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Differentiation of Murine NK Cells into Distinct Subsets Based on Variable Expression of the IL-12Rβ2 Subunit[1]

Habiba Chakir, Allison A. Camilucci, Lionel G. Filion, and John R. Webb[2]

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-12Rβ2^{low} and IL-12Rβ2^{high} subsets after in vitro stimulation with IL-2 in the absence of exogenous polarizing cytokines. Subset development occurs gradually as NK cells expand in vitro and is generally complete by 8–12 days of culture. Once established, IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{high} population. Both IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{high} and IL-12Rβ2^{low} 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-12R. 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 potent 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

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[3] Abbreviations used in this paper: LAK, lymphokine-activated killer; huIL-2, human IL-2; MNC, mononuclear cells.

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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 antiserum

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, ATGGCTAGCCATCAC CATCACCATCACAATATAGATGTGTGCAAGCTT; and 3′ primer, CATGGAATTCTCAAGCCTCATTACTCATGAG. After amplification, the PCR product was digested with *Nhe*I and *Eco*RI and cloned into pET17b digested with the same enzymes. The rIL-12Rβ2 extracellular domain (32 kDa) was then expressed in the BL21DE3(pLysS) host cell and purified to homogeneity by nickel-nitrilotriacetic acid affinity chromatography using standard methods (Qiagen, Valencia, CA). A polyclonal rat anti-murine IL-12Rβ2 antiserum was generated by immunizing female Lewis rats (Charles River Laboratories, Lexington, MA) with 100 μg of rIL-12Rβ2 in CFA. Rats were boosted three times at 4-wk intervals with rIL-12Rβ2 in IFA, and total serum was obtained 7 days after the final boost.

Cytokines and Abs

Human IL-2 (huIL-2) was provided by Dr. Craig Reynolds (National Cancer Institute, Bethesda, MD). Murine IL-12 (2.7 U/ng) was provided by Genetics Institute (Cambridge, MA). FITC-conjugated, PE-conjugated, and biotin-conjugated Abs, including goat anti-rat Ig, anti-CD19 (1D3), anti-murine IFN-γ (XMG1.2), anti-DX-5, anti-NK1.1, anti-B220, anti-Ly-49G2 (4D11), and anti-CD3 (145-2C11), were purchased from PharMingen (San Diego, CA). PE-conjugated anti-NK1.1 (PK136) was purchased from Cedarlane (Hornby, Canada). Biotin-conjugated anti Ly-49A (YE 1/32), Ly-49C (4LO3311), and Ly-49G2 (4LO439) were provided by Dr. Suzanne Lemieux (Institut Armand-Frappier, Laval, Canada).

Cell preparation

Mononuclear cells (MNC) were obtained from spleens of 6- to 8-wk-old female C57BL/6 or BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) by purification over Histopaque-1083 (Sigma, St. Louis, MO). MNC were plated at a density of 1×10^6 cells/ml in RPMI 1640, 10% FCS with 25 mM HEPES, 50 μM 2-ME, and penicillin/streptomycin. Con A blasts were generated from MNC by culture for the indicated times with 2.5 μg/ml Con A plus 10 U/ml huIL-2. Activated NK cells were generated from MNC by culture in the presence of 1000 U/ml huIL-2. $DX\text{-}5^+$ cells were purified from splenic MNC by positive selection using anti-DX-5 microbeads according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Cells obtained by this procedure were routinely >98% $DX\text{-}5^+$. Purified $DX\text{-}5^+$ cells were then expanded by culture in the presence of 1000 U/ml huIL-2 and were subcultured every 2–3 days with the addition of fresh IL-2. IL-12R$\beta2^{low}$ and IL-12R$\beta2^{high}$ subsets of IL-2-activated NK cells were obtained either by FACS (using rat anti-murine IL-12Rβ2 antiserum plus FITC-labeled goat anti-rat Ig) or by magnetic selection using FITC-conjugated anti-Ly49G2 (as a surrogate marker for IL-12Rβ2) plus anti-FITC microbeads (Miltenyi Biotec). Cells were sorted by FACS to >98% purity, and cells were sorted by magnetic selection to >90% purity. After sorting, cells were returned to culture and maintained in the presence of IL-2 (1000 U/ml)

Immunofluorescence and cell sorting

The surface phenotypes of cells were established using two-color flow cytometry. All stains were performed in a stepwise manner in the following order: 1) rat anti-murine IL-12Rβ2 antiserum 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 $DX\text{-}5^+$ 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 $DX\text{-}5^+$ cells of C57BL/6 mice were sorted into IL-12R$\beta2^{low}$ and IL-12R$\beta2^{high}$ 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 $DX\text{-}5^+$ cells of C57BL/6 mice were sorted into IL-12R$\beta2^{low}$ and IL-12R$\beta2^{high}$ 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 ^{51}Cr release assay using YAC-1 cells as targets. Briefly, 5×10^3 ^{51}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. 1*A*). Activated NK

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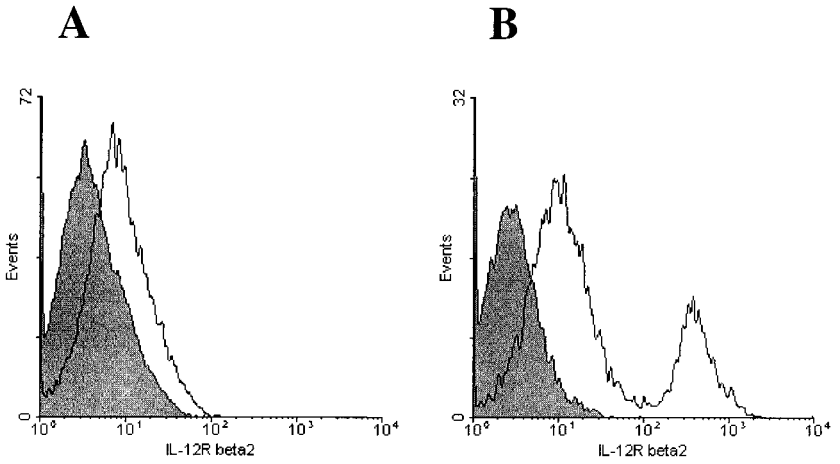

FIGURE 1. Detection of IL-12Rβ2 surface expression on Con A blasts and IL-2 blasts by flow cytometry. Splenic MNC from BALB/c mice were stimulated with Con A (2.5 μg/ml), IL-2 (10 U/ml), and IL-12; 200 U/ml) for 4 days (*A*) or with IL-2 (1000 U/ml) for 8 days (*B*) and then analyzed for surface expression of IL-12Rβ2 by flow cytometry. Cells were stained with rat anti-mouse IL-12Rβ2 antiserum (clear curve) or normal rat serum (shaded curve) and FITC-conjugated goat anti-rat Ig. Cells in *A* were first gated on the $CD3^+$ blast population. Cells in *B* were gated on the NK blast population.

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-12Rβ2 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-12Rβ2 antiserum (Fig. 1*B*). Compared with control samples stained with preimmune serum only, these two populations correspond to IL-12R$\beta2^{low}$ and IL-12R$\beta2^{high}$ subsets of NK cells. This pattern of NK subset distribution based on IL-12Rβ2 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-12Rβ2 antiserum, a competitive binding assay was performed. IL-2-activated $DX-5^+$ NK cells were stained with anti-IL-12Rβ2 antiserum in the absence (Fig. 2*A*) or the presence (Fig. 2, *B* and *C*) of varying amounts of soluble rIL-12Rβ2 as a competitor. At the highest concentration of rIL-12Rβ2 added (500 ng/ml), binding of the rat anti-mouse IL-12Rβ2 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-12Rβ2 antiserum binding to $DX-5^+$ IL-2 blasts by soluble

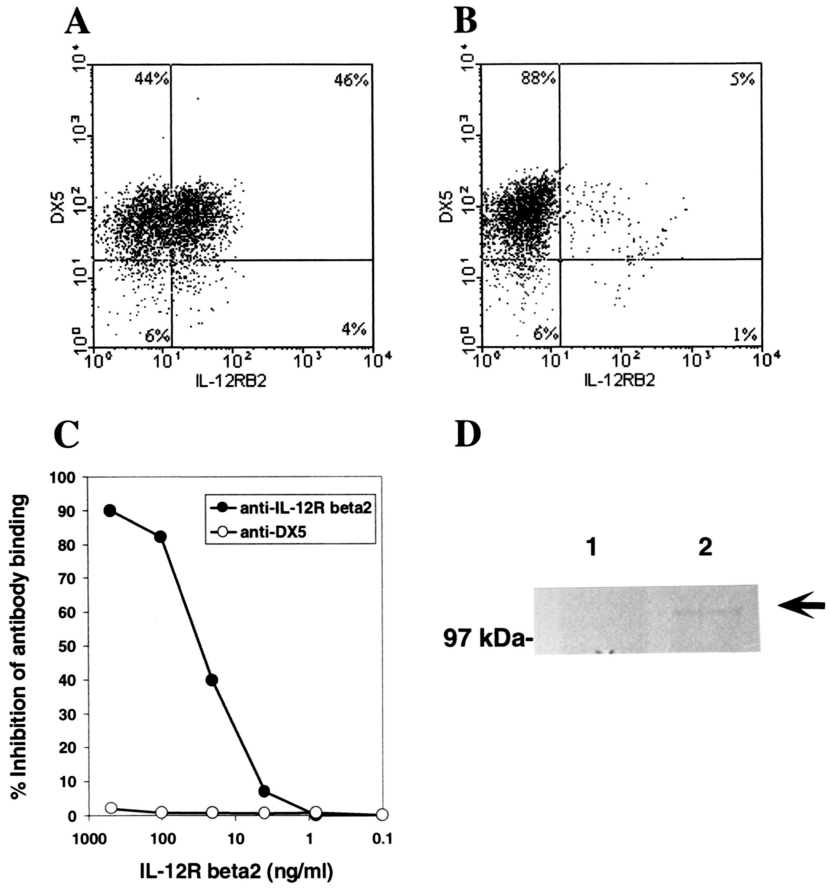

FIGURE 2. Specificity of the rat anti-mouse IL-12Rβ2 antiserum. $DX-5^+$ IL-2 blasts ($DX-5^+$ cells purified from C57BL/6 splenocytes by positive selection and stimulated with IL-2 (1000 U/ml) for 11 days) were stained with rat anti-mouse IL-12Rβ2 antiserum and FITC-conjugated goat anti-rat Ig plus PE-conjugated anti-DX-5 (*A–C*). Specific Ab binding was performed in the absence (*A*) or the presence (*B*) of competitor rIL-12Rβ2 (500 ng/ml). *C*, Titration of competitive inhibition of specific Ab binding shown in *A* and *B*. Data are shown as the percent inhibition of anti-mouse IL-12Rβ2 binding or anti-DX-5 binding in the presence of varying concentrations of rIL-12Rβ2. *D*, Specificity of anti-mouse IL-12Rβ2 antiserum as demonstrated by immunoprecipitation. Lysate from $DX-5^+$ IL-2 blasts (2.5×10^7) was incubated with normal rat serum (*lane 1*) or rat anti-mouse IL-12Rβ2 antiserum (*lane 2*) followed by biotin-conjugated goat anti-rat Ig. Biotin-conjugated complexes were recovered using streptavidin-agarose and were analyzed by Western blot using rat anti-mouse IL-12Rβ2 antiserum. A reactive protein with the expected molecular mass of IL-12Rβ2 is indicated by the arrow.

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rIL-12Rβ2 titrated over the range of rIL-12Rβ2 concentrations tested (Fig. 2*C*). 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. 2*D*). Together these results confirm the specificity and sensitivity of the anti-mouse IL-12Rβ2 antiserum 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-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} to DX-5$^+$, IL-12Rβ2^{high} cells remained stable. To determine whether formation of IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} subsets is an inherent feature of DX-5$^+$ cells upon activation with exogenous IL-2.

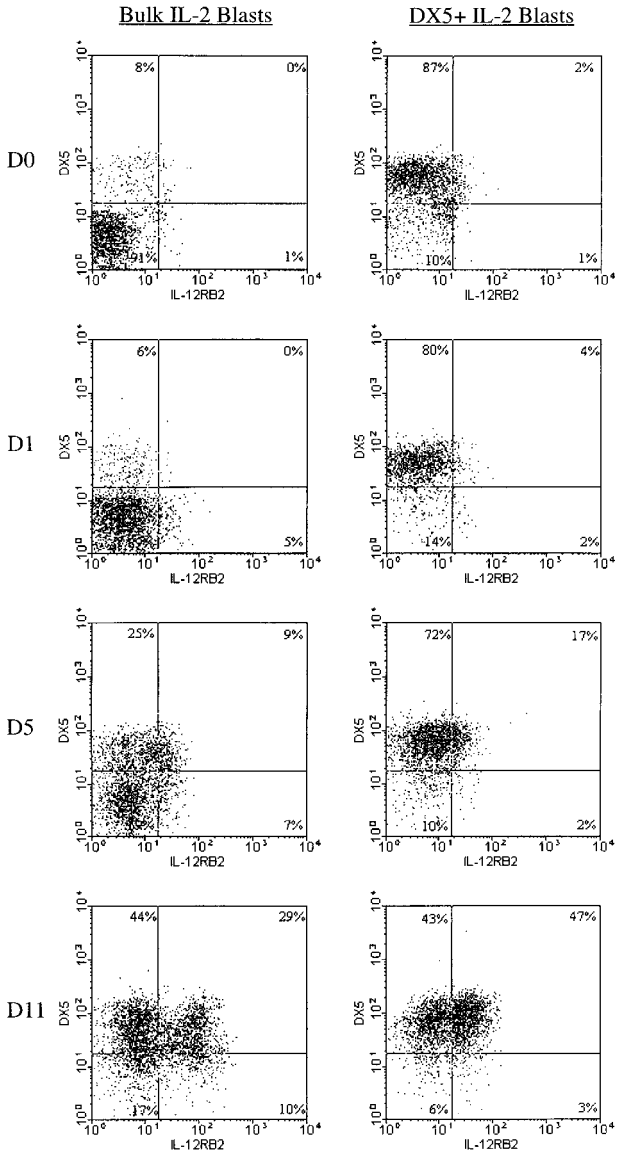

FIGURE 3. Kinetics of IL-12Rβ2 expression on IL-2 blasts. C57BL/6 bulk splenic MNC or DX-5$^+$ splenic MNC (purified from C57BL/6 splenocytes by positive selection) grown in the presence of IL-2 (1000 U/ml) were analyzed by flow cytometry at the indicated time points using rat anti-mouse IL-12Rβ2 antiserum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

Phenotype of IL-12Rβ2^{low} and IL-12Rβ2^{high} IL-2 blasts

To determine whether IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} cells. Furthermore, both the IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} populations. In marked contrast, expression of the Ly-49G2 marker was restricted to the IL-12Rβ2^{high} population, resulting in delineation of NK cells as being either Ly-49G2$^-$IL-12Rβ2^{low} or Ly-49G2$^+$IL-12Rβ2^{high}. B220, which is known to be a marker of activated NK cells (29), was present on both IL-12Rβ2^{low} and IL-12Rβ2^{high} populations, implying that the two subsets do not differ in terms of their activation state. Both the IL-12Rβ2^{low} and IL-12Rβ2^{high} subsets 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-12Rβ2^{low} and IL-12Rβ2^{high} populations. However, in some of the IL-2 blast preparations that we analyzed, the NK T cell population was comprised entirely of IL-12Rβ2^{high} 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 wide variety of mouse strains, although it is reported to have some cross-reactivity with Ly49A (33). Nonetheless, the staining pattern of DX-5$^+$ IL-2 blasts from both C57BL/6 and BALB/c mice (Fig. 5, *A* and *B*, respectively) using mAb 4D11 was identical with the staining pattern obtained using the mAb 4LO439, with precise correlation between IL-12Rβ2 and Ly-49G2 expression in both strains.

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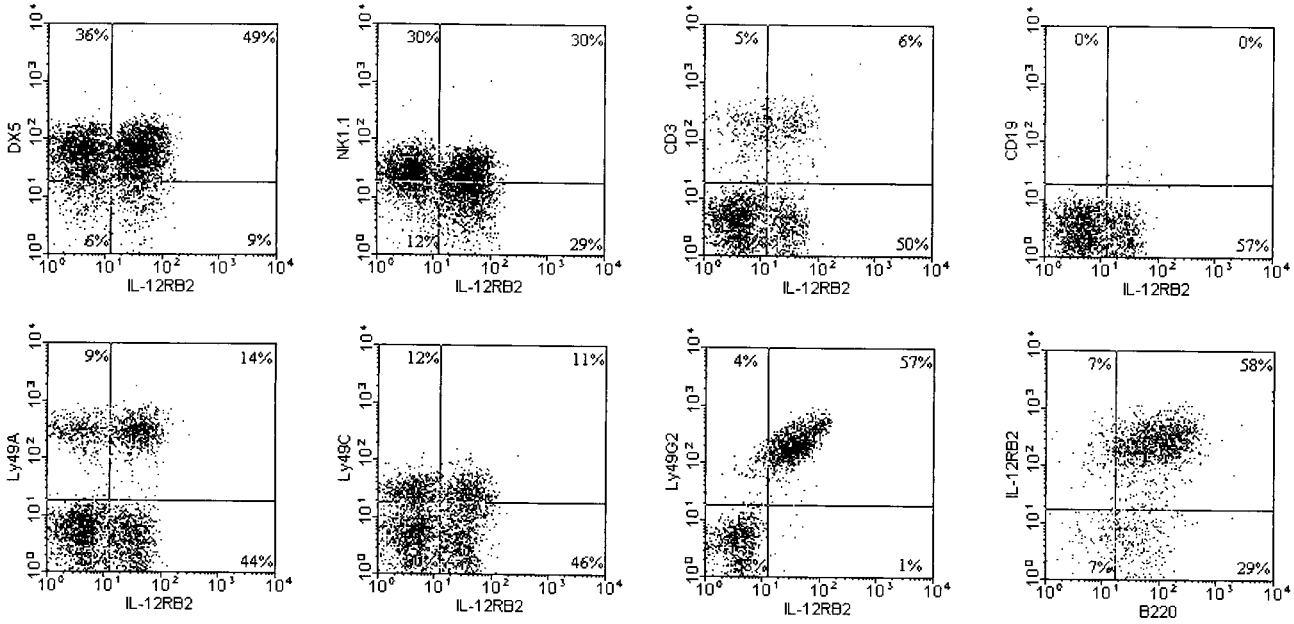

FIGURE 4. Phenotype of IL-12Rβ2^{low} and IL-12Rβ2^{high} murine NK cells. DX-5$^+$ NK cells (purified from C57BL/6 splenocytes by positive selection and stimulated with IL-2 (1000 U/ml) for 11 days) were stained consecutively with rat anti-mouse IL-12Rβ2 antiserum and FITC-conjugated goat anti-rat Ig followed by PE-conjugated anti-DX-5, NK1.1, CD3, and CD19 or biotin-conjugated Ly-49A (YE 1/32), Ly-49C (4LO3311), and Ly-49G2 (4LO439) and PE-conjugated streptavidin or with rat anti-mouse IL-12Rβ2 antiserum and PE-conjugated goat anti-rat Ig followed by FITC-conjugated anti-B220. All cells were blocked with 1% normal rat serum after incubation with goat anti-rat secondary Ab to block any unoccupied Ab binding sites. Cells were then analyzed by two-color flow cytometry.

To determine whether the IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{high} cells; however, the two sorted cell populations were still easily discernable even after 14 days in culture (data not shown).

Cytokine production by IL-12Rβ2^{low} and IL-12Rβ2^{high} DX-5$^+$ IL-2 blasts

We then wished to determine whether IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{high} cells were influencing the IL-12Rβ2^{low} population through an indirect mechanism, the assay was repeated using sorted IL-12Rβ2^{low} and IL-12Rβ2^{high} subsets. In addition, the cells were incubated in decreasing amounts of IL-12 to determine whether the IL-12Rβ2^{high} cells were more sensitive to IL-12 than the IL-12Rβ2^{low} subset. Although the percentage of IFN-γ-producing cells was somewhat higher in the IL-12Rβ2^{high} subset, particularly at higher IL-12 concentrations, both the IL-12Rβ2^{low} and IL-12Rβ2^{high} subsets clearly responded to IL-12 stimulation with increased production of intracellular IFN-γ (Fig. 6*C*). 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-12Rβ2^{low} and IL-12Rβ2^{high} subsets are similar in their capacity to respond to IL-12 stimulation.

To determine whether the IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} subsets, sorted IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-2^d 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 Ly49G2 class I inhibitory receptor on the IL-12Rβ2^{high} subset to see the stimulatory effect of

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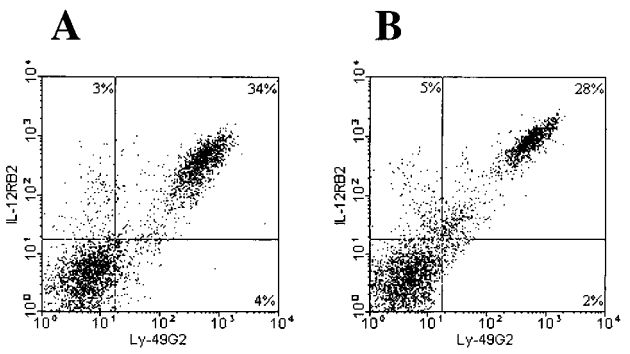

FIGURE 5. Coexpression of IL-12Rβ2 and Ly-49G2 on a subset of activated murine NK cells. DX-5$^+$ NK cells (purified from C57BL/6 (*A*) or BALB/c splenocytes (*B*) by positive selection and stimulated in vitro with IL-2) were stained with rat anti-mouse IL-12Rβ2 antiserum and PE-conjugated goat anti-rat Ig. Following a blocking step in 1% normal rat serum, cells were stained with FITC-conjugated anti-Ly-49G2 mAb (4D11) and then analyzed by two-color flow cytometry.

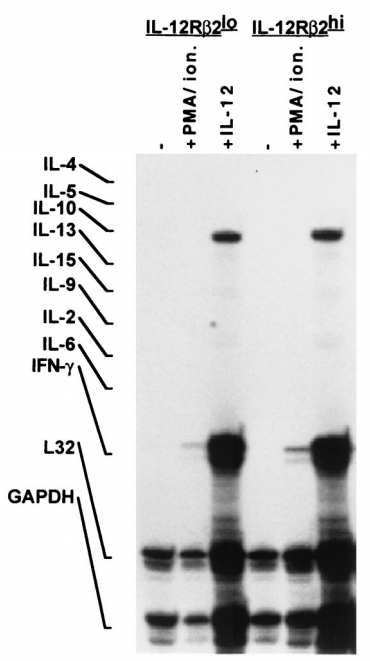

FIGURE 7. Analysis of cytokine gene expression in IL-12Rβ2^{low} and IL-12Rβ2^{high} murine NK subsets. DX5$^+$ IL-2 blasts from C57BL/6 mice (day 18) were sorted into IL-12Rβ2^{low} and IL-12Rβ2^{high} subsets, and cells (2×10^6/well) were cultured overnight in the absence or the presence of IL-12 (1 ng/ml). A third aliquot of cells was stimulated with PMA and ionomycin for the final 3 h of culture. Total RNA was prepared from cells, and cytokine mRNA expression was detected by RNase protection assay using the mouse cytokine set 1 probe set as described in *Materials and Methods*. Significant production of IFN-γ and IL-10 was evident in both the IL-12Rβ2^{low} and IL-12Rβ2^{high} subsets after stimulation with IL-12.

IL-12 (J. Webb, manuscript in preparation). These results provide further confirmation that despite the difference in levels of IL-12Rβ2 surface expression, both IL-12Rβ2^{low} and IL-12Rβ2^{high} subsets of NK cells respond similarly to stimulation with exogenous IL-12.

Discussion

In this report we have analyzed the expression of the IL-12Rβ2 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-12Rβ2 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-12Rβ2 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-12Rβ2 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

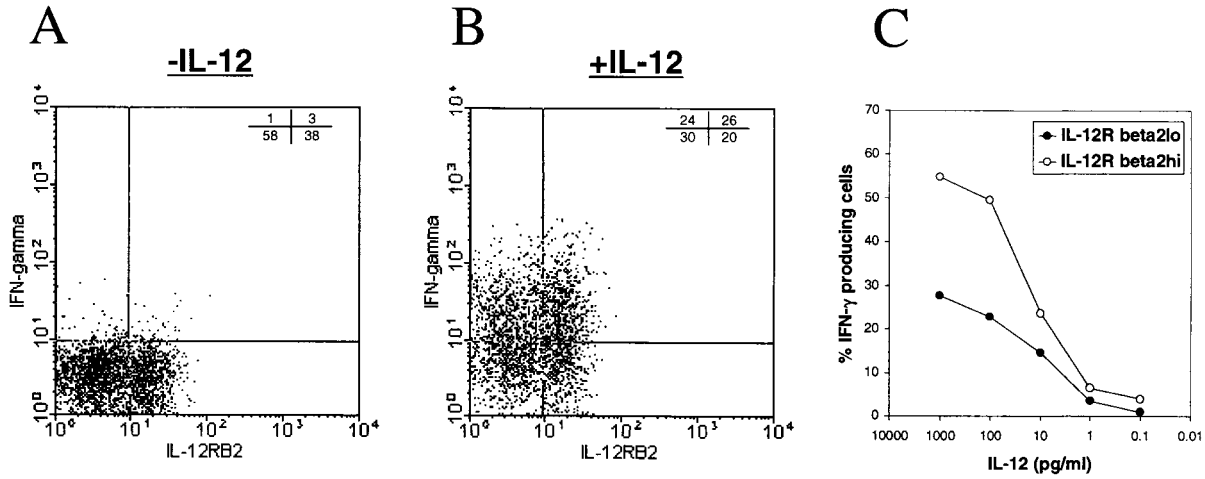

FIGURE 6. IL-12 responsiveness of IL-12Rβ2^{low} and IL-12Rβ2^{high} murine NK cells. DX-5$^+$ NK cells (purified from C57BL/6 splenocytes by positive selection and stimulated with IL-2 (1000 U/ml) for 9 days) were incubated overnight in the absence (*A*) or the presence (*B*) of IL-12 (200 U/ml), and analyzed for intracellular IFN-γ by flow cytometry. Cells were surface stained with rat anti-mouse IL-12Rβ2 antiserum and FITC-conjugated goat anti-rat Ig and then fixed with paraformaldehyde, permeabilized with saponin, and stained with PE-conjugated anti-IFN-γ. *C*, Titration of intracellular IFN-γ production in response to varying amounts of IL-12. Sorted IL-12Rβ2^{low} and IL-12Rβ2^{high} 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.

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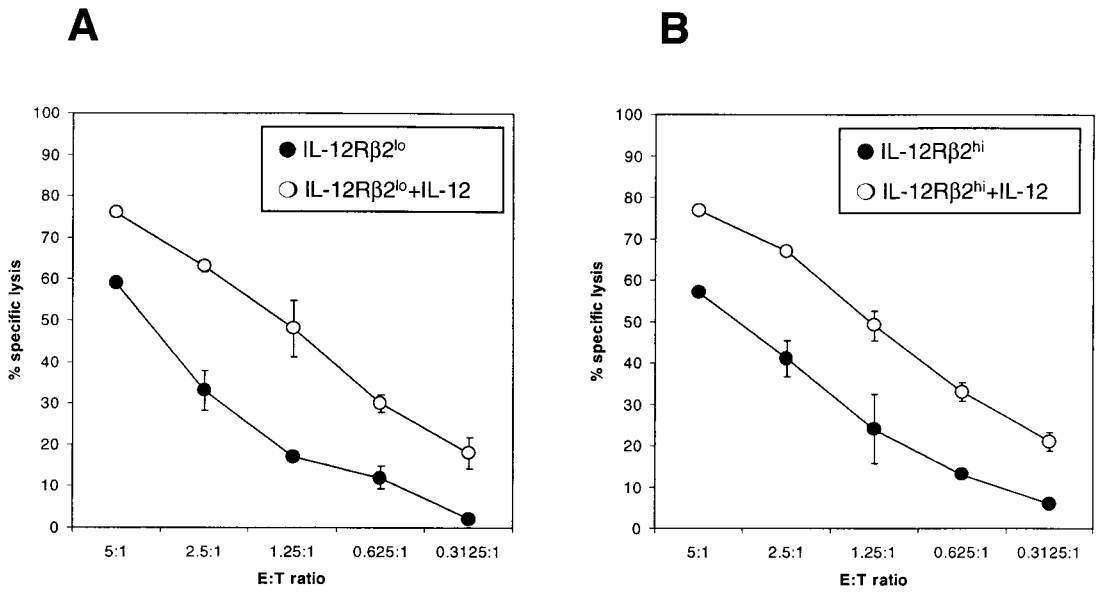

FIGURE 8. Effect of IL-12 on the cytolytic activity of IL-12Rβ2^{low} and IL-12Rβ2^{high} murine NK cells against YAC-1 target cells. DX5$^+$ IL-2 blasts from C57BL/6 mice (day 11) were sorted into IL-12Rβ2^{low} and IL-12Rβ2^{high} subsets, and cells were used as effector cells in a standard 4-h cytotoxicity 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 ^{51}Cr-labeled YAC-1 target cells (5×10^3) at the indicated E:T cell ratios.

production and IL-12Rβ2 expression (26). However, it is important 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-12Rβ2^{low} and IL-12Rβ2^{high} subsets without the addition of exogenous polarizing cytokines. Furthermore, differentiation into IL-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} 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 of 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-12Rβ2^{low} nor the IL-12Rβ2^{high} 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-12Rβ2^{low} 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-12Rβ2^{low} and IL-12Rβ2^{high} NK cells reported herein have a cytokine secretion pattern similar to that of the human NK1 subset, again suggesting that the IL-12Rβ2^{low} 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-12Rβ2^{low} and IL-12Rβ2^{high} populations. This finding implies that IL-12Rβ2^{low} NK cells are capable of responding to IL-12 equally as well as the IL-12Rβ2^{high} 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-12Rβ2^{low} and IL-12Rβ2^{high} 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-12Rβ2^{high} 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

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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-12Rβ2^{high} subset. 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 bears significant sequence homology to receptor molecules for IL-6 and gp130. Perhaps, in NK cells at least, IL-12Rβ2 plays an additional role by contributing to the binding of these or other related cytokines. Currently we do not have access to a mAb against murine IL-12Rβ1; therefore, we cannot asses the level of surface expression of this subunit in the IL-12Rβ2^{low} and IL-12Rβ2^{high} subsets. However, preliminary analysis of IL-12Rβ1 expression at the mRNA level using RT-PCR suggests that both the subsets express the IL-12Rβ1 subunit after activation with IL-2 (data not shown). Regardless, further studies are required to determine the biological significance of IL-12Rβ2^{low} and IL-12Rβ2^{high} NK subsets and their potential roles during an innate immune response.

Staining with a number of NK-associated markers, including the pan NK markers DX5 and NK1.1 as well as the NK cell inhibitory molecules Ly-49A and Ly-49C, confirmed that both the IL-12Rβ2^{low} and IL-12Rβ2^{high} cells were of NK origin. Essentially all cells in both subsets expressed the DX5 and NK1.1 markers, whereas specific subpopulations of IL-12Rβ2^{low} and IL-12Rβ2^{high} cells expressed the Ly-49A and Ly-49C markers. Furthermore, the Ly-49A$^+$ and Ly-49C$^+$ cells were equally distributed between the IL-12Rβ2^{low} and IL-12Rβ2^{high} subsets. In contrast, the Ly-49G2 marker was expressed exclusively on IL-12Rβ2^{high} NK cells. Thus, IL-2 activated murine NK subsets can be further defined as being either Ly-49G2$^-$IL-12Rβ2^{low} or Ly-49G2$^+$IL-12Rβ2^{high}. 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-2D^d and or H-2L^d (32), although inhibition by H-2L^d 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-2D^d (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 coordinated 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-2D^d-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-12Rβ2^{low} and IL-12Rβ2^{high} 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 cells 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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