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Multiple Cytokines Regulate the NK Gene Complex-Encoded Receptor Repertoire of Mature NK Cells and T Cells

Frances Gays, Kimberley Martin, Rupert Kenebeck, Jonathan G. Aust, and Colin G. Brooks

Mature NK cells comprise a highly diverse population of lymphocytes that express different permutations of receptors to facilitate recognition of diseased cells and perhaps pathogens themselves. Many of these receptors, such as those belonging to the NKR-P1, NK-G2, and Ly-49 families are encoded in the NK gene complex (NKC). It is generally thought that these NKC-encoded receptors are acquired by a poorly understood stochastic mechanism, which operates exclusively during NK cell development, and that following maturation the repertoire is fixed. However, we report a series of observations that demonstrates that the mature NK cell repertoire in mice can in fact be radically remodeled by multiple cytokines. Thus, both IL-2 and IL-15 selectively induce the de novo expression of Ly-49E on the majority of mature NK cells. By contrast, IL-4 not only blocks this IL-2-induced acquisition of Ly-49E, but reduces the proportion of mature NK cells that expresses pre-existing Ly-49 receptors and abrogates the expression of NK-G2 receptors while leaving the expression of several NKR-P1 receptors unaltered. IL-21 also abrogates NK-G2 expression on mature NK cells and selectively down-regulates Ly-49F. IL-4 and IL-21 additionally cause dramatic and selective alterations in the NKC-encoded receptor repertoire of IL-2-activated T cells but these are quite different to the changes induced on NK cells. Collectively these findings reveal an unexpected aspect of NKC receptor expression that has important implications for our understanding of the function of these receptors and of the genetic mechanisms that control their expression. The Journal of Immunology, 2005, 175: 2938–2947.

Natural killer cells are an important population of lymphocytes that is thought to play a critical role in protection against infectious (1) and malignant (2) diseases, and may also be involved in certain autoimmune diseases (3) and in the rejection of transplanted tissues (4). More generally, it appears that NK cells have evolved to recognize and eliminate aberrant, stressed, or diseased cells using a series of activatory and inhibitory receptors (5, 6). In murine species, many of these receptors are encoded in the NK gene complex (NKC) located near the distal end of chromosome 6 in mice (7, 8). Prominent among these are the Ly-49 receptors, which comprise ~10 members in the inbred strains of mice that have been examined. Some Ly-49 receptors such as Ly-49A and Ly-49G recognize classical class I molecules and deliver inhibitory signals via ITIMs located in their cytoplasmic domains. These inhibitory signals are thought to counteract low level activatory signals and thereby ensure that healthy cells expressing normal levels of class I molecules are not lysed by NK cells. Other Ly-49 receptors lack ITIMs and instead possess a charged transmembrane residue that allows them to deliver activatory signals (21). The balance of inhibitory and activatory signals depending on whether the NKG2 chain contains ITIMs or a charged transmembrane residue that allows them to deliver activatory signals via their association with DAP12 (9, 10). One of these, Ly-49H, is now known to recognize a class I-like molecule encoded by murine CMV with high avidity (11). Another member of the Ly-49 family is Ly-49E, which differs from the “classical” inhibitory and activatory Ly-49 receptors in a number of ways. Firstly, it has both a cytoplasmic ITIM and a charged transmembrane residue associated with DAP12 (21, 25, 26). The balance of inhibitory and activatory signals may be altered by displacement of the default peptide Qdm (22–24), and can deliver inhibitory or activatory signals depending on whether the NKG2 chain contains ITIMs or associates with DAP12 (21, 25, 26).

A second family of receptors encoded in the NKC are the series of heterodimers composed of a common CD94 chain and an NKG2 chain (21). In mice these CD94/NKG2 receptors recognize the nonclassical class I molecule Qa1 associated with its dominant peptide Qdm (22–24), and can deliver inhibitory or activatory signals depending on whether the NKG2 chain contains ITIMs or associates with DAP12 (21, 25, 26). The balance of inhibitory and activatory signals may be altered by displacement of the default Qdm peptide from Qa1 by peptides derived from “heat shock proteins” induced in stressed cells or encoded by pathogens (27). A third set of receptors encoded in the NKC is the NKR-P1 family that again includes inhibitory and activatory members (28, 29). At least some of the NKR-P1 members interact with C-type lectin-related ligands, which are also encoded in the NKR-P1 region (30, 31), but the significance of these interactions is currently unclear.

CD94/NKG2 receptors together with most of the Ly-49 family members and at least some of the NKR-P1 receptors are acquired in a stochastic manner during NK cell development, thereby generating a complex repertoire of mature NK cells that express NKC-encoded molecules in a random permuted manner (32). Such a highly diverse NK cell repertoire presumably exists to maximize...
the likelihood of recognizing diseased or infected cells, and perhaps pathogens themselves, but at the same time to ensure self-tolerance. It has generally been thought that, like the TCR repertoire, the NKC-encoded receptor repertoire once created during ontogeny remains fixed and immutable on mature NK cells. In support of this, although the level of expression of certain Ly49 receptors can be modulated by class I ligands (33), changes in the frequencies of NK cells expressing particular NKC-encoded receptors have rarely been observed. We report a series of observations that demonstrate that this notion is incorrect, and that the mature NK cell receptor repertoire is subject to extensive and often highly selective regulation by a variety of cytokines. The same cytokines also affect the repertoire of NKC-encoded receptors expressed on activated T cells, but often in quite different ways to their effects on NK cells.

Materials and Methods

Cells and culture conditions

C57Bl/6 female mice were obtained from Harlan Olac and were used at between 8 and 16 wk of age in procedures approved by U.K. licensing authorities. Splenous and thymus cells were obtained by mechanical disruption of tissues, and bone marrow cells were obtained from femurs and tibias. Aliquots of million nucleated cells were cultured in 24-well plates in 10% CO2, at 37°C in 1 ml of DMEM (51200-039; Invitrogen Life Technologies) made up in highly purified water and supplemented with 2× nonessential amino acids, 5× 10−5 M 2-ME, 10% FBS (F-7524; Sigma-Aldrich), and appropriate cytokines: human rIL-2 purchased from Cetus, used at a dose of 350 ng/ml, except where otherwise stated; mouse IL-15 purchased from PeproTech, used at 350 ng/ml; mouse rIL-4 purchased from PeproTech, used at 10 ng/ml; and mouse rIL-21, a kind gift of Dr. D. Foster (Zymogenetics, Seattle, WA) (34), used at 10 ng/ml, except where otherwise stated. At appropriate intervals cells were reseed or subcultured into new wells. In some experiments, spleen cells depleted of red cells by incubation in red cell lysis buffer for 5 min at room temperature were incubated for 10 min in PBS containing 5 μM CFSE. An equal volume of FBS was then added, cells incubated for 10 min at 37°C, and washed three times before setting up in culture. Fetal NK cells were prepared and grown as previously described (35).

Staining and flow cytometry

Aliquots of 2 × 106 cells were incubated at room temperature with appropriate combinations of reagents at predetermined optimal concentrations in HBSS (61200-093; Invitrogen Life Technologies) containing no bicarbonate and supplemented with 2% FBS and 0.2% sodium azide. Staining was analyzed on LSRS2 or FACSscan instruments (BD Biosciences), using forward and side light scatter to gate on single viable cells. Compensation for spectral overlap of dyes was set by running mixtures of unstained cells and cells stained with each fluorochrome singly. Data were analyzed using FCS Express v2 software. Cell sorting was performed on a FACSDiva instrument (BD Biosciences).

The CM4 mAb was generated by repeatedly immunizing rats with the immunizing cell line 1608b. One mAb, CM4, reacted strongly against cells transduced with Ly49E, more weakly against cells transduced with Ly49F, and not at all with cells transfected with Ly49A–D, G, H, or I (Fig. 1A). The specificity of CM4 was further evaluated in competition experiments with other mAbs that are known to react against Ly49E and/or Ly49F, namely 14B11, which recognizes Ly49C, F, H, and I in addition to Ly49E (36), 4D12 which recognizes Ly49C and Ly49E (16), and HBF which recognizes only Ly49F (37). As shown in Fig. 1B, unlabeled Ab completely inhibited the binding of the same labeled Ab to the relevant Ly49. In many cases, unlabeled Abs also inhibited the binding of heterologous labeled Abs, but only partially, indicating that the epitopes recognized by CM4, 4D12, 14B11, and HBF on Ly49E or Ly49F are probably distinct (consistent with the different specificities of the four mAbs) but are sufficiently close to cause steric hindrance of Ab binding. Interestingly, although unlabeled 14B11 strongly inhibited the binding of CM4 to Ly49F it caused little or no inhibition of the binding of CM4 to Ly49E.

The CM4 mAb reacted strongly against the immunizing cell line 1608b, and against other lines and clones of immature NK cells derived from fetal progenitors, but failed to stain any of the major cell populations present in adult thymus, spleen, or bone marrow cells (data not shown). To examine in detail its potential reactivity with mature NK cells, spleen cells were stained with the CM4 anti-Ly49E/F mAb, HBF anti-Ly49F, and mAbs against NK1.1 and CD3 in the presence and absence of unlabeled 14B11. The results of a typical experiment are shown in Fig. 2. In the absence of 14B11, the CM4 mAb stained ~5% of splenic NK cells, but only 1.1% were unambiguously Ly49E+, with a further small percentage perhaps expressing very low levels of Ly49E (Fig. 2B). The majority of cells that stained with CM4, comprising 4.2% of NK cells, were found in a tight population of weakly stained cells that co-stained with HBF and formed a diagonal line on the dot plot. Similarly, most HBF+ cells were weakly stained with CM4. If these double-positive cells expressed both Ly49E and Ly49F, it would imply that in contrast to the normally random coexpression of Ly49 molecules, Ly49E and Ly49F were generally coexpressed on adult splenic NK cells. However, two lines of evidence argue against this. Firstly, most of the cells that were costained by CM4

PCR analysis

RNA was prepared from cells using RNA-Bea (Biogenesis) according to the manufacturer’s instructions. cDNA was prepared using Promega MMLV H− reverse transcriptase according to the manufacturer’s instructions. Appropriate dilutions of cDNA were set up in PCR using BioTag enzyme (BioLine). For Ly49E exon 1, Ly49A, and β-actin transcripts, reactions were run with 2 mM Mg at 95/30 s, 58/30 s, 72/60 s for 40 (Ly49) or 30 (β-actin) cycles using the following primers: Ly49E forward ggtaggagatagccccagagggg, Ly49A forward catggagcggcagcaatctaaagtgac, reverse atggaggaatttacgcg, and β-actin forward atctggcaacctggaatggttcc, reverse tctactctgccgtgtcgt. For Ly49E exon-1a transcripts reactions were run with 1 mM Mg at 95/60 s, 58/60 s, 72/60 s for 40 cycles using the primers 5′-forward ctacccattctgtggtggc and reverse tacaacctgtgttctgcgtc. Reactions were run in 1% agarose gels, stained with ethidium bromide, and quantified on a GelDoc imager (Bio-Rad).

Transfectants

cDNA was prepared by PCR amplification of RNA extracted from cultured NK cells or from plasmid templates kindly provided by Dr. D. Rautel (University of California, Berkeley, CA) or by Dr. K. Kane (University of Alberta, Alberta, Edmonton, Canada). These were cloned into the pNS vector (39) and transfected into rat YB20 or RNK16 cells under G418 selection, followed by selection of clones showing stable high level expression.

Results

Characterization of a novel Ab that recognizes Ly49E

Ab were raised by immunizing rats with the immature NK cell line 1608b. One mAb, CM4, reacted strongly against cells transfected with Ly49E, more weakly against cells transfected with Ly49F, and not at all with cells transfected with Ly49A–D, G, H, or I (Fig. 1A). The specificity of CM4 was further evaluated in competition experiments with other mAbs that are known to react against Ly49E and/or Ly49F, namely 14B11 which recognizes Ly49C, F, H, and I in addition to Ly49E (36), 4D12 which recognizes Ly49C and Ly49E (16), and HBF which recognizes only Ly49F (37). As shown in Fig. 1B, unlabeled Ab completely inhibited the binding of the same labeled Ab to the relevant Ly49. In many cases, unlabeled Abs also inhibited the binding of heterologous labeled Abs, but only partially, indicating that the epitopes recognized by CM4, 4D12, 14B11, and HBF on Ly49E or Ly49F are probably distinct (consistent with the different specificities of the four mAbs) but are sufficiently close to cause steric hindrance of Ab binding. Interestingly, although unlabeled 14B11 strongly inhibited the binding of CM4 to Ly49F it caused little or no inhibition of the binding of CM4 to Ly49E.

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and HBF showed a typical “line of identity” staining pattern that occurs when two Abs react against the same molecule. This was strikingly different from the pattern observed when the two Abs recognize separate molecules on the same cells, as exemplified by costaining with CM4 and A1 (anti-Ly49A) mAbs (Fig. 2E). Secondly, when stained in the presence of 14B11, the CM4 low HBF/H11001 cells that formed the “diagonal” population almost completely disappeared, whereas the population of 1.1–1.2% of cells that was presumed to be truly Ly49E/H11001 remained unchanged (Fig. 2D). These data indicate that 1) in agreement with the results obtained with Ly49-transfected cells, 14B11 blocks the staining of Ly49F on NK cells by CM4 and HBF mAbs but not the staining of Ly49E by CM4, 2) most NK cells that costain with the CM4 and HBF mAbs (and probably all cells within the line of identity) express Ly49F and not Ly49E, and 3) Ly49E-expressing cells can be specifically identified in cell populations either by costaining with labeled CM4 and HBF mAbs or by staining with labeled CM4 in the presence of unlabeled 14B11.

Ly49E expression is progressively and selectively up-regulated following IL-2-induced activation of NK cells

Fig. 3 shows typical results obtained when the expression of Ly49E on different subpopulations of spleen cells was examined before and after culture in IL-2. For simplicity, in this and subsequent figures the percentage of cells expressing Ly49E will be taken as the percentage of cells in the top left quadrant of CM4 vs HBF staining diagrams, ignoring the very small proportion of Ly49E/H11001 cells that also express Ly49F. As previously described, only ~1% of fresh splenic NK cells expressed high levels of

**FIGURE 1.** Specificity of the CM4 mAb. A, YB2 or RNK cells transfected with Ly49 constructs were stained with medium or first layer Abs followed by AF488 goat anti-mouse Ig. Ly49A = A1, Ly49B = 1A1, Ly49C = 4D12, Ly49D = 4E5, Ly49E = 4D12, Ly49F = HBF, Ly49G = 4G11, Ly49H = 3D10, Ly49I = YB1. B, Cross-competition between Abs. YB2 cells transfected with Ly49E (YB2-E) and RNK cells transfected with Ly49F (RNK-F) were incubated with medium or saturating quantities of the unlabeled Ly49 Abs shown on the y-axis. After 20 min, AF488-labeled CM4, 4D12, or HBF Ab was added, and incubation was continued for an additional 20 min. Median fluorescence values were determined by flow cytometry and the percentage inhibition caused by pretreatment with each unlabeled Ab is plotted on the y-axis. The likelihood that the inhibition observed was due to chance variation was determined by Student’s t test (*, p < 0.05, **, p < 0.01, ***, p < 0.001). The experiments shown are representative of three similar experiments of each type that were performed.

**FIGURE 2.** Staining of adult NK cells by CM4. Freshly prepared spleen cells were stained with anti-NK1.1 and anti-CD3 mAbs together with the CM4 mAb and the HBF anti-Ly49F (or A1 anti-Ly49A) (E), in the presence of medium (A and B) or of an excess of unlabeled 14B11 mAb (C and D). Dot plots show the staining patterns of NK1.1+CD3+ cells. The data shown are representative of three similar experiments.

**FIGURE 3.** Expression of Ly49E and Ly49F on fresh and cultured spleen cells. Spleen cells were stained with NK1.1, CD3, CM4, and HBF mAbs before and after culture in 104 U/ml IL-2 or 102 U/ml IL-2 plus 2 µg/ml Con A for 6 days. Dot plots show the percentage of NK1.1+CD3+ NK cells, NK1.1+CD3+ T cells, and NK1.1+CD3+ T cells expressing different receptors. The data shown are representative of more than six experiments.
FIGURE 4. Ly49E is selectively induced on activated NK cells. A–C, Spleen cells were stained with NK1.1, CD3, CM4, and various Ly49 or NKG2 mAbs before and after culture in IL-2 for 0 (◼), 3 (shaded □), 7 (shaded △), or 14 (▲) days. The percentages of cells expressing Ly49 or NKG2 are the mean values from five independent experiments, and error bars represent the SEM. D, NK cells purified by cell sorting were cultured in IL-2 for 60 days. At various time points the total number of cells present (◼) and the percentage expressing Ly49E (●) and Ly49I (▲) was determined.

Ly49E, with another 3–4% expressing low levels (Fig. 3A). Among NK1.1+ T cells the proportion that expressed Ly49E was even lower, and among NK1.1− T cells (and also non-NK/non-T cells, data not shown) Ly49E expressing cells were virtually undetectable. Unexpectedly, following culture of spleen cells in IL-2 for 6 days, the proportion of NK cells that expressed Ly49E increased to >20%, about one-fifth of which expressed high levels of Ly49E, with the remainder at low levels (Fig. 3B). Among the NK1.1+ T cells and NK1.1− T cells present at this time, the expression of Ly49E remained low, but there was a substantial increase in expression of Ly49F. In these experiments IL-2 was used at the normal dose required to efficiently promote the proliferation of mouse NK cells, namely 350 ng/ml corresponding to 104 IU/ml (40, 41). By contrast, when spleen cells were cultured with Con A and a low dose (105 U/ml) of IL-2, very few of the proliferating T cells, even those that were NK1.1+, expressed either Ly49E or Ly49F. Under these conditions, NK cells failed to proliferate or survive (Fig. 3C). It should be noted that when CD8+ T cells are exposed to high concentrations of IL-2 they rapidly acquire many of the characteristics of NK cells (42), including the acquisition of NK1.1 (43) and of various Ly49 receptors (44). Thus, although the NK1.1+ T cells present in fresh spleen cell preparations (Fig. 3A) represent the distinct T cell subset generally known as NKT cells (45), the NK1.1+ T cells present following several days culture in high-dose IL-2 (Fig. 3B) are predominantly activated conventional CD8+ T cells.

Fig. 4 summarizes the results from a series of experiments in which the expression of various NK receptors was monitored on spleen cell subpopulations during culture with IL-2. NK cells displayed a substantial and progressive increase in the expression of Ly49E, and by day 14 ~50% of NK cells were Ly49E+ (Fig. 4A). In line with a recent report (46), there was also an increase in the proportion of cells expressing Ly49G but this increase was relatively small compared with the change in Ly49E expression and was not progressive. There was also little change in the proportion of cells expressing Ly49A, F, H, and I (and also Ly49C and Ly49D, data not shown), or of NKG2. As previously noted, and confirmed in Fig. 4, B and C, T cells cultured in high-dose IL-2 acquired various NK receptors, and these were particularly abundant among those that also acquired NK1.1. Strikingly, however, very few T cells, the vast majority of which in later cultures were conventional CD8+ αβ T cells, acquired Ly49E. More extended analysis of the acquisition of Ly49E by purified NK cells showed that it was closely linked to cell division, the proportion of cells expressing Ly49E, but not Ly49I, increasing progressively during the period of cell expansion, but ceasing to increase as soon as cell expansion ceased (Fig. 4D).

The acquisition of Ly49E by NK cells appeared to occur in a completely random manner with respect to the expression of other NK cell receptors. Thus, as shown in Fig. 5A, the frequency with which Ly49E−, Ly49Elow, and Ly49Ehigh subpopulations of NK cells expressed other Ly49 receptors or high levels of CD94 corresponded closely with the product rule of independent expression (32). By contrast, as shown in Fig. 5B, when purified NK cells were costained with CM4 and HBF mAbs, the Ly49E+ cells formed a line of identity similar to that seen in Figs. 2 and 3. Similarly, when purified NK cells were costained with the CM4 and 4D12 mAbs, the Ly49E+ cells formed a line of identity for CD94, A Sorted NK1.1−CD3− spleen cells were cultured with IL-2 for 3–4 wk then costained with HBF anti-Ly49F mAb, CM4, and a third anti-Ly49 mAb or anti-CD94. Dot plots show the patterns of corexpression of Ly49E with Ly49A, Ly49I, or CD94 among Ly49F− cells. The data shown are representative of those obtained in two experiments. B, Purified NK cells (left and middle) or YB2 cells transfected with Ly49E (right) were costained with CM4 (anti-Ly49E/Ly49F) together with HBF (anti-Ly49F) or 4D12 (anti-Ly49C/Ly49E) mAbs.
whereas the Ly49F+ C− E−, Ly49F+ E/C+, and Ly49F− F− C+ cells formed dispersed populations (Fig. 5B, upper left and right and lower right quadrants, respectively). As expected, staining of Ly49E transfected cells with CM4 and 4D12 mAbs revealed a pure line of identity.

Detailed analyses were also performed on lymphocyte populations in the bone marrow, peritoneum, thymus, and liver. Similar results were found to those with spleen cells, namely that NK cells

and other NK1.1/CD3-defined subpopulations present at these sites contained very few or no Ly49E+ cells, the only exception being that the thymic NK1.1+ CD3+ NKT population contained ~20% Ly49E+ cells, most of which were Ly49Ehigh. These cells frequently also expressed Ly49F (Fig. 6A). Following culture in IL-2, there was a substantial increase in the expression of Ly49E on NK cells from these sites (data not shown), but not on other cells (indeed, the expression of Ly49E on the NK1.1+ CD3+ cells present in 7-day cultures of thymocytes was markedly lower than on the corresponding fresh cells, Fig. 6A). γδ T cells present in thymus, spleen, and bone marrow were also examined and found to be largely devoid of Ly49E expression (Fig. 6, B–D). However, in contrast to αβ T cells, after 7-day culture with IL-2, a substantial proportion of these cells expressed Ly49E.

IL-2 induces the de novo expression of Ly49E on mature NK cells

In principle, the increased proportion of Ly49E-expressing cells found in IL-2-containing cultures could arise through either the selective expansion of pre-existing Ly49E+ cells or the induction

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**FIGURE 6.** Expression of Ly49E on thymic NKT cells and γδ T cells. The CM4 vs HBF staining pattern of NK1.1+ CD3+ thymus cells (A) and of γδ T cells present in thymus, spleen, and bone marrow (BM) populations (B–D). Staining of fresh cells (top panels) and staining after culture (bottom panels) with IL-2 for 7 days is shown. Data are representative of at least three experiments.

**FIGURE 7.** Ly49E+ cells do not arise by selective proliferation. Spleen cells were labeled with CFSE then placed in culture with IL-2 for 3 days. A, Expression of Ly49E and Ly49F on small cells (mostly B cells) and on large cells subdivided into NK1.1− cells (mostly T cells) and NK1.1+ cells (mostly NK cells). (Note that the small cells show artificiately high fluorescence in the PE channel due to dye resonance effects arising from their very high content of CFSE, and the cells are not Ly49F−.) B, CFSE fluorescence of small cells, NK1.1− large cells, and NK1.1+ large cells. C, CFSE fluorescence of the Ly49E−, Ly49EELow, and Ly49EEhigh subpopulations of NK1.1+ cells defined by the regions inset in A (upper right) dot plot. Similar results were found in two other experiments.

**FIGURE 8.** Initiation of Ly49E expression in vitro and in vivo. A, Fresh spleen cells were stained with anti-NK1.1 and 4D12 anti-Ly49C/Ly49E mAbs, and total unfractionated NK1.1high (left) or NK1.1high 4D12− (middle) NK cells were purified by cell sorting. After 12 days culture with IL-2, the cells (>99% of which were NK1.1+ CD3−) were examined for expression of Ly49A, C, E, F, and G. Unfractionated NK1.1high (■ and sorted 4D12− NK1.1high (■) cells are shown. B, Sorted NK1.1high spleen cells were cultured for 5 days with IL-2, then stained with CM4 and HBF mAbs and sorted into Ly49E− Ly49F− and Ly49E+Ly49F− subpopulations (top panels). Unsorted and sorted subpopulations of stained cells were then returned to culture for an additional 14 days and stained again with the CM4 and HBF mAbs (bottom panels). C, Young adult mice were injected i.p. daily for 3 days with PBS or 1 million units of IL-2. Dot plots show CM4 and HBF staining of NK1.1+ CD3− gated cells. All dot plots shown are representative of results obtained in two to three independent experiments.
days culture in IL-2 were the product of no greater number of cell
or at final dilutions of 1/10, 1/100, and 1/1000 in PCR with Ly49E, Ly49A, or β-actin primers. B, cDNA prepared from equivalent numbers of fetal (F) or adult (A) NK cells was set up in PCR with primers for sites in exon 1 plus exon 8 or exon-1a plus exon 4 of Ly49E. Lane 0 shows PCRs with no template.

of Ly49E expression on Ly49E" cells. To discriminate between these two possibilities, spleen cells were labeled with CFSE, and the extent of proliferation of different subpopulations was determined. The results of a typical experiment are shown in Fig. 7. Following 3 days culture in IL-2 the vast majority of small cells (which lacked NK1.1 and were mostly B cells) had undergone little or no proliferation, having a mean CFSE fluorescence of 3041 (Fig. 7, A and B). By contrast, most large cells were the products of extensive proliferation. Those lacking NK1.1 (mostly T cells) were virtually all Ly49E" and had undergone up to six divisions, reducing their mean CFSE fluorescence to 408. Those expressing NK1.1 (mostly NK cells) had divided somewhat less with a mean CFSE level of 580 (data not shown). These results show that the Ly49E" cells present after 3 days culture in IL-2 were the product of no greater number of cell divisions than the Ly49E" cells, and indeed a slightly fewer average number of divisions than the Ly49F" cells or NK1.1" cells. Similar results were found when cells were analyzed after 4 or 5 days of culture.

To further examine the origins of Ly49E-expressing cells, a series of sorting experiments was performed. Fresh spleen cells were stained with NK1.1 and 4D12 mAbs and sorted into either total unfractionated NK1.1" cells or into NK1.1" 4D12" cells, the latter representing a population of pure NK cells depleted of both Ly49C" and Ly49E" cells. As shown in Fig. 8A, both NK populations were ~95% pure, and the proportion of 4D12" cells was reduced by almost 90% in the NK1.1" 4D12" population. Following 12 days culture in IL-2, the proportion of these Ly49C/
Ly49E-depleted NK cells that expressed Ly49C was less than one-quarter of the proportion that expressed Ly49C in control cultures of unfractionated spleen cells, confirming that 4D12+ cells had been severely depleted by the cell sorting. By contrast, the proportion that expressed Ly49E (and also Ly49A, Ly49F, and Ly49G) was very similar in the two cultures. These results demonstrate that 1) the Ly49E-expressing cells found in IL-2-containing cultures arise from pre-existing mature (NK1.1high) NK cells, 2) the expression of Ly49E is initiated de novo in NK cells that previously lacked Ly49E, and 3) the acquisition of Ly49E is an autonomous function of NK cells that does not require the participation of other cell types such as macrophages, dendritic cells, or T cells. The slow progressive manner in which Ly49E is acquired suggests either that Ly49E-expressing cells have a growth advantage, which is a possibility inconsistent with the CFSE and sorting experiments already described, or that the expression of Ly49E in NK cells exposed to IL-2 is generally triggered only after a considerable lag period. To test this important distinction directly, NK cells were sorted from fresh spleen cells. After 5 days culture in IL-2, at which point ~13% of the NK cells already expressed Ly49E, the cells were stained with the CM4 and HBF mAbs, and the Ly49E Ly49F- cells purified by sorting. As shown in Fig. 8B, the Ly49E Ly49F- sorted cells were almost completely devoid of Ly49E+ and Ly49F+ cells. However, following 14 days further culture in IL-2 ~38% of the sorted Ly49E- cells now expressed Ly49E. For comparison, ~49% of control cells that had been stained and returned to culture without sorting expressed Ly49E. A small but clear subpopulation of the control cells also retained expression of Ly49F, but almost no Ly49F- cells had developed in the Ly49E Ly49F- sorted population. These data indicate that Ly49E, but not Ly49F, is acquired by mature NK cells in a progressive and stochastic manner over a prolonged period of time. By contrast, as shown by purifying Ly49E Ly49F- cells from 5-day cultures, Ly49E, once acquired by mature NK cells, is rarely if ever lost even when the cells enter a resting G0 stage (Fig. 8B).

To determine whether the expression of Ly49E on IL-2-activated NK cells correlated with gene transcription, sorted NK cells that had been cultured in IL-2 for 10 days were sorted again into Ly49E+ and Ly49E- populations that were ~98% pure. As shown in Fig. 9A, Ly49E+ cells expressed much higher levels of Ly49E mRNA than did Ly49E- cells, whereas both populations expressed similar levels of β-actin mRNA. Quantitation of band intensities indicated that the purified Ly49E+ cells contained no more than ~2% of the Ly49E transcripts found in Ly49E- cells. Interestingly, not only did purified NK cells cultured in IL-2 express high levels of conventional Ly49E transcripts initiating from the so-called Pro-2 promoter just upstream of exon 1, purified NK cells also expressed transcripts containing exon1a that initiated from the distal upstream promoter termed Pro-1 (47) albeit at somewhat lower levels than found in fetal NK cells that were used as a positive control source of Ly49E Pro-1 transcripts (Fig. 9B). Finally, the acquisition of Ly49E by NK cells was not limited to in vitro systems because within 3 days of administering IL-2 to mice, splenic NK cells showed a substantial up-regulation of Ly49E expression (Fig. 8C). The dose of IL-2 used in these experiments (35 µg/106 IU injected i.p. daily for 3 days) was based on, but was somewhat lower than, the doses reported by others to be required for the activation of mouse NK cells in vivo (48, 49).

Multiple cytokines regulate the expression of NK receptors on mature NK cells and T cells

The discovery that IL-2 selectively induces the expression of Ly49E on mature NK cells led us to explore the effects of other cytokines on the expression of NKC-encoded receptors on NK cells and T cells. IL-15 behaved in a similar manner to IL-2, inducing the appearance of Ly49E on a large proportion of NK cells but on only a very small proportion of NK1.1+ and NK1.1- T cells (Fig. 10). By contrast, αIFN, γIFN, IL-4, IL-12, IL-18, IL-21, or a combination of IL-12 and IL-18, when tested over a range of concentrations, had no noticeable effect on the expression of Ly49E on NK cells even though, as expected, each of these cytokines clearly activated NK cells as judged by an increase in either the number or size of NK cells in culture (data not shown). However, when combined with IL-2, two of these cytokines, IL-4 and IL-21, were found to have unexpected and pleiotropic effects on the expression of NK-encoded receptors.

The results of a large series of experiments are summarized in Fig. 11. IL-4 strongly inhibited the de novo acquisition by NK cells of Ly49E in response to IL-2 (Fig. 11A). Remarkably, however, it also substantially reduced the proportions of NK cells expressing pre-existing Ly49 receptors, typically by 50–70%, and the median level of expression of these receptors on remaining positive cells was greatly reduced (data not shown). For example, the proportion of NK cells that expressed Ly49I following culture with IL-2 alone for 7 days averaged 45% (very similar to the proportion of fresh NK cells that expressed Ly49I), whereas in cultures containing IL-4 it averaged only 20%. Even more dramatically, IL-4 almost completely eliminated the expression of CD94/NKG2 receptors on NK cells, yet had no discernible effect on the expression of NKRPI receptors as determined using the PK136 anti-NKRPI and 10A7 anti-NKRPI/A mAbs. IL-21 exerted quite different effects on NK cells (Fig. 11B). IL-21 did not affect the acquisition of Ly49E in response to IL-2, and had relatively little effect on the pre-existing expression of most Ly49 receptors but almost completely blocked the expression of Ly49F. Like IL-4, IL-21 greatly reduced the proportion of NK cells expressing CD94/NKG2 receptors and had no effect on the expression of NKRPI/A. However, it noticeably reduced the level of expression of NKRPI on NK cells (see Fig. 12).

Both cytokines also affected the expression of NKC-encoded receptors on T cells cultured in IL-2, but often in a very different way to that found on NK cells. IL-4 reduced the proportions of T cells that expressed Ly49 receptors by 50–80% and almost completely blocked the acquisition of NKG2 receptors (Fig. 11C). By

**FIGURE 12.** Effect of IL-4 and IL-21 on the expression of particular receptors. Typical data obtained in one experiment. The expression of NK1.1 (NKRPIC) and CD3 on spleen cells cultured in IL-2 (left column) in the presence or absence of IL-4 or IL-21 is shown. The remaining columns show the expression of Ly49G and NKG2 on the NK1.1-CD3+ NK cell and CD3+ T cell populations.
contrast, it almost doubled the proportion of T cells that acquired NKR-P1C, but caused no significant induction of NKR-P1A/D receptors. The effects of IL-2 were very different (Fig. 11D). It dramatically and selectively increased the proportion of T cells that expressed Ly49E. At the same time it blocked the acquisition of NKR-P1C and, in complete contrast to its down-regulation of NK2G receptors on NK cells, it increased the proportion of T cells that acquired NK2G receptors \( \sim 2 \)-fold. In addition, as shown in Fig. 11E, different receptors were sensitive to different concentrations of IL-2, with the acquisition of NKR-P1C by T cells being inhibited at IL-2 doses 10-fold lower than doses required to inhibit the acquisition of Ly49F by T cells. Typical staining data from one experiment are shown in Fig. 12.

Discussion
The studies described in this report demonstrate that the receptor repertoire of mature NK cells is not fixed during development, but can undergo major alterations in response to environmental signals, in particular in response to certain cytokines. The principal effects described in this study are firstly that Ly49E, which is rarely expressed by mature NK cells from healthy animals, can be selectively induced on the majority of NK cells by IL-2 and IL-15 in the absence of substantial changes in the expression of other NK receptors. Secondly, IL-4 and IL-21 can cause major and highly selective alterations in the repertoire of NKR-P1, NK2G, and Ly49 receptors expressed on mature NK cells and T cells. In view of the very limited number of cytokine combinations examined in the present study it seems likely that many other cytokine combinations will affect the expression of NKC-encoded receptors on NK cells and T cells. In essence, the repertoire of NKC-encoded receptors expressed by mature NK cells is revealed to be highly malleable, presumably providing an adaptive response to diverse threats encountered by the animal.

Current knowledge of Ly49E, particularly its function, is relatively limited. Previous studies have demonstrated that Ly49E is expressed abundantly, and in the absence of other Ly49 family members, by NK cells in fetal and neonatal mice (15, 16), and also by V-y3-bearing subpopulations of \( \gamma \delta \) T cells in vivo (18). Ly49E is acquired in a stochastic manner during the development of NK cells from immature progenitors, both in vitro (17) and in vivo (50), but is lost at some later point in development resulting in its being expressed on only a very small proportion of adult splenic NK cells. The detection of Ly49E in these previous studies relied on the use of the 4D12 mAb, which also reacts with Ly49C and perhaps with other members of the Ly49 family (16). Specific detection of Ly49E by this mAb is rendered difficult by the lack of any commercially available Ly49C-specific mAb. By contrast, the CM4 mAb described in this study appears to react only on Ly49F, which is expressed on <5% of adult NK cells. Costaining with the commercially available HBF mAb that is specific for Ly49F, or blocking of the Ly49F cross-reactivity of CM4 with the commercially available 14B11 mAb, provides two simple methods for the specific detection of Ly49E.

Such studies confirmed that <2% of adult splenic NK cells clearly expressed Ly49E, and showed that Ly49E was expressed by similarly low percentages of bone marrow, peritoneal, and liver NK cells. Ly49E was also expressed by <2% of \( \alpha \beta \) or \( \gamma \delta \) T cells in adult spleen, thymus, and bone marrow, and although rarely expressed by splenic NKT cells was present at high levels on a substantial proportion of thymic NKT cells. In contrast to the generally low or absent expression of Ly49E on fresh NK cells and T cells from normal mice, its expression could be dramatically up-regulated both in vivo and in vitro by exposure to IL-2 or IL-15. This event was highly selective in several ways. First, although IL-2 caused some increase in the proportion of NK cells expressing Ly49G and certain other Ly49 receptors, these changes were small compared with those observed for Ly49E. Next, although IL-2 caused a marked increase in the proportion of conventional \( \alpha \beta \) T cells that expressed most Ly49 receptors (and also CD94/NKG2 receptors), particularly among those that also acquired NK1.1, there was very little increase in the expression of Ly49E. By contrast, \( \gamma \delta \) T cells from a variety of sources readily acquired Ly49E in response to IL-2.

The acquisition but not maintenance of Ly49E expression by NK cells was clearly linked to cell division, increasing progressively throughout the 2-wk period during which NK cells proliferated to IL-2, but failing to either increase or decrease when cells spontaneously returned to the resting state. The linkage to cell division, rather than activation, was further indicated by the inability of NK cell activators that fail to trigger proliferation, such as IFN, IL-18, and IL-12, to induce Ly49E expression. The apparent requirement for proliferation suggested that the increase in the proportion of NK cells that expressed Ly49E might have been due to selective expansion of the pre-existing small subpopulation of Ly49E+ cells. However, this explanation was ruled out by the finding that the cells that acquired Ly49E had not undergone any more rounds of division than those cells that did not acquire Ly49E, and more directly by the demonstration that purified Ly49E+ splenic NK cells acquired Ly49E with a similar efficiency to unfractionated cells.

An important finding was that the acquisition of Ly49E by mature NK cells occurred in a similar progressive and stochastic manner to that previously found for fetal NK cell progenitors (17). Thus, the proportion of mature NK cells expressing Ly49E increased in a roughly linear manner throughout the proliferation phase in IL-2. The acquisition of Ly49E occurred completely independently of the pre-existing expression of other Ly49 receptors, the percentage of cells coexpressing Ly49E and other Ly49 (or NKG2) receptors closely obeying the product rule (32). Even after exposure to IL-2 for 1 wk, many cells that had failed to activate expression of Ly49E up to that point could still do so when exposed to IL-2 for a second week. By contrast, Ly49E, once acquired, was never lost under the conditions used. Expression was clearly controlled at the transcriptional level, as cells that had switched on the expression of Ly49E had much greater quantities of Ly49E mRNA than those that remained Ly49E−. Intriguingly, Ly49E was acquired by NK cells at two different levels, generating a strikingly bimodal expression pattern with distinct subpopulations of Ly49Elow and Ly49Ehigh cells. A similar bimodal expression pattern was seen on \( \gamma \delta \) T cells cultured in IL-2 and has previously been reported on immature NK cells (17) and on \( \gamma \delta \) T cells (51). To our knowledge no other Ly49 receptor has been found to be expressed in this bimodal manner, although CD94 (17, 26) and probably NKG2 (16) are. Ly49E is unusual among Ly49 receptors in having both a cytoplasmic ITIM-like domain and a positively charged transmembrane residue. It is tempting to speculate that high level expression of Ly49E is dependent on the coexpression of an adaptor or partner protein that contains a suitably positioned negatively charged transmembrane residue.

Even more surprising than the discovery that IL-2 and IL-15 could specifically induce the expression of Ly49E on NK cells (and \( \gamma \delta \) T cells) was the finding that two other cytokines from a small selection of cytokines investigated could induce profound and selective changes in the repertoire of NKC-encoded receptors expressed on NK cells and T cells. IL-4 essentially eliminated expression of pre-existing CD94/NKG2 receptors on NK cells and blocked the IL-2-driven acquisition of CD94/NKG2 receptors by T cells. IL-4 also greatly reduced the proportions of NK cells that...
expressed preformed inhibitory and activatory Ly49 receptors, and greatly reduced the proportions of T cells that acquired these receptors in response to IL-2. By contrast, IL-4 had no effect on the proportion of NK cells expressing NKRP1A/D or on the level of expression of NKRP1C (NK1.1) but markedly increased the proportion of T cells that acquired NKRP1C without inducing any significant expression of NKRP1A/D.

Although IL-21, like IL-4, is produced predominantly by Th2 cells (52), it had quite different effects on the expression of NKC-encoded molecules. IL-21 had little effect on the expression of most Ly49 receptors on NK cells, including the IL-2-driven acquisition of Ly49E, but virtually ablated expression of Ly49F. It also had little effect on the proportions of NK cells expressing NKRP1A/D or the level of expression of NKRP1A/D but reduced the level of expression of NKRP1C. Some of these findings are in agreement with results recently reported by Brady et al. (53). However, whereas their study reported that IL-21 enhanced the expression of CD94/NKG2 receptors on NK cells, in our hands it had exactly the opposite effect, greatly reducing the proportion of NK cells expressing CD94/NKG2 receptors. By contrast, it increased the proportion of T cells that expressed CD94/NKG2 and the level of expression of these receptors on T cells. The most dramatic effects of IL-21 on T cells, however, were a substantial and selective enhancement of Ly49E expression and the almost complete inhibition of NKRP1C expression. In addition to these qualitative effects, different doses of IL-21 could differentially affect the expression of different receptors. The reasons for the discrepancy in results obtained by ourselves and Brady et al. (53), particularly concerning CD94/NKG2 receptors on NK cells, are unclear. Whereas Brady et al. (53) used DX5-expressing cells purified from the bone marrow of RAG−/− mice, we used whole spleen cells from normal mice. One possibility is that the cells used by Brady et al. (53) contained a substantial proportion of immature NK cells that may behave differently than mature NK cells. Alternatively, factors present in whole spleen cell cultures may have altered the response of mature NK cells to IL-21.

The discovery that the repertoire of NKC-encoded receptors expressed by mature NK cells is subject to substantial and highly specific regulation by a variety of cytokines has important implications for our understanding of “self tolerance” within the NK cell population and of the biochemical and genetic mechanisms that control the stochastic acquisition of these receptors. It is generally believed that to avoid self reactivity only those immature NK cells that have randomly acquired at least one (and preferably only one) self reactive inhibitory receptor are allowed to mature, and that thereafter the expression of these receptors is retained to maintain self tolerance (32). The finding that mature NK cells can lose expression of inhibitory Ly49 and NKG2 receptors implies a potential loss of self tolerance that could lead to increased tissue destruction and inflammation. Although such an event could be beneficial in a spatially and temporally restricted context, it is surprising that the two cytokines found in this study to promote the loss of inhibitory receptors are selectively produced by the Th2 subset of T cells, which is generally considered to be anti-inflammary. It is possible that potential autoreactivity associated with the loss of inhibitory receptors is mitigated by other changes. These might include the loss of activatory receptors, inactivation of the cytolytic machinery, or the imposition of an anergic state similar to that found among NK cells in class I-deficient mice (54, 55) and for a subpopulation of NK cells in normal mice that lack inhibitory self-class I receptors (56). However, none of these outcomes are compatible with reports that IL-21 enhances cytolytic activity and γIFN production by NK cells (34, 53, 57). Down-regulation of inhibitory receptors may play a significant role in the NK cell-dependent enhancement of tumor rejection by IL-21 (53, 58), and could strongly influence the success of antiviral defenses and normal pregnancy in humans. Both circumstances have recently been reported to be dependent on a critical balance between activatory and inhibitory signals delivered to NK cells (59, 60).

It has frequently been proposed that the stochastic expression of NKC receptors might be controlled by limiting concentrations of key transcription factors. In support of this, Held et al. (61) have demonstrated an intriguing relationship between the number of functional genes for the transcription factor TCF and the frequency of NK cells expressing Ly49A. Signals delivered by cytokine receptors through the Jak/Stat pathways might modulate the production or activity of such key transcription factors, or as exemplified by the regulation of IL-2Rα expression by IL-2 (62), Stat molecules may bind directly to regulatory sequences in NKC genes. Recently, an alternative model for the stochastic acquisition of NKC receptors has been provided by the discovery of a distal upstream bidirectional promoter termed Pro-1. The frequency of expression of Ly49 receptor genes is directly related to the frequency of transcription from the forward and reverse promoter elements of Pro-1 (47). It is possible that changes in the frequency with which mature NK cells express Ly49 receptors, particularly the IL-2/IL-15-driven de novo expression of Ly49E, might involve the reactivation and resetting of this genetic switch, a possibility supported by our finding of Pro-1 transcripts in IL-2-activated mature NK cells. Much work remains to be done to understand the mechanisms that control the expression of NK receptors. The finding that IL-2 and other cytokines can cause major changes in the NKC-encoded repertoire of mature cells provides not only new insights into these control processes, but experimentally tractable models with which to study them.

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