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Differentiation of Murine NK Cells into Distinct Subsets Based on Variable Expression of the IL-12Rβ2 Subunit

Habiba Chakir, Allison A. Camilucci, Lionel G. Filion, and John R. Webb

The cytokine IL-12 manifests its biological activity via interaction with a heterodimeric receptor (IL-12R) present on activated T and NK cells. The cDNAs for two IL-12R subunits have been cloned from human and mouse and designated IL-12Rβ1 and IL-12Rβ2. The expression of IL-12Rβ2 on T cells is influenced by cytokines, particularly IL-4, IL-12, and IFN-γ; however, little is known regarding regulation of IL-12R expression on NK cells. In this study we show that murine NK cells differentiate into IL-12Rβ2low and IL-12Rβ2high subsets after in vitro stimulation with IL-2 in the absence of exogenous polarizing cytokins. Subset development occurs gradually as NK cells expand in vitro and is generally complete by 8–12 days of culture. Once established, IL-12Rβ2low and IL-12Rβ2high subsets are highly stable in vitro and can be maintained for at least 20 days after FACs sorting. Formation of these NK subsets appears to be strain independent. Flow cytometric analyses demonstrate that both subsets express a number of NK-associated markers, including NK1.1, DX-5, Ly-49A, and Ly-49C, but that the Ly-49G2 class I inhibitory receptor is expressed predominantly on the IL-12Rβ2high population. Both IL-12Rβ2low and IL-12Rβ2high NK cells respond to exogenous IL-12 by rapid production of high levels of IFN-γ and increased lytic activity against NK-sensitive YAC-1 target cells. Analyses of cytokine gene expression by RNase protection assay indicated that similar to the recently described human NK1 subset, both IL-12Rβ2high and IL-12Rβ2low murine NK subsets expressed high levels of IFN-γ, whereas neither subset expressed mRNA for the NK2-associated cytokines IL-5 and IL-13. The Journal of Immunology, 2000, 165: 4985–4993.

Interleukin-12 is a 70-kDa heterodimeric cytokine comprised of p40 and p35 subunits that together form the biologically active p70 protein. IL-12 is produced by macrophages, dendritic cells, and some B cells (1–3), typically after stimulation with bacterial preparations such as formalin-fixed Staphylococcus aureus or heat-killed Listeria monocytogenes (4–6). In addition, exposure to bacterial components, such as LPS or bacterial DNA, results in the production of biologically active IL-12 (4, 7). IL-12 is best known for its role in promoting effector CD4+ T cell differentiation toward a Th1 phenotype (8, 9); however it has many other important biological activities. These include induction of proliferation and IFN-γ production from activated T and NK cells (1–3), promoting Ag-specific cytolytic T cell responses (10, 11), and enhancement of lytic activity in NK and lymphokine-activated killer (LAK)3 cells (10, 12–14). These activities are often synergistic or at least additive with other cytokines, in particular IL-2 and TNF-α (for review, see Ref. 2). Knockout mice lacking the IL-12 p40 subunit (8) or the receptor for IL-12 (15) are severely compromised in IFN-γ production, Th1 immune responses, and delayed-type hypersensitivity.

IL-12 manifests its biological functions through interaction with a cell surface IL-12Rb chains. cDNAs for two IL-12R subunits have been cloned from human and mouse and designated IL-12Rβ1 (16, 17) and IL-12Rβ2 (18). Both the IL-12Rβ1 and IL-12Rβ2 subunits are type I transmembrane glycoproteins with molecular sizes of approximately 100 and 130 kDa, respectively. IL-12Rβ1 belongs to the hemopoietin receptor family, and both IL-12Rβ1 and IL-12Rβ2 belong to the gp130 subgroup of the cytokine receptor superfamily (1). The IL-12Rβ2 subunit contains two cytoplasmic cytokine box motifs and is thus thought to be the receptor component that transduces a signal after binding of IL-12 at the surface (19). However, both IL-12R subunits appear to be required for the formation of a high affinity IL-12 binding site, as transfection of COS-7 cells with individual subunits leads to the formation of homodimers/oligomers with low affinity IL-12 binding (16, 18). In contrast, cotransfection of COS-7 cells with both subunits of the human IL-12R results in high affinity binding of human IL-12 (18).

Resting, naive T cells do not express the IL-12R (16, 20, 21); however, activation of T cells with mitogens or anti-CD3 results in rapid, but transient, surface expression of high affinity IL-12 binding sites (for review, see Ref. 1, 2). Furthermore, a number of reports have demonstrated that whereas the IL-12Rβ1 subunit is constitutively up-regulated in CD4+ T cells after TCR cell stimulation, expression of the IL-12Rβ2 subunit is influenced by the presence of key cytokines and accessory molecules at the time of stimulation. In particular, the presence of IL-4 during in vitro stimulation of naive T cells has been shown to result in the down-regulation of the IL-12Rβ2 chain in several different systems (22, 23). This loss of IL-12Rβ2 in emerging Th2 populations is thought to render the cells refractory to the effects of IL-12, thus contributing to the stability of the Th2 phenotype. Conversely, IL-12 itself has a positive regulatory effect on IL-12Rβ2 surface expression on T cells (24). Together, these mechanisms for regulating high affinity IL-12 binding sites on T cells through modulation of the IL-12Rβ2 chain have obvious and profound implications for the development of an ensuing immune response considering the potential ability of IL-12 to drive Th1 differentiation.

As indicated above, IL-12 also has significant biological activity on NK cells and was initially referred to as NK cell stimulatory...
factor (12). Although the regulation of IL-12R expression on NK cells is poorly characterized compared with that on CD4⁺ T cells, it is generally considered to be up-regulated after activation with IL-2 (21, 25). As was observed in CD4⁺ T cells, differential expression of the IL-12Rβ2 chain has recently been reported to occur on human NK cells after in vitro expansion in the presence of the Th1 or Th2 polarizing cytokines IL-12 and IL-4 (26). These polarized cells (referred to as NK1 and NK2) were found to have distinct cytokine production profiles, particularly with respect to the production of IL-5 and IL-13 in the NK2 subset. However, despite the differential level of IL-12Rβ2 surface expression, both subsets retained the capacity to respond to IL-12 by increased IFN-γ production during subsequent restimulation in the presence of IL-12.

In the present study we have generated a polyclonal antiserum against the murine IL-12Rβ2 chain for the purpose of analyzing the regulation of IL-12Rβ2 expression on murine lymphocytes. We report herein that after in vitro activation with IL-2, murine NK cells develop into two distinct populations with differential expression of the IL-12Rβ2 chain. Furthermore, these subsets develop in the absence of exogenous polarizing subsets and exhibit a highly stable phenotype once they are established. Interestingly, expression of IL-12Rβ2 was found to correlate with expression of the NK inhibitory molecule Ly-49G2 (Lgl-1). However, despite the difference in levels of IL-12Rβ2 surface expression both subsets respond similarly to IL-12 in terms of IFN-γ production and augmented cytotoxicity.

**Materials and Methods**

**Polyclonal antisemur**

A portion of the extracellular domain of murine IL-12Rβ2 (aa 24–275) was expressed as a recombinant protein with an N-terminal 6×His affinity tag in Escherichia coli using the pET17b T7 promoter system (Novagen, Madison, WI). Briefly, DNA encoding IL-12Rβ2 was amplified from a plasmid containing the complete murine IL-12Rβ2 gene (provided by K. M. Murphy, Washington University, St. Louis, MO) by PCR. The PCR primers had the following sequences: 5′ primer, ATGGCTAGCCATCACCATCACACCATAAAATGATGTTGCAAGCTT; and 3′ primer, CATGGAATTCCTTCAAGCCTACTTCAAGCT. After amplification, the PCR product was digested with Nhel and EcoRI and cloned into pET17b digested with the same enzymes. The IL-12Rβ2 extracellular domain (32 kDa) was then expressed in E. coli XL-1Blue (Stratagene, La Jolla, CA) cells and purified to homogeneity by nickel-nitrilotriacetic acid affinity chromatography. The purified protein was then used to generate a rat anti-mouse IL-12Rβ2 antisemur and FITC-conjugated goat anti-rat Ig. Additionally, antibody against second surface marker as indicated, and 5) streptavidin-PE (as required) to detect biotin-conjugated Ab in step 4. Flow cytometric analyses were performed on an EPICS analyzer (Coulter, Hialeah, FL) using a minimum of 20,000 events. Cell sorting was performed on an Astra cell sorter (Coulter).

**Immunofluorescence and cell sorting**

The surface phenotypes of cells were established using two-color flow cytochemistry. All stains were performed in a stepwise manner in the following order: 1) rat anti-murine IL-12Rβ2 antisemur or equivalent nonimmune serum, 2) FITC- or PE-conjugated goat anti-rat Ig, 3) 1% normal rat serum to block free goat anti-rat Ig binding sites, 4) biotin-conjugated or PE-conjugated Ab against second surface marker as indicated, and 5) streptavidin-PE (as required) to detect biotin-conjugated Ab in step 4. Flow cytometric analyses were performed on an EPICS analyzer (Coulter, Hialeah, FL) using a minimum of 20,000 events. Cell sorting was performed on an Astra cell sorter (Coulter).

**Intracellular staining for IFN-γ**

IL-2 blasts generated from bulk splenic MNCs or purified DX-5⁺ NK cells were incubated overnight in the presence of murine IL-12 at the indicated concentrations. The next morning, monensin (2 μM) was added, and incubation at 37°C was continued for 4 h. Cells were then stained for surface IL-12Rβ2 using rat anti-murine IL-12Rβ2 antiserum and FITC-conjugated goat anti-rat Ig. After a 15-min fixation with 4% paraformaldehyde plus 0.1% saponin, cells were stained for intracellular IFN-γ by incubation for 30 min with PE-conjugated anti-IFN-γ in the presence of 0.1% saponin.

**RNase protection assays**

IL-2 blasts generated from purified DX-5⁺ cells of C57BL/6 mice were sorted into IL-12Rβ2low and IL-12Rβ2high subsets (as described above) and were grown overnight in the presence or the absence of IL-12 (1 ng/ml). Total RNA was prepared using TRIzol according to the manufacturer’s instructions (Life Technologies, Gaithersburg, MD). Cytokine mRNAs were detected using the mouse cytokine set 1 multiprobe template set according to the manufacturer’s protocols (RiboQuant, PharMingen). RNase-protected probes were resolved on 6% denaturing polyacrylamide gels and were exposed to film (XAR, Eastman Kodak, Rochester, NY) overnight at ~70°C.

**Cytotoxicity assay**

IL-2 blasts generated from purified DX-5⁺ cells of C57BL/6 mice were sorted into IL-12Rβ2low and IL-12Rβ2high subsets (as described above) and were grown overnight in the presence or the absence of IL-12 (1 ng/ml). Cells were then washed and used as effector cells in a standard 31Cr release assay using YAC-1 cells as targets. Briefly, 5 × 10⁶ Cr-labeled target cells and serial dilutions of effector cells were incubated together for 4 h. After this incubation, supernatants were harvested and counted with a gamma counter. The percent specific lysis was calculated as previously described (27).

**Results**

**Two populations of IL-2-activated NK cells based on IL-12Rβ2 expression**

A recombinant protein comprising the extracellular domain of the murine IL-12Rβ2 chain (aa 24–275) fused to an amino-terminal 6×His Tag was expressed in E. coli and was purified to homogeneity by nickel-nitrilotriacetic acid affinity chromatography. The purified protein was then used to generate a rat anti-mouse IL-12Rβ2 polyclonal antiserum for the analysis of IL-12Rβ2 expression on murine splenocytes. As previously observed with PHA-activated human T cells (20, 21), activation of murine splenic MNC with Con A plus IL-2 resulted in low level IL-12Rβ2 expression on the surface of CD3⁺ T cells (Fig. 1A). Activated NK
cells are also known to be responsive to IL-12; however, little is known regarding the regulation of IL-12R on NK cells. Therefore, murine splenic MNC were activated with IL-2 (1000 U/ml) for 7–10 days, and the resulting cells (70% DX5+ NK cells) were analyzed for IL-12Rb2 surface expression by flow cytometry. We repeatedly observed that the DX5+ NK cells in these cultures formed two distinct populations after surface staining with the anti-IL-12Rb2 antiserum (Fig. 1B). Compared with control samples stained with preimmune serum only, these two populations correspond to IL-12Rb2low and IL-12Rb2high subsets of NK cells. This pattern of NK subset distribution based on IL-12Rb2 surface expression was highly consistent and was observed in 10 different preparations of IL-2 blasts. Furthermore, the phenomenon appeared to be strain independent, as a similar staining pattern was observed in IL-2 blasts originating from BALB/c, FVB/N, and C57BL/6 mice.

To further demonstrate the specificity of the anti-mouse IL-12Rb2 antiserum, a competitive binding assay was performed. IL-2-activated DX-5+ NK cells were stained with anti-IL-12Rb2 antiserum in the absence (Fig. 2A) or the presence (Fig. 2, B and C) of varying amounts of soluble rIL-12Rb2 as a competitor. At the highest concentration of rIL-12Rb2 added (500 ng/ml), binding of the rat anti-mouse IL-12Rb2 antiserum to the surface of DX-5+ IL-2 blasts was completely abrogated, whereas binding of anti-DX-5 mAb was unaffected. Competitive inhibition of the anti-IL-12Rb2 antiserum binding to DX-5+ IL-2 blasts by soluble...
rIL-12Rβ2 titrated over the range of rIL-12Rβ2 concentrations tested (Fig. 2C). Furthermore, immunoprecipitation of IL-12Rβ2 from IL-2 blasts using anti-mouse IL-12Rβ2 antisera confirmed that the reactive protein on the surface of IL-2 blasts was of the approximate molecular mass expected for IL-12Rβ2 (Fig. 2D). Together these results confirm the specificity and sensitivity of the anti-mouse IL-12Rβ2 antisemur and demonstrate the utility of this reagent for detecting IL-12Rβ2 by flow cytometry.

To further investigate the two distinct NK subsets, IL-2 blasts prepared from bulk splenic MNC were analyzed on days 0–11 to measure the kinetics of subset development (Fig. 3, left panels). Resting splenocytes (day 0) did not express detectable amounts of IL-12Rβ2. Five days after addition of IL-2 (1000 U/ml), rapid expansion of the DX-5+ compartment was occurring, and differentiation of the DX-5+ cells into distinct subpopulations was evident. The separation of DX-5+ cells into IL-12Rβ2low and IL-12Rβ2high populations was complete by day 11. Although the number of DX-5+ cells continued to increase upon further incubation in IL-2 (for up to 21 days), the proportion of DX-5+, IL-12Rβ2low to DX-5+, IL-12Rβ2high cells remained stable. To determine whether formation of IL-12Rβ2low and IL-12Rβ2high subsets was inherent to the DX-5+ cells or whether other cell types in the initial bulk culture were required, DX-5+ cells were purified from total splenic MNC by positive magnetic selection and were used to generate IL-2 blasts. As was observed with total splenic MNC, purified DX-5+ cells gradually differentiated into an approximately equal mixture of IL-12Rβ2low and IL-12Rβ2high cells over the 11 days of culture in the presence of IL-2 (Fig. 3, right panels). These data demonstrate that the capacity to form IL-12Rβ2low and IL-12Rβ2high subsets is an inherent feature of DX-5+ cells upon activation with exogenous IL-2.

**FIGURE 3.** Kinetics of IL-12Rβ2 expression on IL-2 blasts. C57BL/6 bulk splenic MNC or DX-5+ splenic MNC (purified from C57BL/6 splenocytes by positive selection) grown in the presence of IL-2 (1000 U/ml) were analyzed by flow cytometry at the indicated time points using rat anti-mouse IL-12Rβ2 antiserum and FITC-conjugated goat anti-rat Ig plus PE-conjugated anti-DX-5.

**Phenotype of IL-12Rβ2low and IL-12Rβ2high IL-2 blasts**

To determine whether IL-12Rβ2low and IL-12Rβ2high IL-2 blasts differed from each other phenotypically with respect to other markers in addition to IL-12Rβ2, cells were costained with the anti-IL-12Rβ2 antiserum and with a panel of mAbs specific for DX-5, NK1.1, Ly-49A, Ly-49C, Ly-49G2, B220, CD19, and CD3 (Fig. 4). As shown in Fig. 2, IL-2-activated DX-5+ C57BL/6 splenocytes were comprised of approximately equal populations of IL-12Rβ2low and IL-12Rβ2high cells. Furthermore, both the IL-12Rβ2low and IL-12Rβ2high populations stained positively for NK1.1, further confirming that both subsets are derived from the NK compartment. In agreement with previous findings (28), these NK cells were heterogeneous in terms of the Ly-49 surface markers that were expressed. Specifically, 23% of DX-5+ C57BL/6 IL-2 blasts stained positively for Ly-49A, 23% stained positively for Ly-49C, and 61% stained positively for Ly-49G2. As was observed with the DX-5 and NK1.1 markers, the Ly-49A+ and Ly-49C+ populations were distributed equally between the IL-12Rβ2low and IL-12Rβ2high populations. In marked contrast, expression of the Ly-49G2 marker was restricted to the IL-12Rβ2high population, resulting in delineation of NK cells as being either Ly-49G2–IL-12Rβ2low or Ly-49G2+IL-12Rβ2high. B220, which is known to be a marker of activated NK cells (29), was present on both IL-12Rβ2low and IL-12Rβ2high populations, implying that the two subsets do not differ in terms of their activation state. Both the IL-12Rβ2low and IL-12Rβ2high populations were negative for CD19 staining, demonstrating that IL-2 blasts derived from DX-5+ splenocytes were free of contaminating B cells. Finally, a small number of NK T cells were evident in the DX-5+ blast cultures, as indicated by positive staining with anti-CD3. In the example shown, the DX-5+ CD3+ NK T cells are distributed equally between the IL-12Rβ2low and IL-12Rβ2high populations. However, in some of the IL-2 blast preparations that we analyzed, the NK T cell population was comprised entirely of IL-12Rβ2high cells (data not shown), suggesting that, like conventional T cells, NK T cells may have variable levels of IL-12Rβ2 expression based on their current state of activation.

Because the Ly-49G2-specific Ab that we initially used (4LO439) recognized an allospecific determinant of Ly-49G2 that is restricted to C57BL/6 mice (30), we repeated this analysis using the mAb 4D11 (31, 32). mAb 4D11 recognizes Ly-49G2 from a strain that is restricted to C57BL/6 mice (30). To determine whether formation of IL-12Rβ2low and IL-12Rβ2high subsets was inherent to the DX-5+ cells or whether other cell types in the initial bulk culture were required, DX-5+ cells were purified from total splenic MNC by positive magnetic selection and were used to generate IL-2 blasts. As was observed with total splenic MNC, purified DX-5+ cells gradually differentiated into an approximately equal mixture of IL-12Rβ2low and IL-12Rβ2high cells over the 11 days of culture in the presence of IL-2 (Fig. 3, right panels). These data demonstrate that the capacity to form IL-12Rβ2low and IL-12Rβ2high subsets is an inherent feature of DX-5+ cells upon activation with exogenous IL-2.

**FIGURE 3.** Kinetics of IL-12Rβ2 expression on IL-2 blasts. C57BL/6 bulk splenic MNC or DX-5+ splenic MNC (purified from C57BL/6 splenocytes by positive selection) grown in the presence of IL-2 (1000 U/ml) were analyzed by flow cytometry at the indicated time points using rat anti-mouse IL-12Rβ2 antiserum and FITC-conjugated goat anti-rat Ig plus PE-conjugated anti-DX-5.
To determine whether the IL-12Rβ2low and IL-12Rβ2high NK subsets were stable populations or whether they represented a transient phenotype, day 12 IL-2 blasts generated from C57BL/6 DX-5+ splenic MNC were sorted into IL-12Rβ2low and IL-12Rβ2high subsets by FACS sorting or magnetic selection. Sorted cells were then returned to culture and grown for an additional 7 days in the presence of IL-2. Over this 7-day period the level of IL-12Rβ2 surface expression was monitored, and the staining patterns of both populations were found to be completely stable (data not shown). Beyond 7 days, the level of IL-12Rβ2 began to drop slightly in the IL-12Rβ2high cells; however, the two sorted cell populations were still easily discernable even after 14 days in culture (data not shown).

Cytokine production by IL-12Rβ2low and IL-12Rβ2high DX-5+ IL-2 blasts

We then wished to determine whether IL-12Rβ2low and IL-12Rβ2high NK cell subsets differed in their capacity to produce IFN-γ in response to IL-12. Day 10 DX-5+ IL-2 blasts were incubated overnight in the presence or the absence of 200 U/ml IL-12 and were analyzed for IFN-γ production by intracellular staining. Surprisingly, both IL-12Rβ2low and IL-12Rβ2high cells produced significant levels of IFN-γ after overnight treatment with IL-12 (Fig. 6, A and B). To address whether IL-12 was acting directly on both cell types or whether IL-12Rβ2high cells were influencing the IL-12Rβ2low population through an indirect mechanism, the assay was repeated using sorted IL-12Rβ2low and IL-12Rβ2high subsets. In addition, the cells were incubated in decreasing amounts of IL-12 to determine whether the IL-12Rβ2high cells were more sensitive to IL-12 than the IL-12Rβ2low subset. Although the percentage of IFN-γ-producing cells was somewhat higher in the IL-12Rβ2high subset, particularly at higher IL-12 concentrations, both the IL-12Rβ2low and IL-12Rβ2high subsets clearly responded to IL-12 stimulation with increased production of intracellular IFN-γ (Fig. 6C). Similar results were obtained by ELISA analysis of IFN-γ secreted into the culture supernatants of these same cells (data not shown), indicating that the IL-12Rβ2low and IL-12Rβ2high subsets are similar in their capacity to respond to IL-12 stimulation.

To determine whether the IL-12Rβ2low and IL-12Rβ2high subsets corresponded to the recently described NK1 and NK2 subsets, the cytokine gene expression profile of these cells was also analyzed by RNase protection assay (Fig. 7). Neither subset accumulated detectable amounts of cytokine mRNA in the absence of stimulation; however, both IL-12Rβ2low and IL-12Rβ2high cells up-regulated IFN-γ mRNA in response to stimulation with PMA and ionomycin. Furthermore, both subsets dramatically up-regulated levels of IFN-γ mRNA in response to stimulation with exogenous IL-12. Also, as previously reported (26), IL-10 mRNA appeared to be highly up-regulated in these cells in response to stimulation with exogenous IL-12. Neither subset produced detectable levels of IL-5 or IL-13 mRNA either before or after stimulation, indicating that despite different levels of IL-12Rβ2 surface expression both have the cytokine expression profile of the previously described human NK1, but not the NK2, subset.

To further investigate the biological significance of the IL-12Rβ2low and IL-12Rβ2high subsets, sorted IL-12Rβ2low and IL-12Rβ2high cells were used as effectors in a standard cytotoxicity assay. Both subsets of cells were highly efficient at lysing NK-sensitive YAC-1 target cells even at very low E:T cell ratios (Fig. 8). Addition of exogenous IL-12 augmented this high level of cytotoxicity to a similar extent in both populations, and this effect titrated through several different E:T cell ratios. Similar results were observed when the cytotoxicity assay was repeated using the H-2k P815 cell line as a target (data not shown). However, interpretation of the P815 assay was more complicated due to a requirement for neutralization of the Ly-49G2 class I inhibitory receptor on the IL-12Rβ2high subset to see the stimulatory effect of
IL-12 (J. Webb, manuscript in preparation). These results provide further confirmation that despite the difference in levels of IL-12Rb2 surface expression, both IL-12Rb2low and IL-12Rb2high subsets of NK cells respond similarly to stimulation with exogenous IL-12.

Discussion

In this report we have analyzed the expression of the IL-12Rb2 molecule on murine IL-2 blasts. In prior studies murine IL-2 blasts have been extensively used to study the affinity of IL-12/IL-12R interactions, but a detailed analysis of the regulation of IL-12R expression has been hampered to date by the lack of an anti-IL-12Rb2 mAb. The findings presented herein are consistent with earlier reports suggesting that both T and NK cells require activation to express appreciable levels of the IL-12R (20, 34, 35). Furthermore, our results indicate that after activation, murine NK cells express the IL-12Rb2 at levels significantly higher than those observed on mitogen-activated T cells. However, the presence of two distinct populations of activated murine NK cells with differing levels of IL-12Rb2 is a novel observation. Recently, human NK cell subsets, termed NK1 and NK2, were described that, like their CD4⁺ T cell subset counterparts, are skewed in terms of cytokine production in response to varying amounts of IL-12. Sorted IL-12Rb2low and IL-12Rb2high cells were stimulated overnight in the presence of 10-fold serial dilutions of IL-12, and the data are plotted as the percentage of IFN-γ-producing cells within each subset.
production and IL-12Rβ2 expression (26). However, it isimportant to note that those NK1 and NK2 subsets were generated in the presence of the strongly polarizing cytokines IL-12 and IL-4, respectively, and also in the presence of irradiated feeder cells. In contrast, the murine NK subsets reported herein were found to differentiate into IL-12Rβ2low and IL-12Rβ2high subsets without the addition of exogenous polarizing cytokines. Furthermore, differentiation into IL-12Rβ2low and IL-12Rβ2high subsets occurred in cultures of purified NK cells, providing further evidence that this process occurs independently of IL-4 or IL-12, since NK cells are not known to be a source of either one of these two cytokines. Because of this apparent inherent capacity to develop into IL-12Rβ2low and IL-12Rβ2high subsets in the presence of IL-2, it is tempting to speculate that there are specific subsets of resting NK cells that have a predetermined fate in terms of IL-12Rβ2 surface expression. This hypothesis may be related to the recent observation that stimulation of resting human NK cells with IL-12 results in up-regulation of IL-12R on only a specific subset of CD56+ NK cells (14). Interestingly, this study also reported that the proportion of IL-12R+ NK cells could be further up-regulated by the addition of IL-4 and that these latter cells were the CD56+CD16− subset.

In addition to influencing the level of surface IL-12Rβ2 expression, growth of human NK cells in NK1 or NK2 priming conditions resulted in the preferential accumulation of mRNAs encoding the Th1-associated cytokine IFN-γ or the Th2-associated cytokines IL-5 and IL-13, respectively (26). In contrast, neither the IL-12Rβ2low nor the IL-12Rβ2high murine NK cells reported herein expressed detectable levels of IL-5 or IL-13 mRNA either before or after stimulation with PMA/ionomycin or IL-12. This result suggests that despite similarities in IL-12Rβ2 surface expression, murine IL-12Rβ2low NK cells do not correspond to the human NK2 subset described above. Indeed, after stimulation with PMA and ionomycin, IFN-γ mRNA expression was up-regulated in both subsets. After stimulation with IL-12, IFN-γ as well as IL-10 mRNA expression was dramatically up-regulated in both subsets. IL-12-mediated up-regulation of IL-10 production in NK cells has been reported previously (26, 36), and although the biological consequences of IL-10 on NK activities are not clear, it may have an autoregulatory effect on IL-12 responsiveness. In summary, both the IL-12Rβ2low and IL-12Rβ2high NK cells reported herein have a cytokine secretion pattern similar to that of the human NK1 subset, again suggesting that the IL-12Rβ2low murine NK subset is not likely to be related to human NK2 cells. Interestingly, with the exception of a small amount of IFN-γ mRNA, murine NK cells grown without additional exogenous stimulation (IL-2 only) did not express detectable amounts of mRNA for any of the cytokine genes we assessed. This result may have implications for LAK cell-based therapies, since it suggests that these activated NK cells are primed in the presence of IL-2, but require a further stimulus to achieve their full potential in terms of cytokine production.

The results of the present study indicate that despite differences in IL-12Rβ2 surface expression, stimulation of IL-2-activated NK cells with IL-12 elicited strong IFN-γ production and augmented cytotoxicity in both IL-12Rβ2low and IL-12Rβ2high populations. This finding implies that IL-12Rβ2low NK cells are capable of responding to IL-12 equally as well as the IL-12Rβ2high population. Previous results have shown that very low levels of IL-12R on the surface of activated human T cells (as observed in Fig. 1) renders these cells functionally responsive to IL-12 (37). Similarly, resting human CD56+ NK cells are reported to express IL-12R at levels that are barely detectable by flow cytometry, yet this level of receptor renders these cells responsive to IL-12 as measured by up-regulation of IL-12R (14). Since both IL-12Rβ2low and IL-12Rβ2high murine NK subsets appear to respond equally to IL-12, it is interesting to speculate about the potential role of the high levels of IL-12Rβ2 in the IL-12Rβ2high subset. Firstly, it is possible that this high level expression of IL-12Rβ2 is artificially driven by the high doses of IL-2 used for the in vitro activation of NK cells. In fact, previous studies have indicated that IL-2, at doses much lower than those used herein, has a direct influence on expression of the IL-12R on human NK cells (21). To investigate this possibility we are currently looking at various in vivo NK

FIGURE 8. Effect of IL-12 on the cytolytic activity of IL-12Rβ2low and IL-12Rβ2high murine NK cells against YAC-1 target cells. DX5+ IL-2 blasts from C57BL/6 mice (day 11) were sorted into IL-12Rβ2low and IL-12Rβ2high subsets, and cells were used as effector cells in a standard 4-h cytotoxicity assay. Sorted cells were incubated overnight in the presence or the absence of IL-12 (1 ng/ml) and then washed before being added to 51Cr-labeled YAC-1 target cells (5 × 103) at the indicated E:T cell ratios.
activation strategies, such as poly(I:C) stimulation, to determine whether these same subsets can be derived in vivo. Also, we are investigating whether stimulation of NK cells with IL-15, which shares many of the stimulatory characteristics, receptor molecules, and signaling pathways of IL-2, is capable of promoting the differentiation of these two subsets. Second, it would be interesting to determine the molecular configuration of the IL-12Rβ2 in the IL-12Rβ2low and IL-12Rβ2high subsets. For example, is the IL-12Rβ2 present at the cell surface in monomer form or is it complexed as a homodimer or a heterodimer with IL-12Rβ1 or another as yet to be defined cytokine receptor molecule. In this regard it may be relevant that IL-12Rβ2 plays a significant role during an innate immune response.

Staining with a number of NK-associated markers, including the pan NK markers DX5 and NKI.1 as well as the NK cell inhibitory molecules Ly-49A and Ly-49C, confirmed that both the IL-12Rβ2low and IL-12Rβ2high cells were of NK origin. Essentially all cells in both subsets expressed the DX5 and NKI.1 markers, whereas specific subpopulations of IL-12Rβ2low and IL-12Rβ2high cells expressed the Ly-49A and Ly-49C markers. Furthermore, the Ly-49A+ and Ly-49C+ cells were equally distributed between the IL-12Rβ2low and IL-12Rβ2high subsets. In contrast, the Ly-49G2 marker was expressed exclusively on IL-12Rβ2high NK cells. Thus, IL-2 activated murine NK subsets can be further defined as being either Ly-49G2− IL-12Rβ2low or Ly-49G2+ IL-12Rβ2high.

The Ly-49 multigene family encodes a complex and polymorphic family of NK cell surface receptors, at least some of which recognize class I MHC molecules and send an inhibitory signal that represses the lytic activity of NK cells (28, 33). The lytic activity of the Ly-49G2− subset of NK cells is inhibited by target cells expressing H-2Dd and/or H-2Ld (32), although inhibition by H-2Ld has recently come into question (38). As a consequence of this class I-mediated inhibitory activity, Ly-49G2− LAK cells from C57BL/6 mice are incapable of lysing the LAK-sensitive target P815, which is H-2Dd (32). However, as observed in the present study, both Ly-49G2− and Ly-49G2+ NK cells from C57BL/6 mice are capable of lysing NK-sensitive YAC-1 target cells. The distinct pattern of IL-12Rβ2 expression on these Ly-49G2− and Ly-49G2+ NK subsets is a novel finding that implies the coordination of regulation of IL-12Rβ2 and Ly-49G2 during the activation of these cells. Considering the role of Ly-49G2 in inhibiting the lysis of H-2Dd-expressing targets and the reported ability of IL-12 to augment LAK activity, it would be interesting to determine whether IL-12 plays a role in modulating the class I-mediated inhibition of lytic activity in these cells. We are currently using purified preparations of IL-12Rβ2low and IL-12Rβ2high populations to investigate this possibility.

Also, it may be interesting to examine whether the coordinated expression of IL-12Rβ2 and Ly-49G2 is a universal phenomenon in all strains of mice. Prior analyses found that Ly-49G2 was expressed on NK cells of all strains of mice examined, but that the percentage of Ly-49G2− NK cells varied considerably among different strains (28). If high level IL-12Rβ2 expression on NK cells is restricted to the Ly-49G2+ subset, then it follows that IL-12Rβ2 expression would mirror strain-dependent differences in Ly-49G2 expression. However, it is important to remember that differences in IL-12Rβ2 expression were noted only in NK cells that were activated in vitro with high doses of IL-2; therefore, the significance of this finding to in vivo responses is not yet clear.

In conclusion, we have identified two distinct subsets of murine NK cells that differ in terms of IL-12Rβ2 surface expression after activation with IL-2. Furthermore, these two subsets correlate with the previously described Ly-49G2− and Ly-49G2+ NK cell subsets. Future studies will evaluate the physiological significance of these two subsets both in vitro and in vivo to establish their potential role during an innate and possibly an adaptive immune response.

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