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The Interactions of Multiple Cytokines Control NK Cell Maturation

Jason Brady,∗1 Sebastian Carotta,∗ Rebecca P. L. Thong,∗ Christopher J. Chan,† Yoshihiro Hayakawa,†,2 Mark J. Smyth,† and Stephen L. Nutt∗

Although NK cells are well known for their cytotoxic functions, they also produce an array of immunoregulatory cytokines and chemokines. During an immune response, NK cells are exposed to complex combinations of cytokines that influence their differentiation and function. In this study, we have examined the phenotypic and functional consequences of exposing mouse NK cells to IL-4, IL-12, IL-15, IL-18, and IL-21 and found that although all factors induced signs of maturation, characterized by decreased proliferation and IFN-γ secretion, distinct combinations induced unique cytokine secretion profiles. In contrast, the immunosuppressive factors IL-10 and TGF-β had little direct effect on NK cell effector functions. Sustained IL-18 signals resulted in IL-13 and GM-CSF production, whereas IL-12 and IL-21 induced IL-10 and TNF-α. Surprisingly, with the exception of IL-21, all cytokines suppressed cytotoxic function of NK cells at the expense of endogenous cytokine production suggesting that “helper-type” NK cells were generated. The cytokine signals also profoundly altered the cell surface phenotype of the NK cells—a striking example being the downregulation of the activating receptor NKG2D by IL-4 that resulted in decreased NKG2D-dependent killing. IL-4 exposure also modulated NKG2D expression in vivo suggesting it is functionally important during immune responses. This study highlights the plasticity of NK cell differentiation and suggests that the relative abundance of cytokines at sites of inflammation will lead to diverse outcomes in terms of NK cell phenotype and interaction with the immune system. The Journal of Immunology, 2010, 185: 6679–6688.

Natural killer cells are components of the innate immune system that play a protective role against some tumors and viral infections through their cytolytic and immunomodulatory capabilities. In contrast with B and T lymphocytes where there is abundant evidence for multiple sublineages and functional states, the nature and consequences of heterogeneity in the NK cell lineage are only now emerging. Although it is well established that a predominant pathway of murine NK cell development occurs in the bone marrow, a thymic pathway characterized by a dependence on IL-7 and the transcription factor Gata3 has been revealed (1, 2). Phenotypically distinct NK cell populations are also found in the uterus and gastrointestinal tract (3–5). We have shown that maturing NK cells can be divided on the basis of CD27 and/or KLRG1 expression to distinguish distinct subsets (6, 7). The CD27+ NK cells predominate in the bone marrow and spleen and are selectively recruited into lymph nodes upon immunization, whereas the CD27– subset is found mainly in the blood and lungs. In vitro functional assays indicate that the CD27+ NK cells are more potent effector cells as measured by cytotoxicity and cytokine secretion (6). Recently, CD94 has been proposed as an alternative marker of NK cell heterogeneity; however, the relationship between the subsets defined by CD27 and CD94 is at present unclear (8).

Human NK cells can be divided into CD56bright cells capable of enhanced proliferation and cytokine production and CD56dim cells that display more potent cytotoxic function (9). Some data suggest that the CD56bright NK cells are the equivalent of the thymus-dependent population in the mouse, whereas others have proposed that the CD56bright population derives from hematopoietic progenitors in the lymph node (10–12). The factors that regulate NK cell heterogeneity are poorly characterized, although by analogy to other lymphocyte lineages, it is likely that extrinsic factors such as cytokines play a role.

Mouse NK cell progenitors express IL-2/15Rβ-chain (CD122) in the absence of NK1.1 (13). Thereafter, NK1.1 expression is maintained throughout the lineage and is followed by the rapid acquisition of the NKG2-C and Ly49 receptor families (8, 14). Besides IL-15, which is continually required for cell survival (15, 16), little is known about the requirements of other cytokines in cellular development as mice deficient in one or multiples of IL-2, IL-4, IL-7, IL-12, IL-18, and IL-21 all have relatively normal numbers of peripheral NK cells (17–19).

In contrast with their minor role in NK cell development, cytokines such as IL-12, IL-4, IL-18, IL-21, and TGF-β have been shown to have pronounced effects on human and mouse NK cell function (18, 20–27). IL-12 and IL-18 are produced by activated macrophages and dendritic cells and have an important function in promoting Th1 responses and IFN-γ production and tumor clearance by NK cells (18). IL-21 is a pleiotropic cytokine made by activated CD4+ T cells (28) and NKT cells (29) that enhances maturation from human multipotent progenitors and activates peripheral NK cells (28). IL-21 is a potent inducer of IL-10.

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Abbreviations used in this paper: PI, propidium iodide; TRAIL, TNF-related apoptosis-inducing ligand.

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and IFN-γ and promotes cytotoxicity against a broad spectrum of targets in vitro (22) and in vivo (30, 31).

As the NK cell differentiation pathways elicited by these inflammatory cytokines have many parallels to the Th1/Th2 differentiation of CD4+ T cells, a number of studies have suggested that the NK cell cytokine responses can be categorized into NK1 and NK2 type cells producing Th1 (IFN-γ) or Th2 (IL-5, IL-13) cytokines, respectively (24, 32–34). However, the diversity of cytokines capable of supporting the functional maturation of NK cells suggested to us that this model does not fully explain the breadth of NK cell functions observed. In this study, we have systematically examined the phenotypic and functional consequences of exposing mouse NK cells to inflammatory cytokines and demonstrate that the nature of these cytokine signals profoundly affected the cell surface phenotype and function of the resulting NK cells.

Materials and Methods

Mice

Rag1−/− mice were maintained on a C57BL/6 background. Mice at 5–10 wk of age were used in all experiments.

NK cell isolation and in vitro culture

NK cells were enriched from the spleen of Rag1−/− mice using DX5-conjugated microbeads (purity typically 70–95% CD49b+ cells; Miltenyi Biotec, Auburn, CA). CD49b+ NK1.1+ TCRβ+ NK cells were isolated from C57BL/6 spleens using flow cytometric sorting. NK cells were cultured for up to 7 d in IMDM supplemented with 10% FCS, 2 mM glutamine, and 50 μM 2-ME using the following recombinant cytokine concentrations (R&D Systems, Minneapolis, MN): human IL-15 50 ng/ml and TGF-β 5 ng/ml, mouse IL-21 100 ng/ml, IL-12 2 ng/ml, IL-18 50 ng/ml, IL-10 10 ng/ml, and IL-4 4 ng/ml. The numbers of viable NK cells were enumerated using trypan blue exclusion and annexin V staining/propidium iodide (PI) staining. Cell morphology was examined by cytospin and demonstrated using trypan blue exclusion and annexin V staining/propidium iodide (PI) staining. Cell morphology was examined by cytospin and demonstrated using trypan blue exclusion and annexin V staining/propidium iodide (PI) staining.

Cytotoxicity assays

The cytotoxicity activity of NK cells was assessed using RMA, RMA-S, and RMA-Rae1 tumor cell lines as we have previously described (22). Experiments were performed at least three times in triplicate.

Abs and flow cytometry

Abs to NK1.1 (PK136), CD11b (M1/70), CD16 (2.4G2), Ly49C/I (SW5E6), Ly49D (4E5/E1), Ly49G2 (4D11), IFN-γ (HI170 and XM1G1.2), IL-10 (JE55-2A5.1), GM-CSF (MP1-22E9 and MP1-31G6), and IL-4 (BVD4-1D11 and BVD6-24G2.3) were purified from hybridoma supernatants on protein-G Sepharose columns (Amersham Pharmacia Biotech, Arlington Heights, IL) and conjugated to allophycocyanin (Pro-Zyme, San Leandro, CA) or biotin (Pierce, Rockford, IL) when required. Abs to TNF-α (TN3-19.12), CD94 (18d3), CXCR4 (2B11), NGK2A/C/E (2D5), Ly49F (HBB-719), KLKL1 (2F1), CD122 (TM-61), CD25 (7D4), CD244 (2B4), CD43 (87), c-Kit (2B8), IL-10 (SX-1), CD49b (Hm2α), TCRβ (H57-597), TNF-related apoptosis-inducing ligand (TRAIL), and TNF-α were purchased from BD Pharmingen (San Diego, CA). Anti-perforin (OMAK-D), DNAM1 (10E5), NKp46 (29A1.4), and NKp2D (CX5) were purchased from eBioscience (San Diego, CA). Anti-Jα-Jβ-actin-HRP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rat IgG HRP was purchased from Amersham Pharmacia Biotech. For flow cytometry, appropriate Abs were used to stain single-cell suspensions in PBS containing 2% FCS. Biotinylated Abs were revealed by PE-streptavidin (Southern Biotechnology Associates, Birmingham, AL) Cells were analyzed using an LSR flow cytometer (BD Biosciences, Mountain View, CA), with dead cells excluded by PI staining.

Cytokine production assays

NK cells were cultured in appropriate cytokines for 6 d, washed, and 1 × 106 cells were seeded in fresh cytokine for 24 h after which supernatants were harvested. IFN-γ and IL-10 were assayed by ELISA as previously reported (22). The GM-CSF ELISA used MP1-22E9 for capture and MP1-31G2 biotin for detection, the TNF-α ELISA used TN3-19.12 for capture and rabbit polyclonal anti-mouse TNF biotin for detection, and the IL-4 ELISA used BVD4-1D11 for capture and BVD6-24G2.3 biotin for detection. Recombinant cytokine standards were purchased from R&D Systems or PeproTech (Rocky Hill, NJ).

Western blot analysis

Western blot analysis was performed as previously described (36).

Hydrodynamic gene delivery

Rag1−/− mice were injected with 20 μg pORf-Empty or pORf–IL-4 hydrodynamic vectors in saline (37). The vectors were injected into the mice intravenously in a volume less than 10% of their body weight with a maximum of 2 ml injected over a period of 3–5 s. NK cells and serum IL-4 were analyzed 3 d postinjection.

RT-PCR

Total RNA from NK cells was subjected to semiquantitative or quantitative RT-PCR as previously described (38). For semiquantitative RT-PCR, cDNA was normalized using Hprt1 expression, and 5-fold serial dilutions of the cDNA were prepared. Hprt and Tbx2 primers were previously described (39). Primers were: Gata3: 5′-CTACCGGTTGTCGGATGAAAG-3′, 5′-TGTCATAGCCTCTCCGGTTTC-3′; Ets1: 5′-CAGCGGGTCTCCCTCTCATGTA-3′, 5′-CGTTCCCCGCGCTGTTGATTG-3′; Elf1: 5′-GGGGAGGACGGGAAAGAAGAAA-3′, 5′-GAATTCGAGGCTCTGGGAAAGACTC-3′; Ifi1: 5′-GATGGAACCTGGCTGAGATAG-3′, 5′-TCTCTCTTCTTCTCTTGTTACTG-3′; Ifi2: 5′-GAGTCTACCGGATGCTGCCCTTAGTT-3′, 5′-GTCTCCCCTCTCGTTCTTCTACT-3′; Ifi2: 5′-ACCAGAGACCTGGAGACAAAGTT-3′, 5′-AAAGCTGAGAAGGATCTACCAG-3′.

Results

NK cell maturation is associated with decreased proliferative potential in vitro

To investigate the comparative roles of the cytokines IL-4, IL-12, IL-18, and IL-21 in NK cell differentiation, we isolated mature DX5+ NK cells from the spleens of Rag1−/− mice using magnetic beads. The use of Rag1−/− mice removed T and NKT cells from the cultures, which may themselves produce regulatory cytokines in response to the exogenous factors. The NK cells were then cultured in the presence of optimal concentrations of cytokines for up to 7 d. Culture of NK cells in IL-15 alone resulted in pronounced proliferation and increased cumulative cell number, whereas, as we have previously reported (22), IL-21 antagonized the proliferative effect of IL-15 by ~10-fold (Fig. 1A). The addition of the other cytokines involved in NK cell maturation, IL-4, IL-12, or IL-18, either alone or with IL-21, similarly antagonized the IL-15 proliferative effect (Fig. 1A). Significantly reduced cell numbers were also observed when wild-type NK cells were cultured in the same cytokines over a 7-d time course (Fig. 1B). These cultures showed that statistically significant reductions in NK cell numbers were observed for all NK cell maturation cytokines as early as day 5. A similar decrease in cell yield resulted from the addition of TGF-β to the cultures, whereas IL-10, another immunosuppressive cytokine, had no impact on cell number. To ascertain the mechanism by which the cytokines reduced the cell yields, cell viability and proliferative assays were performed. Although CSFE labeling studies demonstrated that cells in all conditions were proliferating vigorously (data not shown), analysis of the apoptotic rates on days 4 and 5 of the cultures, using a combination of annexin V and PI staining, revealed a pronounced upregulation in the frequency of apoptotic cells with the addition of all cytokines except IL-15 (Fig. 1C).

Besides the effects that the various cytokines had on cell survival, changes in phenotypes indicative of differentiation were observed, including increased cell size. Cytocentrifuge and histological analysis revealed that IL-12, IL-18, and IL-21 all induced dramatic morphological changes in the cultures, including increased granularity.
Distinct cytokine secretion profiles are induced by different culture conditions

To address potential functions of cytokine-differentiated NK cells, we have tested the secretion of a selection of known NK cell products after 7 d culture in the various cytokine combinations. NK cells propagated in IL-15 alone produced baseline levels of IFN-γ, MIP-1α (CCL3), and MIP-1β (CCL4; Fig. 2). These factors were all further induced in vitro in the presence of IL-12, IL-21, and/or IL-18. As previously noted, the combination of IL-12/IL-18 showed pronounced synergy in eliciting cytokine secretion (40). Interestingly, GM-CSF, IL-13, IL-10, and TNF-α showed much more stringent cytokine requirements. GM-CSF and IL-13 were almost exclusive signatures of IL-18 stimulation, whereas IL-10 was induced by IL-12 or IL-21 but completely repressed by IL-18 signaling. TNF-α in contrast showed an intermediate phenotype, being induced strongly by IL-12 and IL-21 but only mildly repressed by IL-18. Finally IL-4, in conjunction with IL-15, had little positive effect on cytokine secretion but did inhibit IL-21 function. In support of these cytokine profiles being a consequence of cellular differentiation, assessment of cytokine production after 1 d in culture revealed a similar profile of IFN-γ secretion; however, IL-10 was not detectable. This observation agrees with a previous report that found that NK cells secrete IL-10 only after prolonged engagement with activating cytokines (25). These data demonstrate that distinct cytokine signatures can be observed in vitro and that hierarchies of activity can be readily discerned.

Impaired cytotoxic function mediated by IL-4, IL-12, and IL-18

NK cells cultured in IL-15 or IL-2 are able to lyse efficiently an array of target cells suggesting that they are already fully active. We and others have reported that IL-21 further boosts this cytolytic capacity against an array of targets (17, 22, 41). To test for synergistic effects of cytokine stimulation, we have tested lytic activity of day 7 cultures against the relatively resistant RMA and sensitive RMA-S (MHC-I−) targets. Whereas IL-15 and IL-15/IL-21 showed the expected killing capacity against these lines, addition of IL-12, IL-18, IL-4, but not IL-10 or TGF-β, significantly impaired their lytic function (Fig. 3A, Supplemental Fig. 1). This inhibitory effect was most pronounced for the IL-15/IL-4, IL-15/IL-12, and IL-15/IL-18 combinations that exhibited almost no specific lysis. In keeping with the cytokine secretion data, these inhibitory effects were dominant over the prolytic IL-21 function. This inhibition of killing function was not the result of impaired expression of perforin as Western blotting revealed high amounts of perforin in all conditions (Fig. 3B) and did not correlate with an ability to degranulate, as assessed by cell surface CD107 staining (data not shown) or expression of the death domain proteins TRAIL or Fas ligand (Fig. 4 and data not shown).

Modulation of the NK cell surface phenotype by cytokine-driven differentiation

We have previously reported that culturing NK cells in the presence of IL-21 modulates the expression of a number of important NK cell surface receptors (22). We have therefore examined the NK cell receptor expression for Rag1−/− and wild-type NK cells cultured as in Fig. 1 using a panel of 25 markers (Figs. 4, 5, Supplemental Figs. 2, 3, Supplemental Tables I, II). These markers represented an array of molecules that can be used to define the maturation state of NK cells in vivo, as well as their activation and ability to respond to activating and inhibitory signals. As one of our aims was to define the maturation states induced by various cytokine combinations, this analysis was also expected to provide flow cytometric signatures for particular NK cell states that can be further tested in vivo.

The pan-NK cell marker NK1.1 was significantly downregulated in some conditions, whereas other markers, such as CD122 (IL-2/15β), NKp46 (CD335), and CD244 (2B4), were uniformly posi-
tive and confirmed that these cells were NK cells (Fig. 5, Supplemental Table I). The NK cell maturation markers CD11b (Mac1), CD43, and c-Kit (CD117) were also downregulated in vitro and could not be used to determine NK cell differentiation status, although each again showed specific patterns, as the combination IL-12/IL-18 maintained CD43 expression whereas CD11b expression was silenced in the presence of IL-18.

More decisively, a number of receptors that modulate NK cell function or reflect activation status were strongly affected by particular culture conditions. KLRG1, a receptor we have recently found to be expressed specifically on a subset of the most mature NK cell in vivo (7, 42), was significantly induced by combinations of IL-12 and IL-18, suggesting that a similar maturation process occurred in vitro (Fig. 4, Supplemental Table I). A similar profile was observed for CD25 (IL-2Rα), whereas CD16, a receptor that is modulated on human NK cell subsets, was specifically repressed by IL-12/IL-18. One striking example was the expression of the homing receptor, CXCR4, which was expressed on splenic NK cells ex vivo but was strongly downregulated in all conditions except IL-12/IL-18 (Fig. 4).

The analysis of NK cell receptors was also informative. The CD94/NKG2 heterodimer was induced by IL-21 in the presence of IL-15 or IL-15/IL-12. Notably, IL-21 had no obvious effect in the presence of IL-18, demonstrating again a hierarchy in the activity of these cytokines. Most surprisingly, culture in IL-4 or IL-12/IL-18 markedly downregulated this receptor complex as well as the unrelated NKG2D molecule (Fig. 4). Culture of NK cells in the presence of TGF-β resulted in some modest suppression of the expression of several NK cell markers, including NK1.1, NKG2D, and CD25, whereas IL-10 had little effect on receptor expression (Supplemental Fig. 2, Supplemental Table I).

Finally, we examined the expression of a panel of NK cell receptors encoded by the Ly49 cluster (Supplemental Fig. 2, Supplemental Table I). Expression of the Ly49 family was generally decreased in all conditions except IL-15 ± IL-21. The only exception was Ly49G2, which was maintained in all conditions. The modulation of Ly49 receptors by cytokine treatment has recently also been reported for Ly49E, which is induced by IL-15 or IL-2 (43).

It was possible that many of the changes in cell surface Ag expression observed were a consequence of the differential outgrowth of a subset of NK cells with a distinct phenotype. To exclude this scenario, we have performed experiments where NK cells were first activated and cultured in IL-15 for 4 d and then

**FIGURE 2.** Cytokine production from cultured NK cells. *Rag1−/−* splenic NK cells were cultured for 6 d in the indicated conditions. Viable cells (1 × 10⁵) were washed and reseeded in fresh cytokine for 24 h, after which supernatants were harvested and examined for cytokine production using an ELISA. Data are the mean ± SEM of triplicate wells from a representative of at least three experiments. IL13 expression levels were determined by quantitative real-time PCR and are shown relative to Hprt.
further differentiated in the indicated cytokines for a further 3 d. Fig. 6A shows a representative experiment for the downregulation of NKG2D. Comparable results were obtained for the other NK cell receptors assayed in Figs. 4 and 5, suggesting that the phenotypic diversity observed was elicited from a common starting pool of mature NK cells (data not shown).

Downregulation of NKG2D by IL-4

The downregulation of the critical activating receptor, NKG2D, by IL-4 and the combination, IL-12/IL-18, was surprising as only TGF-β had previously been shown to decrease mouse and human NKG2D activity (26, 44, 45) (Supplemental Fig. 2), although IL-21 (46), IL-12, and type I IFNs (27) are reported to decrease human NKG2D. To assess the functional consequences of the NKG2D downregulation, we tested the ability of cultured NK cells to lyse RMA-Rae1 b cells that express an activating NKG2D ligand and are predominantly killed by this pathway. In line with the decreased NKG2D expression, NK cells cultivated in the presence of IL-4 or IL-12/IL-18 did not kill Rae1 b + cells (Fig. 6B). In contrast, the combination IL-15/IL-18, IL-15/TGF-β, and IL-15/IL-10 maintained NKG2D expression and cytotoxic activity, whereas IL-15/IL-21 strongly promoted killing. IL-15/IL-12–treated NK cells were relatively inefficient killers despite maintaining NKG2D expression, indicating that the inhibition of lytic function occurred through a different mechanism.

To investigate whether IL-4 has the capacity to downregulate NKG2D expression in vivo, we have used the hydrodynamic gene therapy to express transiently mouse IL-4 (Fig. 7A, B). Three days after the introduction of the IL-4 expression vector into Rag1 −/− mice, ELISA could readily detect IL-4 in the serum (Fig. 7B). Importantly, the NK cells from mice that received the pOrf–IL-4 construct showed a clear downregulation of several NK cell receptors, including NK1.1, CD49b, CD94/NKG2, and NKG2D (Fig. 7A, Supplemental Fig. 4), demonstrating that IL-4 downregulated specific NK cell receptors including NKG2D in vivo.

To address if IL-4 had any function during earlier stages of NK cell development, we have taken advantage of the NK cell differentiation capacity of Pax5−/− pro-B cells (35). Pax5−/− pro-B cells are maintained on OP9 stromal cells in the presence of IL-7; however, transient exposure to notch signaling via coculture with OP9 cells expressing the notch ligand Δ-like 1 (OP9-DL1) results in NK cell commitment (step 1) (35). These Pax5−/−–derived NK cells can then be further matured by culture on OP9 cells in the presence of IL-15 (step 2), resulting in NK1.1+CD49b+NKG2D+CD94+ NK cells (Fig. 7C, D). Introduction of IL-4 into...
these cultures during step 1 resulted in lower NKG2D and reduced cell yields (Fig. 7C, 7D), whereas addition of IL-4 only during step 2 produced a phenotype reminiscent of the in vivo IL-4–treated mice; that is, decreased NK1.1, CD49b, NKG2D, and CD94. These data indicated that IL-4 had a strong effect on proliferation and gene expression in NK cells from an early stage in differentiation.

Transcriptional regulation of NK cell differentiation in vitro

It is well established that the distinct cytokine profiles produced from in vitro-differentiated CD4+ T cells are the result of the activity of a number of lineage-promoting transcription factors, most notably, T-bet in Th1 cells (47), Gata3 in Th2 cells (48), and RAR-related orphan receptor γ t in Th17 cells (49). To examine if the distinct NK cell fates we have observed in vitro are produced by differential transcription factor expression, we analyzed NK cells cultivated in various cytokine conditions for the expression of a panel of factors that are essential for NK cell differentiation in vivo.

Transcription factors were examined, first by semiquantitative RT-PCR and then by real-time PCR (Fig. 8). Several factors, including Elf4 (Mef), Ets1, Irf1, Idr2, and Irf2, were similarly expressed in all conditions, whereas Gata3 and Tbx21 (encoding T-bet) showed some heterogeneity. Gata3, whose expression is required for thymic and liver NK cell differentiation (10, 50), was significantly downregulated in the presence of IL-12, IL-21, and IL-18. Tbx21 in contrast was significantly decreased only in the presence of IL-15/IL-18 and strongly upregulated in IL-12/IL-18 conditions that also induce the T-bet target gene Ifng. These data suggest that despite the strong phenotypic and functional differences between the distinct cytokine-differentiated NK cells, the NK cell transcription factor profile was remarkably stable and not the predominant cause of the distinct effector profiles.

Discussion

The development and peripheral survival of mouse NK cells absolutely depends on the cytokine IL-15 (13, 15). IL-15 can also promote the survival and proliferation of NK cells in vitro; a function that can also be provided by high concentrations of IL-2. A number of other cytokines have been shown to influence NK cell maturation and effector function, including IL-12, IL-18, IL-4, IL-21, and TGF-β. Importantly, none of these factors have been shown to be essential for NK cell development or homeostasis in vivo (17–19). In this study, we have used primary NK cell cultures to address the interactions between these NK cell modulating cytokines in controlling NK cell differentiation and effector function. We found that distinct cytokine combinations showed striking phenotypic, cellular, and functional consequences in vitro and in the case of IL-4 in vivo. These findings suggest that the cytokine milieu present in situ during the early stages of an immune response are likely to have strong influences on the functional properties of the resulting NK cells.

![FIGURE 5. Cytokine modulation of NK cell maturation marker expression. Rag1−/− or wild-type (DNAM1, NKp46) splenic NK cells were cultured for 7 d in the indicated conditions and analyzed for differentiation markers by flow cytometry. Freshly isolated Rag1−/− NK cells (gated as NK1.1+CD49b+) were used as a comparison (ex vivo). Data are representative of 4–10 experiments.](http://www.jimmunol.org/)

![FIGURE 6. Downregulation of NKG2D expression by IL-4 in vitro. A, Rag1−/− splenic NK cell cultures were cultured for 7 d in the indicated cytokines (upper panel) or for 3 d in IL-15 and then transferred into differentiation conditions for 4 d as indicated (lower panel). B, NK cells were isolated as in A and cultured for 7 d in the indicated cytokines and subjected to a standard 51Cr release assay using RMA-Rae1β+ (NKG2D ligand+) target cells. Data are the mean of triplicate wells ± SEM and are representative of at least three experiments. The p values compare the indicated culture conditions: *p < 0.05; **p < 0.01.](http://www.jimmunol.org/)
We have previously reported that culture of mature NK cells in IL-15 and IL-21 promoted the differentiation of mouse NK cells into effector cells that were strongly cytotoxic and secreted IFN-γ and IL-10 (22). We have now addressed the role of IL-21 in combination with other cytokines, including IL-12, IL-18 (18, 23, 25, 40), and, to a lesser extent, IL-4 and TGF-β (23, 24, 26, 27, 43, 51, 52), which have been reported to influence NK cell functions. As we predominately focused on understanding the cellular differentiation process, we have not assayed the short-term effects of cytokine activation that may have distinct functions compared with that in longer-term cultures (53). A striking example of the difference between short- and long-term cultures is IL-10 expression. Whereas Il10 mRNA is rapidly induced in activated NK cells, IL-10 is not secreted until much later in an immune response (54).

All culture conditions, except IL-15 alone, decreased cell proliferation and increased the proportion of cells undergoing apoptosis. This finding, along with the marked changes in cell size, morphology, and receptor expression, suggests that the predominant consequence of exposure to these inflammatory cytokines is to induce the NK cell lineage terminal differentiation. Of the immunosuppressive cytokines, IL-10 had very little effect on NK cell phenotype and function, whereas TGF-β very strongly antiproliferative but left NK cell effector function largely intact. In agreement with this concept, exposure of NK cells to either IL-4, IL-12, IL-18, or IL-21 resulted in increased production of a range of effector cytokines. One group of cytokines including IFN-γ, TNF-α, MIP-1α, and MIP-1β were induced in all conditions and represent generic activation responses, although in all cases further activation could be induced by specific stimuli such as IL-12/IL-18. Other NK cell products were however specifically produced by distinct cell populations, with IL-13 and GM-CSF being a signature of IL-18 stimulation, whereas IL-10 was induced by IL-21 and IL-12. Diagnostic hierarchies of cytokine secretion were also apparent where IL-4 and IL-18 acted to inhibit IL-21 function. Surprisingly, of all the cytokine combinations, only IL-21 promoted cytolytic function, whereas IL-12, IL-18, and IL-4 actively suppressed target killing. The mechanism for this reduced killing activity remains to be determined as the cytokine-inhibited NK cells retained high perforin expression and underwent some target-dependent degranulation. Moreover, the expression of other cytotoxic effector molecules such as TRAIL and Fas ligand did not correlate with lytic potential. Taken together, these data suggest that, with the exception of IL-21, the inflammatory cytokines favored the differentiation of NK cells with a “helper-type” capacity to produce immunoregulatory cytokines. Similar helper NK cells have been proposed for human NK cells (23, 25).

These pronounced functional differences between the differentiated NK cells also correlated with dramatically altered cell surface profiles and repertoires of activating and inhibitory NK receptors. Of particular interest, many pan-NK cell lineage markers
The regulation of NKG2D was particularly striking. Under inflammatory conditions, the expression of NKG2D was lost upon culture in IL-2 (56), few studies have systematically examined NK cell gene expression in response to differentiation signals. The downregulation of many markers commonly associated with the NK cell lineage, such as NK1.1 and CD49b, raise the possibility that prolonged exposure to inflammatory cytokines may make NK cells difficult to identify using standard FACS conditions in situations such as infection or inflammatory cytokines may make NK cells difficult to identify using standard FACS conditions in situations such as infection or autoimmunity. Indeed, IL-21 is known to repress NK1.1 expression in vivo (22), and here we show a similar effect of IL-4 expression is associated with high risk of progression of myelodysplastic syndrome to acute myeloid leukemia (59). The suppressive effects of IL-4 and IL-12/IL-18 on NKG2D in vitro do not appear to be mediated through autocrine secretion as TGF-β levels were low and unchanged in all NK cell culture conditions tested, and exogenous TGF-β had only a modest effect on cell surface NKG2D. IL-21 was also reported to decrease NKG2D on human NK cells (46); however, we observed no effects of IL-21 on either NKG2D expression level or cytotoxic function in mouse cells, suggesting that some species-specific differences may also occur in the function of IL-21 in the NK cell lineage.

There is increasing evidence for diversity of the NK cell lineage. This includes anatomically distinct developmental and differentiation sites, such as thymus, uterus, and gut, as well as the generation of functionally distinct subsets (1–3, 9). Our data describe potential cytokine signals by which NK cell subsets can be produced in vitro and, potentially, in vivo. It is well established that distinct cytokine profiles are produced from in vitro-differentiated CD4+ T cells as a result of the activity of a number of lineage-promoting transcription factors. These factors function to directly regulate the differentiation of these lineages as well as the diagnostic effector profiles, IFN-γ in the case of T-bet and IL-4 for Gata3 (47, 48). Based on the production of Th1/2 type cytokines, a similar NK1 and NK2 model has been proposed for human and mouse NK cells (24, 33). The studies presented here highlight the diversity of NK cell helper and effector functions and indicate that NK cells do not fit into a clear NK1/NK2 model. Indeed, in the past few years it has become apparent that CD4+ T cell responses are much more diverse and flexible than the Th1/Th2 paradigm allows as other sublines such as Th17 cells (regulated by RAR-related orphan receptor γ t), regulatory T cells (controlled by Foxp3), and most recently T follicular helper cells (Bcl6) have emerged (60). This diversity is further complicated by the existence of Th1/Th2 type programs within the Foxp3 expressing regulatory T cell lineage (61, 62).

Although our data are compatible with a similar diversity of NK cells, our analysis of the expression of several transcriptional regulators implicated in NK cell maturation found an overall very similar expression profile from these otherwise distinct NK cells. This included identical expression of Elf4, Ets1, Irf1, Irf2, and Id2, all known to be important for NK cell differentiation (63). Small changes were observed for Gata3 (decreased in IL-12 and IL-18) and Tbx21 (increased in IL-12/IL-18); however, these changes were modest compared with the strongly polarized gene expression in similarly cultured CD4+ T cells (32). Although these findings may simply represent the fact that the crucial polarizing transcription factor(s) has yet to be found, it may also reflect a greater plasticity of NK cells that possess a much more limited capacity to clonally expand in response to Ag compared with T cells. This plasticity thus enables the NK cells to respond rapidly to the distinctive cytokines produced as a reaction to particular challenges in an immunologically appropriate manner.

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Disclosures
The authors have no financial conflicts of interest.
References


SUPPLEMENTARY FIGURE 1. Cytolytic activity of cultured NK cells. (A-C) Rag1\textsuperscript{−/−} splenic NK cells were cultured in the indicated cytokines for 7 days and subjected to a standard \textsuperscript{51}Cr release assay at the indicated Effector:Target (E:T). Target cells were (A) RMA, (B) RMA-S and (C) RMA-Rae1\textbeta. Data are the mean of triplicate wells ± s.e.m. and are representative of at least three experiments. (D) FACS sorted CD49b\textsuperscript{+}NK1.1\textsuperscript{+}TCR\textbeta\textsuperscript{−} NK cells isolated from C57/Bl6 spleens were cultured in the indicated cytokines for 7 days and subjected to a standard \textsuperscript{51}Cr release assay using RMA-Rae1\textbeta cells as targets. Data are the mean of triplicate wells ± s.e.m. and are representative of two experiments.
SUPPLEMENTARY FIGURE 2. Cytokine modulation of NK cell receptor expression.

FACS sorted CD49b⁺NK1.1⁺TCRβ⁺ NK cells isolated from wild type C57/Bl6 spleens were cultured in the indicated cytokines for 7 days and analysed for the NK cell receptor expression by flow cytometry. Data are representative of 4 experiments.
SUPPLEMENTARY FIGURE 3. Cytokine modulation of Ly49 receptor expression. 

*Rag1*<sup>−/−</sup> splenic NK cells were cultured for 7 days in the indicated conditions and analysed for the NK cell receptor expression by flow cytometry. Freshly isolated *Rag1*<sup>−/−</sup> NK cells (gated as NK1.1<sup>+</sup>CD49b<sup>+</sup>) were used as a comparison (*ex vivo*). Data are representative of at least three experiments.
SUPPLEMENTARY FIGURE 4. Down-regulation of NK cell receptor expression by IL-4 in vivo. Rag1^-^ mice were injected with empty vector (pORF) or an IL-4 expressing vector (pORF-IL-4) using the hydrodynamic gene transfer. At day 3 spleens were analysed for CD122, CD49b and NK1.1 expression. Note the lower expression of CD49b and NK1.1 in the CD122^+ NK cells after IL-4 treatment. Data shows two independent mice from each treatment and is representative of 6 mice of each group. MFI, mean fluorescence index.
<table>
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**Supplementary Table 1.** Regulation of NK cell receptor expression by cytokines *in vitro*.

TCRβCD49b+NK1.1+ NK cells were isolated from C57Bl/6 spleens by flow cytometry and cultured for 7 days in the indicated cytokine combinations. The mean fluorescence index (MFI) of cells stained for the indicated NK cell receptors was determined by flow cytometry. Specific MFI was calculated as the value obtained from a given marker subtracted with the MFI of unstained cells cultured in the same conditions. Data is the MFI (x10³) ± SEM from 3 or 4 experiments for each data point. Values that are
significantly difference to cells cultured in IL-15 alone are indicated by * p<0.1, ** p<0.05, or *** p<0.01.

| % Change after in vitro culture | % NK ex vivo | IL-15 | IL-15 IL-15 IL-15 IL-15 IL-15 IL-15 IL-12 IL-12 IL-12 IL-18 IL-18 IL-18 IL-4 IL-4 IL-18 IL-18 IL-18 IL-12 IL-12 | IL-21 IL-21 IL-21 IL-21 IL-21 IL-21 IL-21 IL-21 IL-21 IL-21 IL-21 IL-21 |
|-------------------------------|-------------|-------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Ly49A                         | 12.4        | 132.3 | 115.0 63.1    | 73.5 86.8     | 86.0 48.4      | 33.1 29.8      | 25.4           | Ly49C+I        | 25.7           | 119.5 164.9 75.7   | 110.7 26.2     | 28.4 28.3 49.8 | 6.4 13.0  |
| Ly49D                         | 47.7        | 144.4 | 96.9 122.6 122.7 | 56.8 41.9 22.9 | 16.0 38.8 33.5 | Ly49G2         | 51.9 105.4 111.0 | 108.7 115.2 98.5 | 94.4 76.1 85.1 96.5 93.1 |
| Ly49F                         | 7.1         | 60.2  | 65.4 33.7 17.7 | 36.3 24.6     | 22.1 14.2 9.2 | 10.6           |                |                |                |                |                |                |                |                |

**Supplementary Table 2.** Regulation of Ly49 family expression by cytokines *in vitro.*

The percentage of NK cells positive for various Ly49 family receptors either *ex vivo* (CD49b+NK1.1+) or after 7 days culture in the indicated cytokine combinations is shown. For cultured NK cells the percentage change after 7 days was calculated relative to the proportion of Ly49-marker positive cells *ex vivo* (set to 100%). Gray background indicates cytokine conditions that result in >50% reduction in the proportion of NK cells expressing particular Ly49 family members. Data is representative of at least three experiments.