptoethanol in a humidified atmosphere at 5% CO\(_2\) at 37°C. Cells and culture SN were harvested and assessed for the expression of CD40L or assayed for cytokine concentration.

**Antibody capture assay for IL-12 activity**

The assay system was essentially the same as described by Gately and Chizzonite (28). Briefly, each well of 96-well microculture plates was coated with 10 \(\mu\)g of purified anti-IL-12 mAb (C15.1) (29). Culture SN or serum together with standard mouse rIL-12 solution was incubated in wells of the mAb-coated plates. After washing, cells (1.5 \times 10\(^3\) cells/well) with [\(\text{H}\)]thymidine were cultured in triplicate. The absolute concentration of IL-12 was determined by extrapolation from a standard curve generated using known amounts of rIL-12 (30). Results were expressed as the mean ± SE of triplicate cultures for culture supernatant or of six animals for serum. The detection limit of this assay was 1 pg/ml.

**Measurement of IL-12 p40 concentration**

IL-12 p40 concentration was measured by ELISA: the mouse IL-12 p40 ELISA system was prepared using two types of anti-mouse IL-12 p40 mAb (29) (C15.6 and biotinylated C17.8) (purchased from PharMingen, San Diego, CA).

**Measurement of IL-4 concentrations**

The concentrations of IL-4 were measured by ELISA: mouse rIL-4 was purchased from R&D Systems (Minneapolis, MN). The IL-4 ELISA was constructed using anti-mouse IL-4 mAb [11B11 and biotinylated BVD4-1D11 (BVD4-1D11 mAb was purified from the corresponding hybridoma and biotinylated in our laboratory)]. 11B11 and BVD4-1D11 hybridomas were obtained from ATCC.

**Detection of CD40L expression by flow cytometry**

To detect the expression of CD40L on CD4\(^+\) and CD4\(^–\) NK1.1\(^+\) T cells, four-color flow cytometric analysis was performed. Cells were first incubated with anti-FcyRIII/II mAb (2.4G2) to prevent the staining mAb from binding with the Fc receptors. These treated cells were then stained directly with PE-conjugated anti-CD40L and biotinylated anti-NK1.1.1 followed by FITC-conjugated anti-TCR\(\alpha\)\(\beta\), Red670-conjugated streptavidin and allophycocyanin-conjugated anti-CD4. The stained cells were analyzed by FACSciAlibur (Becton Dickinson, Mountain View, CA). CD40L expression was detected by gating on a CD4\(^+\) or CD4\(\alpha\)TCR\(\alpha\)\(\beta\)\(\text{NK1.1}^+\) population.
Earlier studies demonstrated that stimulation of V\textsubscript{\alpha}14\textsuperscript{+} NKT cells with α-GalCer plus APC results in the production of IL-4 (16) and IL-12 (18). We examined whether IL-4 and IL-12 production is comparable in cultures from wild-type B6 and IFN-γ\textsuperscript{−/−} B6 mice stimulated with α-GalCer. Figure 1 shows that the level of IL-4 production is comparable in cultures from wild-type B6 and IFN-γ\textsuperscript{−/−} B6 splenocytes following α-GalCer stimulation, indicating that the activation of V\textsubscript{\alpha}14\textsuperscript{+} NKT cells with α-GalCer occurs similarly irrespective of whether they have the IFN-γ-producing capacity. In contrast, IL-12 was produced in cultures from wild-type splenocytes, whereas IFN-γ\textsuperscript{−/−} splenocytes produced no IL-12 (Fig. 2A). Thus, the presence of IFN-γ was an absolute requirement for IL-12 production induced by interaction between APC and α-GalCer-stimulated V\textsubscript{\alpha}14\textsuperscript{+} NKT cells.

Comparable levels of expression of CD40L on V\textsubscript{\alpha}14\textsuperscript{+} NKT cells from wild-type and IFN-γ\textsuperscript{−/−} mice

IL-12 production by APC collaborating with activated T cells depends on the interaction between CD40 and CD40L expressed on the respective cell types (26,31). CD40L is induced after TCR triggering. This was the case with conventional CD4\textsuperscript{+} T cells (26) and CD4\textsuperscript{+} NKT cells (18), but not with conventional CD8\textsuperscript{+} T cells (25) and CD4\textsuperscript{+} NKT cells (18). We compared the expression levels of CD40L on CD4\textsuperscript{+} NKT cells from wild-type and IFN-γ\textsuperscript{−/−} mice. B cell-depleted splenocytes from wild-type (B6) mice and those from IFN-γ\textsuperscript{−/−} mice were stimulated with immobilized anti-CD3 for 3 h. Cells were stained with anti-TCR\textsuperscript{\beta}, anti-NK1.1, anti-CD4, and anti-CD40L mAb as described in Methods. The CD4\textsupersitivity of NKT cells (TCR\textsuperscript{\beta}\textsuperscript{+}NK1.1\textsuperscript{+} cells) is shown. An analysis gate for CD40L was set on the TCR\textsuperscript{\beta}\textsuperscript{+}NK1.1\textsuperscript{+}CD4\textsuperscript{+} and NK1.1\textsuperscript{+}CD4\textsuperscript{+} (conventional CD4\textsuperscript{+} T cell) population.

We have also compared CD40 expression between CD11c\textsuperscript{+}
GalCer-stimulated culture of wild-type splenocytes did not produce IL-12. We further examined whether there is a defect in IL-12 production by positive selection, and stained triply with anti-CD11c, anti-CD8α and anti-CD40. An analysis gate for CD40 was set on CD11chighCD8α- and CD11chighCD8α+ DC populations.

Dendritic cell (DC) populations from wild-type and IFN-γ−/− spleen cells. A DC population consists of CD8+ and CD8− cells which differ in the cellular lineage and function (32–34). Therefore, DC-enriched populations were stained doubly with anti-CD11c and anti-CD8, and the levels of CD40 expression were examined in CD8+CD11c+ and CD8−CD11c+ subsets. As shown in Fig. 4, the levels of CD40 expression were comparable in these two subsets between wild-type and IFN-γ−/− mice. IL-12 is produced by CD11c+ DC as well as F4/80+ macrophages. CD40 expression was also compared between F4/80+ macrophage-enriched populations from wild-type and IFN-γ−/− splenocytes. The frequency of F4/80+ macrophage in spleens as well as their CD40 expression levels did not greatly differ (data not shown).

To further investigate whether CD40 on APC (DC/macrophage) from IFN-γ−/− mice can respond to CD40L, B cell-depleted splenocytes were cultured with CD40L-expressing CHO cells (Fig. 2B). APC from wild-type splenocytes produced a large amount of IL-12. APC from IFN-γ−/− splenocytes exhibited apparently reduced albeit positive levels of IL-12 production. Together, the results indicate that there is no substantial difference between wild-type and IFN-γ−/− mice in the expression of CD40L and CD40 that are indispensable molecules for IL-12 production by T/NKT-APC interactions. The results also suggest that an alteration in CD40-mediated signaling may exist in IFN-γ−/− APC and such an alteration is more manifest when CD40L stimulation is weaker.

Restoration of IL-12 production in IFN-γ−/− splenocytes by supplementing exogenous IFN-γ

We determined whether the failure of α-GalCer-stimulated IFN-γ−/− splenocytes to produce IL-12 can be corrected by supplementing exogenous IFN-γ. rIFN-γ (10 ng/ml) was included in culture of IFN-γ−/− splenocytes stimulated with α-GalCer. As shown in Fig. 5(A), addition of rIFN-γ to α-GalCer-stimulated culture of wild-type splenocytes did not enhance IL-12 production compared to that by these cells in the absence of rIFN-γ. In contrast, IL-12 production by IFN-γ−/− splenocytes was restored by addition of rIFN-γ and the level of the restored IL-12 production was comparable to or slightly higher than that of wild-type splenocytes. rIFN-γ was found to enhance IL-12 production by wild-type splenocytes stimulated with CD40L-expressing CHO cells (Fig. 5B). This enhancement was also observed for IFN-γ−/− splenocytes and the levels of IFN-γ-enhanced IL-12 production were comparable. The effects of rIFN-γ on IL-12 p40 production by wild-type and IFN-γ−/− splenocytes were essentially the same as those on IL-12 (p70) production (data not shown). Taken together, the results confirm that IFN-γ co-stimulation is more critical in IL-12 production stimulated by CD4+ NKT cells than by CD40L-CHO cells.

Restoration of IL-12 production in IFN-γ−/− mice by administering exogenous IFN-γ

We further examined whether there is a defect in IL-12 production in vivo in IFN-γ−/− mice following injection of α-GalCer. (A) B6 (wild-type) or IFN-γ−/− B6 mice (three mice per group) were given i.p. 2 µg α-GalCer. (B) Portions of wild-type and IFN-γ−/− mice were administered 1 µg/mouse rIFN-γ along with i.p. injection of α-GalCer. Six hours after α-GalCer injection, the mice were sacrificed and serum was individually harvested. The concentration of bioactive IL-12 was measured by an antibody capture assay and expressed as the mean ± SE.
simultaneous injection of rIFN-γ (Fig. 6B). Together, these results indicate that IFN-γ is an absolute requirement for IL-12 production induced by the participation of Vα14+ NKT cells.

Discussion

Our earlier study demonstrated that stimulation of Vα14+CD4+ NKT cells with α-GalCer plus splenic APC results in the production of IL-12 along with IL-4. In conjunction with these observations, this study using IFN-γ−/− mice showed that IL-12 production induced in association with α-GalCer activation of these NKT cells depends on the presence of IFN-γ.

IL-12 production by DC/macrophages is induced through two different pathways of stimulation: one that occurs when the cells were exposed to various pathogens or their products (35,36) and the other when DC/macrophages as APC interact with activated T cells (26,31). The latter pathway is mediated via molecular interactions between CD40 constitutively expressed on DC/macrophages (APC) and CD40L that is induced on TCR-stimulated T cells (26,31). Thus, IL-12 is produced by DC/macrophages through T cell-independent (bacterial stimulation) and T cell-dependent (CD40L stimulation) pathways. Initial studies concerned with the interaction between DC/macrophages and conventional T cells, and revealed that among these, only CD4+ T cells can express CD40L upon activation and stimulate IL-12 production of DC/macrophages (26). Subsequently, this interaction was shown to be applied for the Vα14+CD4+ NKT cell subpopulation (18). Thus, two different subpopulations, Vα14+CD4+ NKT cell and conventional CD4+ T cell subpopulations, that function in innate and acquired immunity respectively are responsible for the stimulation of DC/macrophages for IL-12 production.

The production of a given cytokine is regulated by the cumulative effect of other cytokine signals (37–42). IL-12 production has also been shown to be influenced by other cytokines such as IFN-γ and IL-10 (19–21,35,36,43,44). While IL-12 stimulates IFN-γ production of NK/NKT and T cells (45,46), IFN-γ has the capacity to up-regulate IL-12 production of DC/macrophages (19–21). However, this IFN-γ effect has been recognized mainly for the above-mentioned T-independent (bacterial stimulation) pathway. Because CD40L-expressing CHO cells can directly stimulate DC/macrophages for IL-12 production in the absence of exogenous IFN-γ (26,31), IFN-γ has been regarded to be not an absolute requirement for IL-12 production in the CD40L-stimulation pathway. This was confirmed in a recent paper (47). Nevertheless, the requirement for IFN-γ in IL-12 production in the two pathways appears to be more complicated as below discussed.

Heinzel et al. examined whether IFN-γ deficiency alters the in vivo IL-12 production occurring in the mouse model of acute endotoxemia (48). Their results showed that IFN-γ−/− mice produced as much IL-12 p70 as wild-type mice did following injection with LPS, indicating that IFN-γ is not required for IL-12 production in the bacterial (LPS) stimulation pathway. This apparently contrasts with the well-accepted view of IFN-γ requirement in this pathway (19–21).

Contrary to the effect of IFN-γ on IL-12 production in the bacterial stimulation pathway, the present results demonstrated that the IFN-γ-deficient state fails to induce IL-12 production in the CD40L-stimulation pathway involving the activation of Vα14+CD4+ NKT cells instead of conventional CD4+ T cells. It is obvious that Vα14+CD4+ NKT cells are activated with α-GalCer in IFN-γ−/− splenocyte cultures at similar degrees to those observed in wild-type cultures, because IL-4 production and CD40L expression were similarly induced in both types of cultures. Moreover, DC prepared from wild-type and IFN-γ−/− mice exhibited comparable levels of CD40 expression. Nevertheless, IL-12 production in IFN-γ−/− splenocyte cultures was at almost zero levels. IL-12 production of IFN-γ−/− DC/macrophages stimulated directly by CD40L-expressing CHO cells was also reduced compared to that of wild-type DC/macrophages, although it was not decreased to zero level. Thus, IFN-γ deficiency alters IL-12 production in the T cell-dependent (CD40L stimulation) pathway. It should be noted that such an alteration is much more evident in the process of IL-12 production involving Vα14+CD4+ NKT cells as a DC/macrophages-stimulating population.

The consideration will be necessary regarding how IFN-γ is required for the process of DC/macrophages IL-12 production in which Vα14+ NKT cells participate. In this context, our previous study (18) showed that IL-12 production in wild-type splenocyte cultures was not affected by addition of anti-IFN-γ mAb. These observations suggested that IFN-γ is not required for IL-12 production in α-GalCer-stimulated cultures, which appears to be not compatible with the present observations. However, our previous and present results regarding the requirement of IFN-γ may not necessarily be discordant. It is possible that DC/macrophages from wild-type mice have already been exposed to endogenous IFN-γ before harvesting and rendered responsive to CD40-mediated signaling, whereas DC/macrophages from IFN-γ−/− mice have not. Considering that DC themselves are capable of producing IFN-γ (49), it is likely that there exists a difference between wild-type and IFN-γ−/− DC in terms of whether they have acquired responsiveness to CD40 stimulation in vivo. In this hypothesis, IL-12 production induced by the α-GalCer-initiated NKT–DC interaction does not greatly differ between cultures of wild-type splenocytes with and without exogenous IFN-γ (Fig. 5) or with and without anti-IFN-γ mAb (18). In contrast, IFN-γ deficiency would critically influence α-GalCer-stimulated IL-12 production but can be restored by addition of rIFN-γ. Contrary to α-GalCer, CD40L-CHO cells also stimulate macrophages that may not produce IFN-γ. In this case, addition of rIFN-γ may enhance IL-12 production even in wild-type splenocyte cultures. It is also possible that this type of stimulation, which is much stronger than α-GalCer stimulation, induces some levels of IL-12 production even in IFN-γ deficiency. Further studies will be required to investigate the mechanism by which the presence of IFN-γ or IFN-γ pre-exposure promotes the CD40L-mediated IL-12 production.

Because the relative amounts of IL-12 and IL-4 influence the balance of Th1/Th2 differentiation, it would be critical how each of these cytokines is produced and which amount of each is produced. The present results indicate that IFN-γ deficiency alters CD40L-stimulated IL-12 production particularly in the innate immune system involving the activation of Vα14+ NKT cells. These results suggest that Th1/Th2 development is modulated in individuals with a reduced IFN-γ-producing capacity.
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Abbreviations

\[\text{α-GaLcer \ (α-galactosylceramide)}\]

APC \ (antigen-presenting cell)

CD40L \ (CD40 ligand)

DC \ (dendritic cell)

PE \ (phycoerythrin)

SN \ (supernatant)

References

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