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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 Kenefek, 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, NKG2, and Ly49 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 Ly49E on the majority of mature NK cells. By contrast, IL-4 not only blocks this IL-2-induced acquisition of Ly49E, but reduces the proportion of mature NK cells that expresses pre-existing Ly49 receptors and abrogates the expression of NKG2 receptors while leaving the expression of several NKR-P1 receptors unaltered. IL-21 also abrogates NKG2 expression on mature NK cells and selectively down-regulates Ly49F. 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 Ly49 receptors, which comprise 10 members in the inbred strains of mice that have been examined. Some Ly49 receptors such as Ly49A and Ly49G 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 Ly49 receptors lack ITIMs and instead possess a charged transmembrane residue that allows them to deliver activatory signals via their association with DAP12 (9, 10). One of these, Ly49H, is now known to recognize a class I-like molecule encoded by murine CMV and to participate directly in the innate immune response to murine CMV (11). Another member of the Ly49 family is Ly49E, which differs from the “classical” inhibitory and activatory Ly49 receptors in a number of ways. Firstly, it has both a cytoplasmic ITIM and a charged transmembrane residue (12), although whether Ly49E associates with DAP12 or other adaptor proteins is unknown. Secondly, the Ly49e gene is present in all mouse strains so far examined and the coding sequence appears to be more highly conserved than that of other Ly49 receptors (13, 14). Thirdly, Ly49E is expressed abundantly on fetal or immature NK cells (15–17) and on certain γδ T cells (18), but at barely detectable frequencies on adult splenic NK cells (16, 19). Finally, its failure to bind to a variety of soluble or cell bound MHC class I molecules (20) or to be modulated by class I molecules in vivo (16) indicates that its natural ligand is not an endogenous class I molecule.

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 Ly49 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. Spleen 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 2×10−3 M HEPES, 10% FBS, and β-actin transripts. 2

Staining and flow cytometry

Aliquots of 2×107 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 LS2R 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).

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 BioTaq 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 cg tagggatatagcagc, reverse tctgccctagcaagaggag; Ly49A forward catggcagcaactaaggtaac, reverse atggaggttactccag; and β-actin forward ttcagacaagttgac, reverse tctgcccttgctgtc. For Ly49E exon 1-α transcribed reactions were run with 1 mM Mg at 95/60 s, 58/60 s, 72/60 s for 40 cycles using the primers Pro1 forward tcaacctctcctggg and reverse tctcagagtccggtgat.

Results

Characterization of a novel Ab that recognizes Ly49E

Abs 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 or Ly49B. This 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 costained 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 coexpressed 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
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
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 10^4 IU/ml (40, 41). By contrast, when spleen cells were cultured with Con A and a low dose (10^5 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+. 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
whereas the Ly49F⁺ C⁻ E⁻, Ly49F⁺ E/C⁺, and Ly49E⁻ F⁻ C⁺ cells formed dispersed populations (Fig. 5, 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 Ly49E⁺ high. 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
of Ly49E expression on Ly49E<sup>−</sup> 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<sup>+</sup> 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<sup>+</sup> (mostly T cells) were virtually all Ly49E<sup>−</sup> and had undergone up to six divisions, reducing their mean CFSE fluorescence to 408. Those expressing NK1.1<sup>+</sup> (mostly NK cells) had divided somewhat less with a mean CFSE fluorescence of 673. Among the NK1.1<sup>+</sup> cells, the Ly49E<sup>−</sup>, Ly49E<sup>low</sup>, and Ly49E<sup>high</sup> subpopulations had mean CFSE fluorescence levels of 722, 712, and 940, respectively (Fig. 7C), whereas the Ly49F<sup>+</sup> subpopulation had a mean CFSE level of 580 (data not shown). These results show that the Ly49E<sup>−</sup> cells present after 3 days culture in IL-2 were the product of no greater number of cell divisions than the Ly49E<sup>+</sup> cells, and indeed a slightly fewer average number of divisions than the Ly49F<sup>−</sup> cells or NK1.1<sup>−</sup> 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<sup>+</sup> cells or into NK1.1<sup>high</sup> 4D12<sup>−</sup> cells, the latter representing a population of pure NK cells depleted of both Ly49C<sup>+</sup> and Ly49E<sup>+</sup> cells. As shown in Fig. 8A, both NK populations were ~95% pure, and the proportion of 4D12<sup>+</sup> cells was reduced by almost 90% in the NK1.1<sup>high</sup> 4D12<sup>−</sup> 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, which 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 exposed to IL-2 is generally triggered only after a considerable lag period. To test this important distinction directly, NK cells exposed to IL-2 was initiated de novo in NK cells that previously lacked Ly49E, and 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.

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, IFNγ, IFNβ, 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 Ly49E-depleted NK 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, IFNγ, IFNβ, 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 Ly49E-depleted NK 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, IFNγ, IFNβ, 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 Ly49E-depleted NK cells.
contrast, it almost doubled the proportion of T cells that acquired NKRP1C, but caused no significant induction of NKRP1A/D receptors. The effects of IL-21 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 NKRP1C and, in complete contrast to its down-regulation of NKG2 receptors on NK cells, it increased the proportion of T cells that acquired NKG2 receptors ~2-fold. In addition, as shown in Fig. 11E, different receptors were sensitive to different concentrations of IL-21, with the acquisition of NKRP1C by T cells being inhibited at IL-21 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 NKRP1, NKG2, 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 Vγ3-bearing subpopulations of γδ 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 cross-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 αβ or γδ 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 αβ 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, γδ 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 Ly9 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 γδ T cells cultured in IL-2 and has previously been reported on immature NK cells (17) and on gut γδ 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 γδ 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 NKRPIA/D or on the level of expression of NKRPIC (NK1.1) but markedly increased the proportion of T cells that acquired NKRPIC without inducing any significant expression of NKRPIA/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 NKRPIA/D or the level of expression of NKRPIA/D but reduced the level of expression of NKRPIC. 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 NKRPIC 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-inflammatory. 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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