Brief Definitive Report

Interferon γ Production by Natural Killer (NK) Cells and NK1.1⁺ T Cells upon NKR-P1 Cross-linking

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Summary

Natural killer (NK) cells play an important role in immune response by producing interferon γ (IFN-γ) as well as exhibiting cytotoxic function. IFN-γ produced by NK cells has been suggested to be involved in differentiation of T helper cells. On the other hand, the NKR-P1 molecule was recently identified as one of the important NK cell receptors, and it recognizes certain kinds of oligosaccharides on target cells and triggers NK cells for cytotoxicity. In the present study, we found that NK cells produce great amounts of IFN-γ upon cross-linking of the NKR-P1 molecule. In contrast, stimulation of NK cells with IL-2 induced proliferation without producing IFN-γ. Similar to NK cells, NK1.1⁺ T cells also produced IFN-γ upon NKR-P1 cross-linking. NK1.1⁺ T cells produced IFN-γ but not interleukin 4 (IL-4) upon NKR-P1 cross-linking, whereas they secreted both IFN-γ and IL-4 upon T cell receptor cross-linking. These results indicate that NKR-P1 is a receptor molecule on NK and NK1.1⁺ T cells that induces not only cytotoxicity but also IFN-γ production. Our findings provide a new pathway for IFN-γ production by NK and NK1.1⁺ T cells through NKR-P1 molecules; it may be essential for immune regulation.

NK cells exhibit cytotoxicity against certain tumor cells and virus-infected cells without prior sensitization and seem to represent unique lymphocytes that are involved in host defense (1–3). NK cells do not express TCR and exhibit non–MHC-restricted cytotoxicity, unlike cytotoxic T cells. Susceptibility to NK cell cytotoxicity is in part correlated with the expression of sugar chain or MHC class I antigen on target cells. From these observations, NK cells were thought to recognize target cells by using unknown molecules different from TCR and BCR. The rat NKR-P1 molecule was the first receptor protein that was thought to be involved in target cell recognition by NK cells (4, 5). The NKR-P1 molecule contains a lectin-like domain, and cross-linking of NKR-P1 induced cytotoxicity in NK cells (4, 6). NK1.1 antigen, which has been used as a specific marker for mouse NK cells, has been shown to be one of the mouse NKR-P1 molecules and to induce cytotoxicity in NK cells upon cross-linking with anti-NK1.1 mAb (6–8). Recently, Bezouska et al. (9) clearly demonstrated by using a soluble form of NKR-P1 molecule and a series of oligosaccharides that NKR-P1 is a key molecule that induces cytotoxicity of NK cells by recognizing oligosaccharides expressed on target cells. This finding established that NKR-P1 is a receptor protein of NK cells that recognizes target cells and induces cytotoxicity.

On the other hand, NK cells are known to secrete a high level of IFN-γ, and the produced IFN-γ is thought to be important for the immune response against pathogens (10–12). Recently, IL-12 produced by macrophages was found

to be a most potent inducer of IFN-γ from NK cells (13, 14), indicating that IL-12 plays a major role in NK cell activation. However, analysis of IFN-γ receptor–deficient mice showed that IFN-γ was required for IL-12 production upon infection with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) or LPS stimulation (15). Therefore, cells producing IFN-γ at the initial phase of immune defense are particularly important for immune response.

Recently, we and others have found a novel T cell population expressing NKR-P1 (NK1.1) molecule and TCRα/β in CD4⁺ T cells (16–20) and CD4⁻ CD8⁻ T cells (19–24). NK1.1⁺ T cells express an invariant TCR Vα and Vβ repertoire and seem to recognize the β2-microglobulin–associated CD1 molecule (25, 26). Furthermore, we (17) and others (27) have found that NK1.1⁺ T cells are the major population that produces high amounts of IFN-γ and IL-4 upon primary stimulation through TCR. Therefore, NK1.1⁺ T cells are considered to be a regulatory T cell population that is involved in the determination of T helper cell differentiation (17, 27–30). However, the function of the NKR-P1 molecule on the activation of NK1.1⁺ T cells remains unclear.

In the present study, we found that cross-linking of one of the mouse NKR-P1 molecules, NK1.1 Ag, with immobilized anti-NK1.1 mAb induced IFN-γ production from NK cells and NK1.1⁺ T cells in the absence of IL-12. Furthermore, NK1.1⁺ T cells did not produce IL-4 upon NKR-P1 cross-linking, unlike upon TCR cross-linking. These findings suggest that oligosaccharide recognition by

NK cells and NK1.1$^+$ T cells through NKR-P1 molecules is involved in the regulation of IFN-γ production in immune response.

Materials and Methods

Mice. C57BL/6 mice were obtained from the Shizuoka Laboratory Animal Corporation (Hamamatsu, Shizuoka Prefecture, Japan).

Cell preparation. NK cells and NK1.1$^+$ T cells were purified as previously described (19, 31). In brief, NK cells were purified from spleen of 6–8-w-old C57BL/6 mice. Splenocytes were mixed with anti-CD4 mAb (GK1.5) and anti-CD8 mAb (53.6.7) and incubated with magnetic beads (Advanced Magnetics, Inc., Cambridge, MA) coupled with goat anti–mouse IgG Ab and goat anti–rat IgG Ab (Organon Teknika Co., West Chester, PA) to remove surface Ig (sIg)$^+$ B cells and CD4$^+$ and CD8$^+$ T cells. The residual cells were then incubated with PE–anti-NK1.1 mAb and FITC–anti-CD3 mAb (PharMingen, San Diego, CA). The stained cells were sorted into NK1.1$^+$ CD3$^-$ cells by FACStar-Plus® (Becton Dickinson, Mountain View, CA). NK1.1$^+$ T cells were prepared from thymus. Thymocytes were treated with anti-HSA mAb (J11d) followed by complement treatment. The resultant cells were incubated with anti-CD8 and anti–MEL-14 mAbs. Thereafter, CD8$^+$ MEL-14$^+$ cells were removed by use of magnetic beads coupled with goat anti–rat IgG Ab. The residual cells were then incubated with PE–anti-NK1.1 mAb and FITC–anti-CD3 mAb. The stained cells were sorted into NK1.1$^+$ CD3$^+$ cells. The purity of the sorted cells was always more than 99%.

Cell Culture and Stimulation. Basically, purified cells were cultured in RPMI 1640 supplemented with 10% FCS, kanamycin (100 µg/ml), and 5 × 10^{-5} M 2-ME. NK cells were expanded by culturing freshly purified NK cells for 3 d in the presence of 1,000 U/ml human recombinant IL-2 (kindly provided by Dr. J. Hamuro, Ajinomoto Co., Inc., Kawasaki, Japan). 10^5 NK cells and NK1.1$^+$ T cells were stimulated with immobilized anti-NK1.1 or anti–TCR-β mAbs, or with recombinant mouse IL-12 (Pharmingen, 500 pg/ml IL-12 induced almost maximum production of IFN-γ) for certain periods. Anti-NK1.1 mAb or anti–TCR-β mAb was immobilized on 96-well flat-bottomed culture plate (Costar, Cambridge, MA) by incubating for 2 h at 37°C at certain concentrations in PBS. In some experiments, NK cells were stimulated in the presence of anti–IL-12 mAb (clone C17.8, generous gift of Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA). After the cultures, [^{3}H]thymidine incorporation and concentration of IFN-γ in the culture supernatant were determined. All data are presented as mean ± SD from triplicate cultures.

Measurement of IFN-γ and IL-4. IFN-γ and IL-4 were measured by ELISA by standard protocol. The mAbs used to capture IL-4 and IFN-γ were anti–IL-4 (BVD4-1D11) and IFN-γ (R4-6A2) mAbs, respectively. The mAbs used to detect captured IL-4 and IFN-γ were biotinylated anti–IL-4 (BVD6-24G2) and biotinylated anti–IFN-γ (XMG1.2) mAbs, respectively. These antibodies were purchased from PharMingen. Concentrations of IL-4 and IFN-γ were determined with recombinant IFN-γ and IL-4 as standard.

Results

IFN-γ Production by NK Cells upon Cross-linking of NKR-P1. To analyze the function of NKR-P1 on mouse NK cells, NK1.1$^+$ CD3$^-$ cells were isolated by FACS® sorting as

the NK cell population from spleen (1, 7). Fig. 1 *A* shows the purity of NK cells used in this study; >99% of the sorted cells were NK1.1$^+$ and CD3$^-$. FACS®-purified NK cells were expanded for 3 d in the presence of IL-2 and then were harvested and analyzed for IFN-γ production and proliferative response upon NKR-P1 cross-linking with immobilized anti-NK1.1 mAb. The purity of the NK cells was retained during the 3-d culture period (data not shown).

When NK cells were stimulated with immobilized anti-NK1.1 mAb in the presence of 1,000 U/ml IL-2, they produced a considerable level of IFN-γ (Fig. 2 *A*), the amount being dependent on the concentration of anti-NK1.1 mAb used. Stimulation with IL-2 alone did not induce production of IFN-γ from NK cells. On the other hand, NK cells produced only a small amount of IFN-γ in the absence of IL-2. Production of IFN-γ upon NKR-P1 cross-linking was dependent on the concentration of IL-2 (Fig. 2 *B*). Stimulation of NK cells with immobilized anti–TCR-β mAb (H57.597, 100 µg/ml) did not induce IFN-γ production (data not shown). Furthermore, the amount of IFN-γ produced upon NKR-P1 cross-linking was as high as that produced upon IL-12 stimulation (Fig. 2 *C*). Similar to the stimulation via NKR-P1, IFN-γ production by the stimulation with IL-12 was also dependent on the presence of IL-2. Only a low level of IFN-γ was produced upon IL-12 stimulation in the absence of IL-2. On the other hand, the presence of neutralizing anti–IL-12 mAb did not affect IFN-γ production upon NKR-P1 cross-linking (Fig. 3), indicating that IFN-γ production by NK cells upon NKR-P1 cross-linking is not due to the effect of IL-12. These results showed that NKR-P1 transduces signals for IFN-γ production in NK cells in a manner independent of IL-12.

Regulation of NK Cell Proliferation by NKR-P1. The effect of cross-linking of NKR-P1 on the proliferation of NK cells was examined next. In the absence of IL-2, cross-
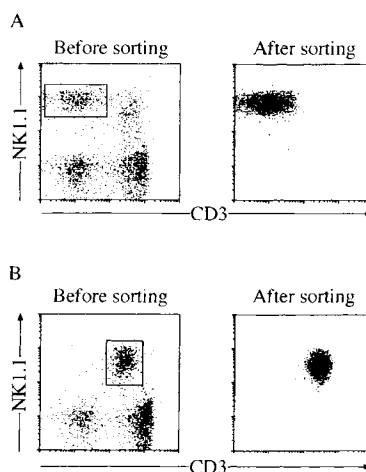
Figure 1. Isolation of NK cells and NK1.1$^+$ T cells. Cells were stained for NK1.1 and CD3 and sorted into NK1.1$^+$ CD3$^-$ population (NK cells) (*A*) or NK1.1$^+$ CD3$^+$ population (NK1.1$^+$ T cells) (*B*). The purity of sorted cells was more than 99%.
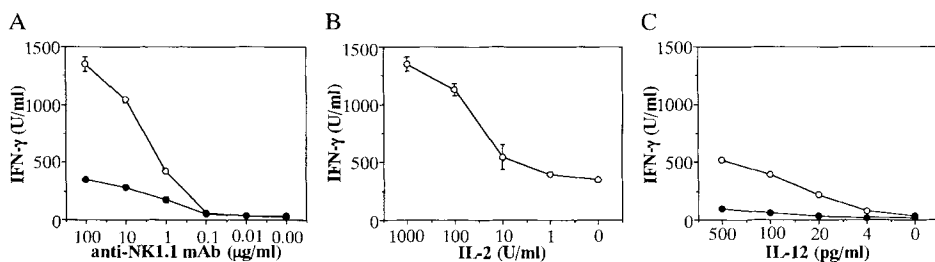
Figure 2. Production of IFN-γ by NK cells upon cross-linking of NKR-P1 or IL-12 treatment. (*A*) NK cells were stimulated for 2 d by NKR-P1 cross-linking with anti-NK1.1 mAb immobilized at indicated concentrations in the presence (*open circles*) or absence (*closed circles*) of 1,000 U/ml IL-2. (*B*) NK cells were stimulated by NKR-P1 cross-linking with immobilized anti-NK1.1 mAb (100 μg/ml) at graded concentrations of IL-2. (*C*) NK cells were stimulated for 2 d with IL-12 at various concentrations in the presence (*open circles*) or absence (*closed circles*) of 1,000 U/ml IL-2. Representative data from three independent experiments are shown.

linking of NKR-P1 as well as IL-12 showed significant [³H]thymidine uptake at 18 h after stimulation. However, when [³H]thymidine uptake was analyzed at 48 h after stimulation, only weak [³H]thymidine uptake was observed (Fig. 4 *A*). Although NK cells proliferated well in the presence of IL-2, the [³H]thymidine uptake was significantly reduced upon NKR-P1 cross-linking (Fig. 4 *B*). Inhibition of NK cell proliferation by NKR-P1 cross-linking was also observed even with low concentrations of IL-2 (Fig. 4 *C*), suggesting that the reduction of [³H]thymidine uptake upon NKR-P1 cross-linking was not due to the overgrowth of NK cells upon stimulation with IL-2 and NKR-P1 cross-linking. Similarly, stimulation of NK cells with IL-12 also reduced the [³H]thymidine uptake induced by IL-2 (Fig. 4 *D*). Exogenous IFN-γ showed no effect on the proliferative response of NK cells to the stimulation with IL-2 (data not shown). These results demonstrated that signals via NKR-P1 regulate NK cell proliferation, and also that NKR-P1 cross-linking exerts a different effect on the proliferation of NK cells dependent on the presence of IL-2.

IFN-γ but Not IL-4 Production by NK1.1⁺ T Cells upon NKR-P1 Cross-linking. Next, to analyze the function of the NKR-P1 (NK1.1) molecule expressed on NK1.1⁺ T cells, we purified NK1.1⁺ CD3⁺ T cells from thymus by cell sorting (see Fig. 1 *B*). Similar to NK cells, when NK1.1⁺ T cells were stimulated by NKR-P1 cross-linking in the presence of IL-2, these cells produced a significant amount of IFN-γ during 3 d culture (Fig. 5 *A*). However, these cells hardly produced IL-4 upon NKR-P1 cross-linking (Fig. 5 *B*). IL-12 also induced the production of IFN-γ but not IL-4 from NK1.1⁺ T cells. On the other hand, consistent with our previous report (17), when NK1.1⁺ T cells were

stimulated with anti–TCR-β mAb, both IL-4 and IFN-γ were equally produced. Even when cells were stimulated with low concentrations of anti–TCR-β mAb, IL-4 was readily detected at an equal level to IFN-γ (data not shown). These results demonstrate that NK1.1⁺ T cells have two functional receptors for IFN-γ production: TCR and NKR-P1.

Discussion

In the present study, we found that signaling via NKR-P1 is involved in IFN-γ production by NK cells and NK1.1⁺ T cells. NKR-P1 cross-linking induced a high level of IFN-γ from NK cells compared with that produced by IL-12 stimulation. This suggests that signaling via NKR-P1 is one of the most effective signals for IFN-γ production by
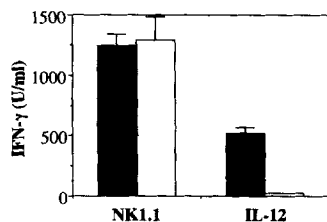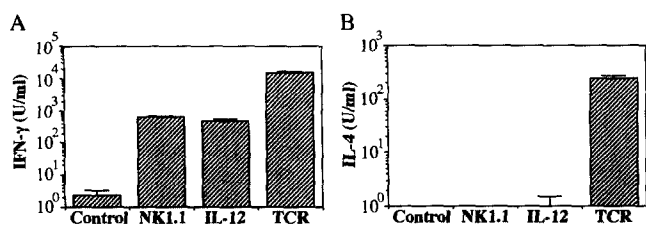

Figure 3. Production of IFN-γ by NK cells upon cross-linking of NKR-P1 in the presence of neutralizing anti–IL-12 mAb. NK cells were stimulated with immobilized anti-NK1.1 mAb (100 μg/ml) or IL-12 (100 pg/ml) in the presence (*open histogram*) or absence (*closed histogram*) of 50 μg/ml anti–IL-12 mAb. We observed that 0.5 μg/ml anti–IL-12 mAb was enough to neutralize 1 ng/ml IL-12. Representative data from three independent experiments are shown.
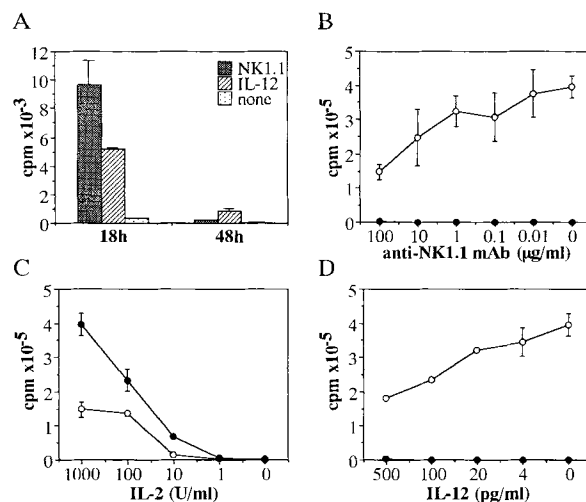
Figure 4. Regulation of proliferative response of NK cells by cross-linking of NKR-P1 or IL-12. (*A*) NK cells were stimulated for indicated periods with immobilized anti-NK1.1 mAb (100 μg/ml) or 5 ng/ml IL-12 in the absence of IL-2 and analyzed for [³H]thymidine uptake. (*B*) NK cells were stimulated for 2 d with anti-NK1.1 mAb immobilized at graded concentrations in the presence (*open circles*) or absence (*closed circles*) of 1,000 U/ml IL-2. (*C*) NK cells were cultured at graded concentrations of IL-2 in the presence (*open circles*) or absence (*closed circles*) of immobilized anti-NK1.1 mAb (100 μg/ml). (*D*) NK cells were stimulated for 2 d with IL-12 at graded concentrations in the presence (*open circles*) or absence (*closed circles*) of 1,000 U/ml IL-2. Representative data from three independent experiments are shown.

A

B

Figure 5. Production of IFN-γ by NK1.1⁺ T cells upon NKR-P1 cross-linking, IL-12 treatment, and TCR cross-linking. NK1.1⁺ T cells were stimulated with immobilized anti-NK1.1 mAb (100 μg/ml), anti-TCR-β mAb (100 μg/ml), or IL-12 (500 pg/ml) in the presence of IL-2 (1,000 U/ml) for 3 d, and the amounts of IFN-γ (A) and IL-4 (B) in the culture supernatant were analyzed by an ELISA assay. Representative data from three independent experiments are shown.

NK cells. Recently, the ligand for NKR-P1 was identified as certain kinds of oligosaccharides. Recognition of these oligosaccharides on target cells by NKR-P1 was essential for induction of NK cell cytotoxicity (9). Therefore, it is likely that NK cells produce IFN-γ upon recognition of such oligosaccharides by the NKR-P1 molecule. Although the distribution of the oligosaccharides as the ligand of NKR-P1 is unclear at present, it is likely that IFN-γ produced by NK cells upon stimulation with oligosaccharides on virus-infected cells or tumor cells regulates the immune response against these cells. Indeed, IFN-γ produced by NK cells is involved in immune defense against certain kinds of pathogens (10–12).

Although NK cells produced significant amounts of IFN-γ even in the absence of IL-2, exogenous IL-2 was found to significantly increase the production of IFN-γ upon NKR-P1 cross-linking as well as stimulation with IL-12. Although the exact mechanism of the effect of IL-2 on the NKR-P1–induced IFN-γ production is unclear, one possible role of IL-2 in the production of IFN-γ is to prevent NK cells from death in vitro, because NK cells die readily in vitro in the absence of IL-2. Since NK and NK1.1⁺ T cells constitutively express β and γ chains of the IL-2 receptor, signaling via IL-2R may be involved in the maintenance of NK cells in vivo.

Another unique effect of NKR-P1 cross-linking on NK cells was the regulation of the proliferative response. Whereas the signal via NKR-P1 induced proliferation of NK cells in the initial phase after stimulation in the absence of IL-2, the same stimulus inhibited the IL-2–induced proliferative response. A similar effect was observed when NK cells were stimulated with IL-12. Since exogenous IFN-γ did not influence NK cell growth, the growth inhibition upon stimulation through NKR-P1 was not mediated by IFN-γ produced upon NKR-P1 cross-linking. Inhibition of IL-2-induced proliferation has also been observed when

T cell clones are activated via TCR, and it is thought to be involved in the prevention of hyperresponsiveness of T cells (32). Although the physiological meanings of the growth inhibition upon NKR-P1 cross-linking have to be determined, NKR-P1 transduces signals important for regulating growth of NK cells.

We found that not only NK cells but also NK1.1⁺ T cells produced IFN-γ upon cross-linking of NKR-P1. Interestingly, whereas NK1.1⁺ T cells produced both IL-4 and IFN-γ upon TCR stimulation, stimulation of NKR-P1 did not induce IL-4 production from NK1.1⁺ T cells. It is unlikely that this effect was due to weak signals transduced through NKR-P1 as compared with those through TCR, because both IL-4 and IFN-γ were equally produced upon stimulation with a low concentration of anti-TCR mAb (data not shown). These observations suggest an important role of NKR-P1 in the differentiation of Th1/Th2 cells in immune response by regulating lymphokine production by NK1.1⁺ T cells (27, 29, 30). The different pattern of lymphokine production upon cross-linking of these two different Ag receptors, TCR and NKR-P1, suggests the existence of distinct signaling pathways mediated through these two receptors.

Cross-linking of NKR-P1 is known to trigger a Ca^{2+} influx and inositol phosphate metabolism (9, 33). Furthermore, cytoplasmic domains of NKR-P1 molecule have a consensus motif (Cys-X-Cys-Pro) involved in the association of CD4 and CD8 molecules with the tyrosine kinase p56[lck]. However, the mechanism of the signaling pathway via NKR-P1, including the association between NKR-P1 and p56[lck], has not been investigated. Because the ligand for NKR-P1 was identified to be certain oligosaccharides and NKR-P1 triggers not only cytotoxicity but also IFN-γ production by NK cells and NK1.1⁺ T cells, the analysis of signal transduction through NKR-P1 is crucial for elucidating the activation mechanism of NK cells and NK1.1⁺ T cells.

Finally, in the present study, we showed that signals via NKR-P1 effectively induced IFN-γ production both from NK cells and NK1.1⁺ T cells. IFN-γ produced by NK cells is known to play a role in determining Th cell differentiation (2, 10). While IL-12 exerts a remarkable effect on the induction of IFN-γ by NK cells, IFN-γ seems to be required for IL-12 production by macrophage after BCG infection (15). Therefore, there is the possibility that IFN-γ production by NK cells via NKR-P1, independent of the IL-12 pathway, may have an important role in immune regulation. The analysis of the distribution of oligosaccharides, as the ligand of NKR-P1, and the contribution of NKR-P1 to the immune response will clarify the physiological role of IFN-γ production via NKR-P1.

Note added in proof: IFN-γ production from NK cells was also observed even when NKR-P1 was cross-linked by immobilized $(Fab')_2$ fragment of anti-NK1.1 mAb. This excludes the involvement of Fc receptor in the IFN-γ production observed in this study.

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