Expression of IFN-γ Upon Triggering of Activating Ly49D NK Receptors In Vitro and In Vivo: Costimulation with IL-12 or IL-18 Overrides Inhibitory Receptors[1]

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NK cells can express both activating and inhibitory Ly49 receptors on their cell surface. When cells expressing both receptors are presented with a ligand, inhibition dominates the functional outcome. In this report we demonstrate that costimulation of the activating Ly49D murine NK cell receptor with IL-12 or IL-18 is capable of over-riding the inhibitory Ly49G2 receptor blockade for cytokine production both in vitro and in vivo. This synergy is mediated by and dependent upon Ly49D-expressing NK cells and results in significant systemic expression of IFN-γ. This would place NK cells and their activating Ly-49 receptors as important initiators of microbial, antiviral, and antitumor immunity and provide a mechanism for the release of activating Ly49 receptors from inhibitory receptor blockade. *The Journal of Immunology,* **2003, 170: 1763–1769.**

The inhibitory Ly49 receptors (Ly49A, -C, -G, and -I) inhibit NK cell function upon binding of class I ligands on target cells (1–3). These Ly49 inhibitory receptors as well as inhibitory KIRs contain cytoplasmic immune receptor tyrosine-based inhibitory motifs that are phosphorylated upon stimulation, leading to the recruitment of SHP-1 phosphatase and the attenuation of intracellular signals (2, 4, 5). In contrast, the activating receptors (e.g., Ly49D and Ly49H) do not contain any immune receptor tyrosine-based inhibitory motif in their cytoplasmic domains (6–9). Ly49D has been shown to mobilize intracellular $^{2+}$Ca and mediate reverse Ab-dependent cellular cytotoxicity in the presence of specific mAb (10, 11). Activating Ly49 molecules have been shown to associate with a 12-kDa homodimeric protein, DAP12, that contains an immunoreceptor tyrosine-based activation motif critical for positive signaling by these receptors (10, 11).

Recently, we have (8, 12, 13) examined the breadth of gene expression via microarray analysis following Ly49D cross-linking and provide pharmacological analysis of the biochemical pathways that are activated upon Ly49D signaling.

Circulating NK cells expressing activating Ly49s also express coreceptor paired inhibitory Ly49s. Effector cells that express the activating Ly49D receptor that binds H2-D^d as a ligand also co-express, at very high levels, the inhibitory Ly49G2 or Ly49A (14) receptors, whose ligand is also H2-D^d, and dominate the activating function. Thus, engagement of activating Ly49 NK receptors in vivo appears constantly at odds with inhibitory forces.

As T cells require two signals to induce sufficient cellular activation, we postulated that NK cells may require two positive signals to over-ride the inhibitory receptor blockade. Thus, we sought to examine the secretory function of activating Ly49 NK receptors that might be triggered by coreceptor function both in vitro and in vivo. In this study we demonstrate that the cytokines IL-12 and IL-18 costimulate with cross-linking or ligand binding of Ly49D receptors and overcome the effects of Ly49 inhibitory receptors.

Materials and Methods

NK cell isolation

Liver NK cells were isolated from C57BL/6 (B6) mice and grown for 7–10 days in 1000 IU/ml rIL-2 (Chiron Corp., Emeryville, CA) (15). Liver NK cells were isolated from IL-2-treated mice (16). Liver mononuclear cells were used either fresh or after IL-2 expansion and were 35–70% CD3$^-$, NK1.1$^+$. In experiments involving sorted NK cells, the population of Ly49D$^+$, G2$^+$ NK cells contains 12–22% Ly49 C/I-positive NK cells. Experiments with freshly isolated NK cells were performed with cells isolated by negative selection with anti-CD3 and CD19 and/or specific Ly49s (e.g., Ly49C/I).

Abs used

The mAb 4E5 (Ly49D) has been previously described (17). Rat IgG (BD Biosciences/BD PharMingen, San Jose, CA) was used as a control for flow cytometric and functional studies. Rabbit F(ab′)$_2$ anti-rat IgG was used as a cross-linking reagent. NK1.1-PE, DX-5-PE, and CD3e-PcP (BD Biosciences/BD PharMingen) as well as 4E5-FITC were used for flow cytometric analysis. 3D10 (Ly49H) was provided by Dr. W. Yokoyama (Washington University, St. Louis, MO). IFN-γ cytoplasmic detection was performed using kits purchased from BD Biosciences/BD PharMingen.

Flow cytometry analysis

Cells were stained as previously described (15) and analyzed on an LSR flow cytometer (BD Biosciences). Cells were directly stained using FITC-, PE-, PerCP-, and allophycocyanin-labeled primary abs. Cells were sorted using a Cytomation AMoFlo cytometer, then were expanded in IL-2 to allow regeneration of surface receptors.

In vitro activation

Activations with cytokines in vitro for analysis were performed in RPMI 1640 medium containing 5% FCS and the following concentrations of cytokines or Abs: Ly49D (4E5), 1 μg/10^6 cells; Ly49G2 (4D11), 1 μg/10^6 cells; IL-2, 1000 IU/cc; IL-12, 100 ng/μl; and IL-18, 100 ng/μl.

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Receptor cross-linking and cytokine measurement

Cytokines were measured using IFN-γ and chemokine ELISA kits (R&D Systems, Minneapolis, MN). Cell stimulations were performed at cell concentrations of 1–5 $\times$ 10^6 cells/ml. Abs (anti-Ly49D or Ly49G2) were added at a concentration of 1 μg/10^6 cells for 30 min at 4°C. Cells were then washed and plated on 24-well Costar (Corning, NY) plates that were precoated with 2 μg/well rabbit $F(ab')_2$ anti-rat IgG and blocked with medium containing 10% FCS. Unless otherwise stated, samples collected after 5- to 6-h incubation (37°C, 5% CO_2) were measured in duplicate against the assay standard curve, and were reported as picograms per milliliter. In all assays the SD was $<$5 pg/ml.

RNase protection assay

The multiprobe RNase protection assay was performed using the mck-1 or mck-5 template set (BD PharMingen, San Diego, CA) (18).

STAT4-PO_4 detection and inhibitors

A flow cytometric detection technique was used to evaluate tyrosine-phosphorylated STAT-4. Briefly, cells were stimulated, fixed, permeabilized, and stained with Abs to STAT and STAT4-PO_4 as previously described (19). The mean channel expression of STAT-PO4 indicated the degree of STAT-4 phosphorylation. Specific inhibitors of mitogen-activated protein (MAP)[3] kinases ((PD98059 (catalogue no. 513000; 2′-amino-3′-methoxyflavone) and SB 203580 (catalogue no. 559389; 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole)) were purchased from Calbiochem (San Diego, CA).

Mice and tumor cells

C57BL/6 mice were obtained from the animal production area of the National Cancer Institute (Frederick, MD). Mice were maintained in a dedicated pathogen-free environment and were used between 8 and 10 wk of age. Animal care was provided in accordance with the procedures outlined previously (19a). The transplantable mouse leukemia line L5 Mf22 (H-2^b), which was transfected with a neo-empty vector (L5-neo) and a vector expressing H-$2D^d$ (L5-Dd; H-$2^{b/Dd}$), was used as previously described (20). Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23).

Reagents

Recombinant murine IL-18 was purchased from PeproTech (Rocky Hill, NJ). For in vivo administration, aliquots of stock IL-18 (100 ng/μl) were diluted with HBBS containing 0.1% (v/v) sterile-filtered C57BL/6 mouse serum. Recombinant human IL-2 was provided by Chiron (Emeryville, CA). After reconstitution with sterile water, IL-2 (1000 IU) was diluted with HBSS containing 0.1% C57BL/6 mouse serum. Recombinant murine IL-12 was provided by Genetics Institute (Cambridge, MA) and Hoffmann-La Roche (Nutley, NJ) and was used at a stock concentration of 100 ng/μl. For in vivo drug administration, stock aliquots were diluted with PBS containing 0.1% (v/v). For those experiments 200 μl of a 1/2 dilution of anti-NK1.1 ascites fluid was injected i.p. on days 4 and 9 after the injection of 3LL tumor, and the effects of this regimen were analyzed 6 days after the last injection of Ab.

Tumor models and in vivo treatment

Cohorts of four to six mice per group were used in the present studies unless otherwise noted. To generate liver tumor burden, a modification of a previously described renal protocol was used (21). Tumor cells (5×10^5) were injected intrasplenically into mice. To investigate the impact of the administration of IL-18 and IL-12 on the in vivo activation of NK cells in a tumor-bearing host, IL-12 (1 μg) (22), IL-18 (2.5 μg) (23), or vehicle alone was administered i.p. 1 h after intrasplenic tumor was injected and after splenectomy. These doses were based on previous studies (22, 23) demonstrating that this protocol generated significant and sustained levels of serum cytokine. Liver tissue was obtained at the indicated time points and processed for RNA isolation or cytologic analysis. Cohorts of control mice received tumor without IL-12 or IL-18 and/or IL-12 or IL-18 alone. Mice from the respective treatment groups were euthanized, and serum samples were obtained for cytokine evaluation from individual mice via tail bleeding.

[3] Abbreviations used in this paper: MAP, mitogen-activated protein; MIP, macrophage inflammatory protein.

In vivo depletion experiments

NK cell subsets were depleted in vivo by i.p. injection of mAbs directed against Ly49D (anti-4E5) under conditions that led to $>$95% depletion of each subset. Two doses of Ab (100 μg/dose) were administered on days 3 and 1 before the beginning of therapy. Control depletions were performed with isotype-matched rat Ig.

Results

In vitro synergy between Ly49 receptor ligation and cytokine receptor engagement

To examine whether NK receptors could cooperate with other agents that are known to mediate potent second signals, we examined mRNA expression using IL-2-expanded liver NK cells treated with Ly49D cross-linking and/or cytokines. IL-2 expansion permitted us to obtain more highly purified cells in sufficient numbers for analysis. Fig. 1 demonstrates a representative result of such an experiment. As previously shown (14), upon cross-linking of the Ly49D-activating receptor, NK cells expressed numerous cytokines (TNF-α, IFN-γ, lymphotactin) and chemokines (macrophage inflammatory protein-1α (MIP-1α) and MIP1β) relative to control cells. However, when these same cells were costimulated with IL-2, IL-12, and IL-18, a selective synergy in IFN-γ mRNA was observed. The degree of synergy observed with Ly49D receptor ligation and IL-12 or IL-18 was similar to that seen with IL-12 and IL-18 combined, a known potent coactivation model of IFN-γ expression. However, stimulation of NK cells upon receptor ligation did not induce NK cells to express IL-12, IL-18, or IL-23 mRNA or increase IL-12 or IL-18 receptor mRNA levels (not shown). In contrast to IFN-γ, other cytokines and chemokines were not further activated by additional coligation of IL-2, IL-12, or IL-18. This activation was selective, since IL-2, a known potent activator of NK cell lytic function, has little or no effect on IFN-γ mRNA when combined with Ly49D activation. Likewise, IL-4 suppressed the activation via Ly49D, a result previously shown in human NK cells for generation of lymphokine-activated killer activity (24, 25). The kinetics of mRNA and IFN-γ production under these conditions are shown in Fig. 1, *B* and *C*. The data indicate a rapid and sustained activation of mRNA and production of IFN-γ when cells were stimulated with both IL-12 and receptor ligation. The data in Fig. 1*A* show that IFN-γ induction (20-fold) was further induced to 60- to 70-fold by IL-12 and IL-18, but not by IL-2. Stimulation by IL-2, IL-12, or IL-18 alone was very modest compared with that in control cells (see Fig. 1*A*). Thus, a potent and specific synergy was observed between Ly49D and IL-12/IL-18 for IFN-γ gene expression.

Activation of primary and cultured NK cells: synergy with IL-12 or IL-18

To determine whether the synergy observed in Fig. 1 is also seen with primary NK cells, freshly isolated leukocytes from mouse liver (14% NK, 23% NKT, 45% T) were analyzed using a similar experimental scheme. NK cells were coligated with either Ab specific for Ly49D (not present on either NKT or T cells) with or without coligation of IL-2, IL-12, and IL-18. Both fresh and IL-2-expanded NK cells demonstrated a potent mRNA synergy (15- to 30-fold) with IL-12 or IL-18 and a potent induction of IFN-γ protein not seen with MIP-1α (Fig. 2).

IL-12 over-rides inhibitory receptors

These data indicate that the combination stimulation of NK cells by Ly49D and IL-12 results in a synergistic induction of cytokine gene expression. However, a key question is whether such a costimulation can over-ride the negative signal delivered by Ly49G2 during the recognition of H2-D^d. To test this hypothesis, sorted

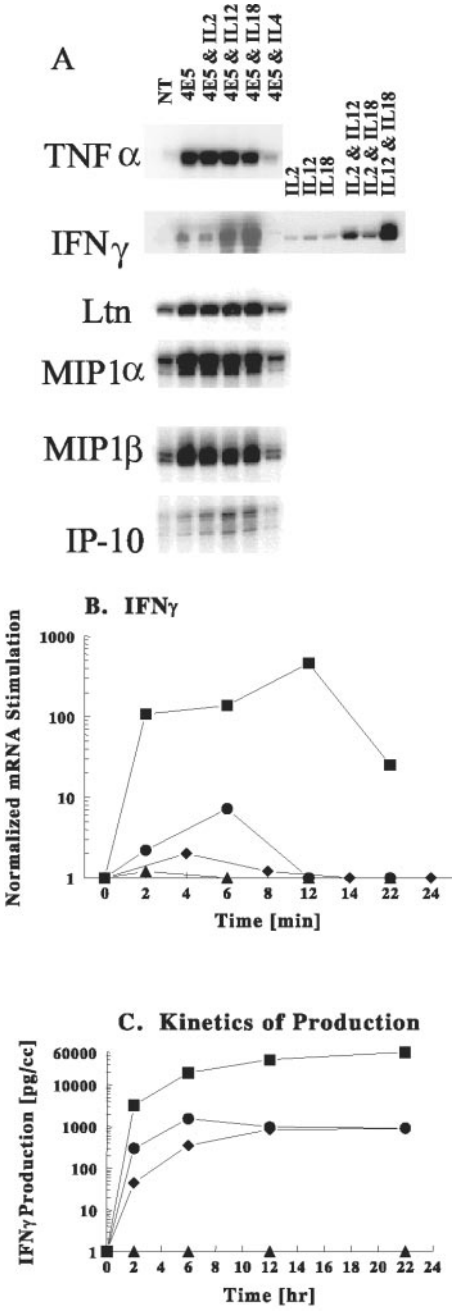

FIGURE 1. *A*, RNase protection analysis (RPA) of cytokine/chemokine mRNA expression. IL-2-induced NK cells from mouse liver (70% $NK1.1^+$, $CD3^-$) were stimulated by cross-linking Ly49D (4E5) and combinations of IL-2, IL-12, IL-18, and IL-14 for 2 h. The kinetics of IFN-γ mRNA (*B*) and protein (*C*) are shown for $Ly49D^+$, $G2^+$ NK cells untreated (▲) or treated with IL-12 (♦), anti-Ly49D (●), or both (■). Cells or cell supernatants were examined for mRNA expression by RPA or supernatant IFN by ELISA. Total RNA (1 μg) was hybridized to the mck-1 and mck-5 multiprobe templates (BD PharMingen) or the chemokine custom template (Torrey Pines Biolab, Houston, TX) as described in *Materials and Methods*. The data shown represent five independent experiments. Ltn, lymphotactin.

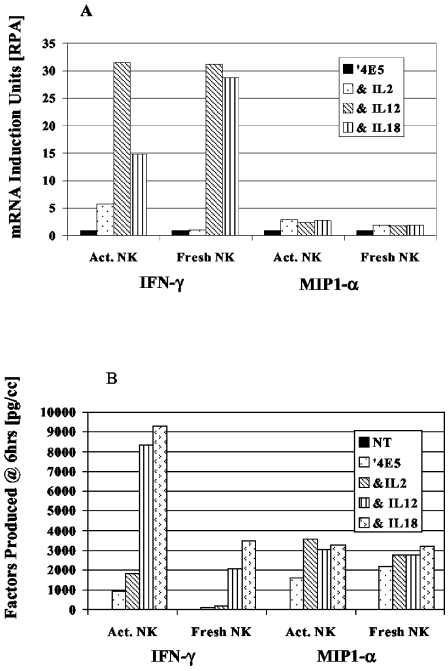

FIGURE 2. *A*, RNA quantitation for fresh or IL-2 cultured NK cells. RNase protection analysis of cytokine/chemokine mRNA expression was performed on freshly isolated (45% $NK1^+$, $CD3^-$) or IL-2-induced NK cells from mouse liver (65% $NK1.1^+$, $CD3^-$) stimulated by cross-linking Ly49D (4E5) and combinations of IL-2, IL-12, IL-18, and IL-4 for 2 h. Density analysis of RPA bands as total density units is shown for IFN-γ and MIP-1α. *B*, Cytokine/chemokine production by fresh or IL-2-cultured NK cells. Supernatants from cells analyzed in *A* were collected after 6 h and analyzed for supernatant factors by ELISA. Production (picograms per cubic centimeters) is shown for IFN-γ and MIP-1α. The data shown represent three independent experiments.

NK cells that coexpressed both Ly49D and Ly49G2 (>95% double-positive) were evaluated by in vitro cosignaling using receptor cross-linking with Ab (Table I*A*) and targets that express the ligand for both Ly49G2 and Ly49D, H-$2D^d$. When untreated NK cells were stimulated through their activating receptor, activation by anti-Ly49D could be strongly inhibited if the inhibitory Ly49G2 were coengaged (Table I*A*). However, if NK cells were cotreated with either IL-12 or IL-18, this strong inhibition was not observed. Both IL-12 and IL-18 treatment resulted in 80% of the expected IFN-γ production. In contrast, stimulation of NK cells with H-2^b targets with or without IL-12 did not result in significant IFN-γ production, whereas targets expressing the ligand for Ly49D (H-$2^{b/Dd}$) generated IFN-γ when costimulated with IL-12, but failed to produce cytokine in the absence of IL-12. In addition to H-$2D^d$, CHO cells have been shown to be a ligand for both Ly49D (26) and Ly49G2 (27). When CHO cells were used, the strong inhibition of IFN-γ production and MIP-1α observed in the untreated cells was overcome by addition of IL-12 (Table I*B* and data not shown). Thus, receptor cross-linking that occurs with either Ab or target ligand and that normally results in diminished or lack of a positive signal due to the inhibitory Ly49 receptors can be overcome by addition of IL-12.

In vivo synergy of Ly49D and IL-12 or IL-18

The results in Table I suggest that IL-12 and IL-18 costimulation can over-ride the inhibitory blockade of both receptor cross-linking and target interaction in vitro. To test this hypothesis in vivo, an intrasplenic tumor cell model system (mouse B cell leukemia, L5 MF22 (H-2^b) and L5 M-D105 (H-$2^{b/Dd}$)) that results in rapid migration of tumor cells into the liver was employed. Animals were splenectomized and after 1 h were injected with PBS, IL-12, or IL-18. As shown in Fig. 3*A*, there was significant production of IFN-γ observed only when L5 M-D105 (H-$2^{b/Dd}$) was injected with IL-12 (serum protein levels peaked at 24 h). Neither agent

Table I. *Reversal of inhibitory effect in Ly49G2$^+$, Ly49D$^+$ NK cells by IL-12*[a]

Treatment	Control		IL-12		IL-18		IL-12/IL-18
	IFN-γ	% Control response	IFN-γ	% Control response	IFN-γ	% Control response	IFN-γ
Ab cross-link[a]							
Control	0		83		429		10,500
Anti-Ly49D (4E5)	879	100	7,731	100	2,404	100	
Anti-Ly49G2 (4D11)	29	3	266	3	801	33	
Both	348	40	6,367	82	1,952	81	
Target-induced effect[b]							
Targets added	NT		+IL-12				
L5M H-2^b	0		0				
L5-D4 H-2$^{b/Dd}$	0		240				
CHO	0		1,723				

[a] NK cells were pretreated at 4°C with 1 μg/10^6, of rat Ab to the designated Ly49 molecules, washed, and activated on a plastic dish coated with F(ab′)$_2$ goat anti-rat Fc Ig for 6 h at 37°C. Supernatants were collected and evaluated for cytokine production.

[b] NK cells were mixed with target cells at a 2:1 ratio for 6 h; supernatants were collected and evaluated for cytokine production. This is a representation of more than five experiments.

alone was able to induce measurable IFN-γ. As shown in Fig. 3*B*, the kinetics of IFN-γ were very rapid when IL-18 was injected; however, the magnitude was 10-fold less. Again, the induction of IFN-γ production was only seen when targets expressing a ligand for Ly49D were coinjected with IL-18. Injection of L5 MF22 did not induce endogenous IL-12 or IL-18 serum expression. Serum IL-12 or IL-18 was only elevated in groups of mice receiving i.p. injection of the appropriate cytokine. These results indicate that the induction of circulating IFN-γ was not a result of in vivo synergy of endogenous IL-12 with injected IL-18 or endogenous IL-18 with injected IL-12.

In situ production of IFN-γ

Although the above data demonstrate a strong induction of circulating IFN-γ through coengagement of the Ly49D ligand and cytokine receptors, the cellular source of IFN-γ was not identified. Cytoplasmic staining of IFN-γ was performed on liver leukocytes using four-color analysis to simultaneously analyze CD3, NK1.1, and Ly49 expression. As shown in Table II, the injection of L5 M-D105 (H-2$^{b/Dd}$) induced ~1% of the cells to make IFN-γ; however, this level was increased to 3.9 and 3.5%, respectively, with IL-12 or IL-18. Animals injected with L5 MF22 (H-2^b) parental cells demonstrated little or no IFN-γ production. These data demonstrate that virtually all the increased IFN-γ was being produced by CD3$^-$ leukocytes, suggesting that NK cells were the source of IFN-γ. When NK, NKT, and T cells were compared, NK cells expressed >80% of the IFN-γ, as detected by cytoplasmic staining (not shown). To evaluate whether the Ly49D-positive cells were being activated by the H-2D^d ligand, subset analysis was performed by selecting for CD3$^-$, NK1.1$^+$, Ly49D$^+$, or Ly49H$^+$ NK cells. Table III demonstrates that injection of L5 M-D105 (H-2$^{b/Dd}$) induced selective increases in the percentage of Ly49D$^+$ NK cells expressing IFN-γ over basal IFN-γ production only when animals were coinjected with IL-12 or IL-18. Ly49H$^+$ NK cells did not demonstrate this pattern despite the fact that this surface molecule is a potent activating Ly49 receptor. A basal induction of IFN-γ by both the parental and H-2D^d-transfected line was seen in all NK cells, suggesting that other NK receptors can induce some low level of IFN-γ, but this induction did not change with the addition of IL-12 or IL-18. These data demonstrate that in vivo activation of the Ly49D receptor can trigger cytokine gene expression only when circulating cytokines are present, thus over-riding negative regulation.

To directly evaluate the cells responsible for serum IFN-γ, mice were depleted of Ly49D-expressing NK cells in vivo by the administration of Abs (4E5; Fig. 3*C*). In our in vivo model with H-2D^d-expressing target ligand for Ly49D, the control mice only produced significant IFN-γ when both Ly49 ligand-expressing targets and IL-12 were injected. However, depletion of this NK subset ablated production, indicating that the Ly49D-expressing NK cells are the source of serum IFN-γ. These results rule out the possibility that the increased IFN-γ production in vivo was the result of generalized expression of circulating cytokines (e.g., IL-12 or IL-18) in response to injected IL-12 or IL-18.

Mechanism of activation

Next we evaluated the potential mechanism leading to increased gene expression resulting from this coreceptor activation. It is well established that NK receptor ligation results in rapid activation of the coreceptor DAP12. Evaluation of the DAP12 phosphorylation status in NK cells ligated with either anti-activator (Ly49D) or anti-inhibitor (Ly49G2) with IL-12 pretreatment did not alter the DAP phosphorylation kinetics (not shown). Thus, the immediate and proximal membrane signaling through the Ly49D receptor did not change. Since IL-12 signaling has been reported to involve STAT4 (28) and the p38 MAP kinase pathway, whereas Ly49D involves p42/44 MAP kinase (29), we examined these downstream pathways during coreceptor ligation. Fig. 4*A* demonstrates that IL-12 and activating NK receptor ligation result in an increased and sustained phosphorylation of STAT4. However, sustained activation of STAT4 was also seen in cells simultaneously coligated with Ly49G2 inhibitory receptors. Examination of STAT3, -5, and -6 phosphoprotein levels demonstrated no activation or role in IL-12 and/or coreceptor ligation (not shown). Thus, the increased and sustained levels of phospho-STAT4 could explain the increased levels of IFN-γ mRNA. Interestingly, levels of T-bet mRNA did not increase upon costimulation (not shown), thus implicating a direct role for STAT4 in increasing IFN-γ mRNA levels. It is not yet possible to test this hypothesis in STAT4 knockout mice as these mice do not express the C57BL/6 Ly49 alleles (J. R. Ortaldo, unpublished observation).

The role of MAP kinase in the signaling pathways was next evaluated (Fig. 4*B*). With either activating Ly49D receptor ligation or IL-12 induction, the expected inhibition with p42/44 or p38 MAP kinase inhibitors, respectively, was observed. The addition of both inhibitors had little increased effect on IFN-γ production.

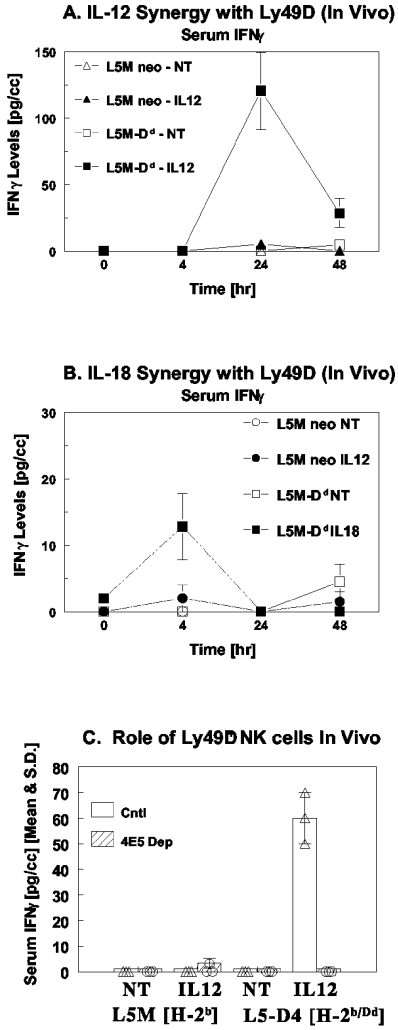

FIGURE 3. In vivo production of serum IFN-γ was measured at the indicated times by ELISA. Animals were injected intrasplenically with 5×10^{-5} L5 MF22 (H-2^b) or L5-D104 (H-$2^{b/Dd}$), the spleen was removed after 3 min, then 1 h later animals were injected i.p. with PBS, IL-12, or IL-18. Values represent the mean and SD of four mice per group. *A*, Coinjections with IL-12; *B*, coinjections with IL-18; *C*, parallel experiment in which mice were injected on days −3 and −1 with either rat IgG (control) or anti-Ly49D (4E5). Mice were verified for >90% depletion of $4E5^+$, $NK1.1^+$ $CD3^-$ cell by flow cytometry (not shown). Serum levels of IFN-γ were measured 24 h after the administration of tumor cells and/or IL-12. Values represent the mean and SD of three mice per group.

However, upon coreceptor treatment there was no effect of the p38 inhibitor and a minimal effect of the p42/44 inhibitors, and even the presence of both MAP kinase inhibitors resulted in a significant level of IFN-γ production (>100,000 pg/cc). Thus, synergistic and signaling cross-talks occur during IL-12 and anti-Ly49D ligation, and these dual signals over-ride the ability of inhibitory Ly49G2 to block IFN-γ gene expression through the activation of two distinct signal transduction pathways.

Discussion

One of the classic hallmarks of adaptive immunity is the strong synergy observed upon coreceptor activation. Here we demonstrate that mouse activating NK receptors can mediate a coreceptor activation in a similar fashion and induce strong expression of IFN-γ. Our findings have major implications in a number of relevant systems. NK cells have been previously shown to be important innate producers of IFN early in virus infections (30, 31). Recent studies (30, 32) have defined the importance of NK cell Ly49H expression and cytokine production in murine CMV infection for the development of $CD8^+$ T cells that mediate Ag-specific clearance of virus. These results support the hypothesis that early, Ag-independent IFN-γ production is dominated by Ly49H-secreting NK cells and is critical for a successful host response. Local and systemic synergy of Ly49H activation with locally produced IL-12 or IL-18 would be consistent with our current data and would provide a specific local advantage in the development of adaptive immunity.

Recent data have suggested that the expression cytokines and chemokines by NK cells could play an important role in the development of other adaptive responses. It is well established that the ability of professional APCs to mediate many of their coactivating functions is through the production of soluble mediators such as IL-12. The ability of NK cells to rapidly secrete 1) IFN-γ,

Table II. *Expression of IFN-γ by $CD3^+$ and $CD3^-$ liver leukocytes*[a]

Group	Cytokine Treatment	% Positive: IgG $CD3^-$	IgG $CD3^+$	IFN-γ $CD3^-$	IFN-γ $CD3^+$
L5neo	NT	0.1	0.2	0.2	0.1
	IL-12	0.1	0.1	0.2	0.1
	IL-18	0.1	0.1	0.2	0.2
L5-Dd	NT	0.1	0.2	1.0	0.5
	IL-12	0.1	0.1	3.9	1.2
	IL-18	0.1	0.1	3.5	0.8
No Ab	IL-12	0.2	0.1	0.3	0.2
No Ab	IL-18	0.1	0.1	0.2	0.1

[a] Animals were treated in a manner similar to that described in Fig. 4, A and B. Livers were harvested at 2 h, and leukocytes were isolated in the presence of 1 μM brefeldin A. Isolated leukocytes were cultured for an additional 4 h at 37°C in complete medium in the presence of 1 μM brefeldin A. Cells were then washed, stained with $CD3^-$ PerCP to identify T cells, and analyzed for their intracellular IFN-γ. Cells from animals injected with L5MF22 (H-2^b) or L5D104 (H-$2^{b/Dd}$) alone or with IL-12 or IL-18 are shown with IgG-PE control or anti-IFN-γ on the CD3-expressing or negative subset. Values represent the mean of three mice per group.

Table III. *IFN-γ expression by activating NK subsets of $CD3^-$, $NK1^-$ liver leukocytes*[a]

Group	Cytokine Treatment	% Positive of $NK1.1^+$, $CD3^-$: IgG $Ly49D^-$	IgG $Ly49H^+$	IFN-γ $Ly49D^-$	IFN-γ $Ly49H^+$
L5neo	NT	0.01	0.02	4.54	0.99
	IL12	0.02	0.01	4.78	2.01
	IL18	0.11	0.10	4.01	2.13
L5-Dd	NT	0.11	0.22	4.47	3.72
	IL12	0.15	0.17	8.58	3.74
	IL18	0.16	0.15	10.29	4.03
No Ab	IL12	0.21	0.10	2.26	1.68
No Ab	IL18	0.18	0.11	2.68	2.62

[a] Liver cells were 19.59% $NK1^+$, $CD3^-$, of which 35% were $Ly49D^+$ and 28% were $Ly49H^+$. Using four-color analysis, IFN-γ expression was quantified in liver leukocytes from cells prepared as described in Table II (underlined). After leukocytes were isolated from livers, cells were surface-stained with CD3 PerCP, NK1.1-aliophycocyanine, and anti-Ly49D (4E5-F) or anti-Ly49H (3D10-F). Cells were then analyzed for their intracellular IFN-γ as described above. Animals were injected with tumor cells (L5MF22 (H-2^b) or L5-D104 (H-$2^{b/Dd}$)) with PBS (NT), IL-12, and IL-18 in combination or with cytokines alone. Values represent the average of four animals per group that were pooled prior to evaluation.

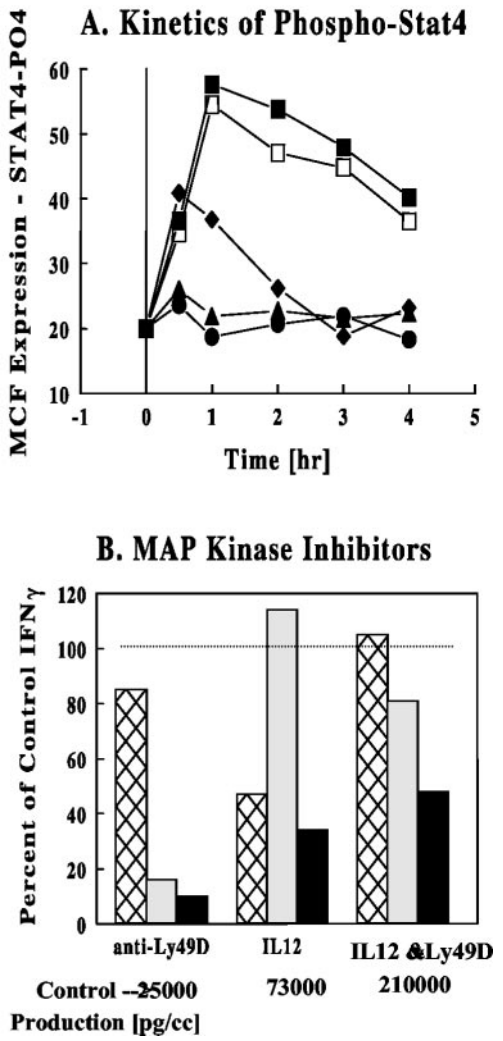

FIGURE 4. Mechanism of IL-12 synergy. Ly49D$^+$, G2$^+$ NK cells (*C*) were examined for the expression of phospho-STAT4 using intracellular detection with rabbit anti-STAT4-PO_4. Ly49D$^+$, G2$^+$ NK cells were untreated (▲) or were treated with IL-12 (◆); anti-Ly49D (●); anti-Ly49D, anti-Ly49G2, and IL-12 (□); or anti-Ly49D and IL-12 (■). Receptor cross-linking was performed using a goat F(ab′)$_2$ anti-Rat Fc specific secondary Ab. *B*, IFN-γ production in the presence of MAP kinase inhibitors (IC_{50} dose) for p38 (SB203580, 600 nM; (▨) or p42/44 (SB98059, 2 μM; ⊡) or both (■). Values are expressed as a percentage of control production (shown below chart).

a key molecule in the development of adaptive immunity, and 2) chemokines (MIP1α) that can retain or recruit selective subsets of leukocytes, suggest that another role of NK cells is to modulate the delicate balance of immune development in response to viral or microbial challenge. There have been a number of tumor model systems that demonstrate the important role NK cells play in early tumor clearance, later leading to adaptive immunity. However, the mechanisms involved in these responses have not been elucidated. Since IFN-γ has been shown to play an important role in the modulation of key surface receptors or ligands (e.g., MHC class I, Fas, and TRAIL), our data are consistent with the hypothesis that innate NK cell IFN-γ production will provide early immune regulation that can alter the outcome and quality of the adaptive immune response.

Another critical quandary addressed by our findings is the ability of activating Ly49 receptors to function in vivo in the presence of dominant, inhibitory Ly49 receptors. Previous in vitro studies have demonstrated that most activating/inhibitory pairs are dominated by the negative signal. Thus, understanding how a ligand-bound activating receptor would function in the presence of an inhibitory signal has been perplexing. Mere ligand interaction in vivo in the absence of a local immune activation would not result in NK activation, whereas Ly49D interaction in the context of systemic or local immunity would bridge the threshold and allow development of immune activation. This hypothesis is supported by the data in Table I. Cross-linking of inhibitory receptors by either Ab or with target ligand in highly purified Ly49G2$^+$, D$^+$ NK cells failed to result in significant IFN-γ production; however, these same cells could be strongly induced by cotreatment with IL-12. Although the detailed biochemical mechanism for this effect is unknown, IL-12 can reverse the inhibitory signals, and the two signals (i.e., cytokine and Ly49D cross-linking) result in elevated and sustained biochemical activation of STAT4 and IFN-γ. mRNA. As recently demonstrated (30), IL-12 and IL-18 synergistic induction of IFN-γ mRNA in a murine T cell line results from a STAT4-dependent increase in AP-1 binding to the IFN-γ promoter, and a similar mechanism may occur here. It is also possible that receptor cross-linking in the presence of cytokines increases the IFN-γ mRNA half-life. Consistent with this possibility is the fact that IFN-γ mRNA was maintained for 24 h in vitro (Fig. 1) when both activating signals were present. These results have implications in vivo, since the local immune response can be dependent upon specific NK responses. Our data now provide a model for how the Ly49 receptors and cytokines function together to modulate the host immune response.

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