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*J Immunol* 2009; 182:6267-6277; doi: 10.4049/jimmunol.0801933
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A Programmed Switch from IL-15- to IL-2-Dependent Activation in Human NK Cells

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IL-2 and IL-15 differentially control the development, activation and proliferation of human NK cells, although they share common signal-transducing receptor chains CD122 and common γ. To explore this issue, we analyzed in detail the kinetics of cytokine receptor expression, cytokine binding, and signaling responses in human NK cells treated with common γ-chain family cytokines. We provide evidence for the sequential expression of IL-15Rα and IL-2Rα at the surface of cytokine-stimulated human NK cells, independent of the cytokine used for stimulation (IL-2, IL-15, or IL-7). Binding experiments confirmed the switch of high-affinity receptor from IL-15R to IL-2R between 18 and 48 h after stimulation. Consequently, phospho-STAT5 signaling responses to IL-15 were efficient in human NK cells pretreated with cytokines for 18 h, but were abolished at 48 h. Functional NK cell responses to IL-15, including IFN-γ secretion and CD107a expression, followed a similar pattern, indicating the physiological relevance of the cytokine receptor switch. Importantly, maturation of colocalized monocytes and dendritic cells, innate immune response against pathogens, in the activation and in the early differentiation of a Th1-adaptive response after infection.

Development, survival, proliferation, and effector functions of NK cells are critically dependent on cytokines of the common γ-chain (γc) family (2, 9). In vitro studies indicate that IL-2, IL-15, and IL-7 can all support NK cell activation (10). IL-15 is essential for NK cell differentiation from T/NK progenitor cells (11) and for NK cell function (2, 12–14). IL-2 also potentiates NK cell function for NK cell differentiation from T/NK progenitor cells (11) and for IL-7 can all support NK cell activation (10). IL-15 is essential for NK cell differentiation from T/NK progenitor cells (11) and for NK cell function (2, 12–14). IL-2 also potentiates NK cell function

Institut Pasteur, Département d’Infection et d’Épidémiologie, Unité d’Immunogénétique Cellulaire, Paris, France

Received for publication June 13, 2008. Accepted for publication March 17, 2009.

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1 This work was supported by the Institut Pasteur. A.-H.P. is the recipient of a fellowship from the Ministère de l’Éducation Nationale et de la Recherche.

2 Address correspondence and reprint requests to Dr. Thierry Rose, Unité d’Immunogénétique Cellulaire, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris, Cedex 15, France. E-mail address: rose@pasteur.fr

3 Abbreviations used in this paper: DC, dendritic cell; γc, common γ-chain; MFI, mean fluorescence intensity.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0801933

The Journal of Immunology

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activation status, while in return DC activate NK cells and promote their cytotoxic activity (6, 7). These studies have led to an increasing appreciation of the role of NK cells in shaping the initiation of DC-dependent adaptive responses.

IL-2 and IL-15 signal through three major pathways: Jak/STAT, MAPKs, and PI3K/Akt (26–29). It should be noted that at high cytokine concentrations, the intermediate-affinity receptor recapitulates signaling events transduced through high-affinity receptors (9, 30). Activation of the Jak/STAT pathway depends on conformational rearrangement of receptor chains, leading to phosphorylation of receptor-associated Jak1 and Jak3 kinases, which in turn phosphorylate STAT5α and STAT5β and to a lesser extent STAT1β and STAT3 transcription factors. The phosphorylated STATs dimerize, diffuse into the nucleus, and activate the transcription of multiple target genes (26). To date, no difference has been characterized in NK cells between the patterns of protein phosphorylation induced after IL-2 or IL-15 stimulation (31). However, these two cytokines clearly induce different effects on NK cells (10, 32–34). IL-15 has mostly pro-survival effects (35) and induces a stronger proliferative response than IL-2 (36, 37). In contrast, IL-2 induces NK cell apoptosis under some conditions in vivo (35). The molecular basis for these functional differences still remains a matter of debate (9, 32). Several mechanisms have been proposed to differentially control IL-2 and IL-15 action, including cytokine compartmentalization (10), differential expression of the high-affinity receptors (9), and differential activation of cell growth and survival pathways (38).

To address this issue, we investigated the kinetics of expression of all of the chains comprising the IL-2 and IL-15 receptors, as well as the binding characteristics and signaling responses of the receptor complexes expressed by human NK cells upon activation. We demonstrate a tight temporal regulation of IL-15Rα and IL-2Rα expression in cytokine-stimulated NK cells, resulting in an initial and transient responsiveness to free IL-15, which is replaced at later stages by a dependency on IL-2 and trans-present IL-15. Thus, NK cells activation can initially be sustained by a variety of IL-15-producing cells, but is restricted at a later stage to effectors of the adaptive responses that produce IL-2 and/or trans-present IL-15. This programmed switch in cytokine responsiveness may have profound consequences on the regulation of NK cell activity in innate and adaptive immune responses.

Materials and Methods

Surface expression of cytokine receptor chains in human NK cells

Venous blood was obtained from healthy volunteers through the Établissement Français du Sang, Centre Cabanel (Paris, France). PBMC were purified by density gradient centrifugation on Lymphoprep solution (Axis-Shield). For cytokine activation, PBMC were resuspended at 107/ml in RPMI 1640 medium (Cambrex) complemented with 5% FBS (complete medium) in 24-well plates were treated with 5 nM IL-2 (Chiron-Novartis) or 2 nM IL-15 (Abcys) and cultured for 12, 24, or 48 h in 5% CO2 at 37°C. For flow cytometry analysis with labeled Abs, PBMC were harvested and resuspended in 50 μl of FACS buffer (PBS with 0.02% sodium azide and 5% FBS) and labeled for 1 h at 4°C with Abs to CD56-allophycocyanin, CD3-allophycocyanin-AF750 (eBioscience), CD132-PE (BD Biosciences), and IL-15Rα (goat IgG1; R&D Systems) and used at the same concentration as free IL-15. The membrane was saturated with BSA, incubated with either anti-γ, rat TUGh4 (BD Pharmingen) or goat anti-γ, polyclonal Abs (R&D Systems), and washed in TBS/0.5% Tween 20 buffer before being incubated with HRP-coupled anti-rat or anti-goat Abs (Amersham Biosciences). Proteins were then revealed by ECL-plus Western blotting detection reagents (Amersham Biosciences).
STAT5 molecules phosphorylated at Y694. Analysis was carried on a cyan flow cytometer, on a minimal number of 500,000 events in the NK cell gate. The mean fluorescence intensity (MFI) of p-STAT5 after cytokine stimulation was determined by subtracting the p-STAT5 MFI after the 18 or 48 h pretreatment from the p-STAT5 MFI after cytokine restimulation. Each experiment included fluorescence minus one controls in which the p-STAT5 mAb was replaced by an isotypic control Ab (mouse IgG1/H9260; BD Biosciences).

Analysis of intracellular IFN-γ production and cell surface CD107a induction

In brief, 10⁶ PBMC were prestimulated with cytokines for 18 or 48 h following the same protocol as for the STAT5 phosphorylation assay. Cells were then incubated for 1 h at 37°C in 5% CO₂ with brefeldin A (Sigma-Aldrich) at a final concentration of 0.5 μg/ml to prevent exocytosis of expressed cytokines stored in vesicles. Six hours after restimulation, cells were permeabilized with a staining buffer set (eBioscience) and labeled with anti-CD-3-allophycocyanin-AF750 (eBioscience), CD-56-allophycocyanin (Beckman Coulter), anti-IFN-γ-PE-Cy7, and anti-CD107a-AF488 (eBioscience). Cells were analyzed by flow cytometry on a minimal number of 500,000 events in the NK cell gate. The MFI of IFN-γ and CD107a after cytokine stimulation were corrected by subtracting baseline values after 18 or 48 h of cytokine pretreatment from the MFI postcytokine restimulation.

Results

IL-15 activates quiescent NK cells more efficiently than IL-2

We monitored the induction of CD69, an early activation marker, in NK cells treated for 48 h with increasing concentrations of the cytokines IL-2 and IL-15. Surface expression of CD69 was measured by flow cytometry in CD3⁻CD56⁺ NK cells from five healthy blood donors (Fig. 1). CD69 was induced more efficiently by IL-15 than by IL-2, with maximum expression on NK cells reached at 2 nM IL-15 instead of 5 nM IL-2. In addition, the point of half-transition was 150 pM for IL-15 and 800 pM for IL-2, indicating that IL-15 was 5- to 6-fold more potent than IL-2 at activating quiescent NK cells. The maximum CD69 expression level was comparable for both cytokines at high concentrations, as expected for cytokines targeting the same intermediate-affinity receptor CD122/γc.

Rapid induction of the intermediate-affinity receptor at the surface of NK cells

The expression kinetics of individual IL-2R and IL-15R chains were followed on human NK cells from six healthy blood donors after in vitro stimulation with IL-2 or IL-15. CD122 was constitutively expressed at high levels by most quiescent NK cells (>90%) (Fig. 2A). After IL-2 and IL-15 stimulation, the percentage

![FIGURE 1](http://www.jimmunol.org/)

CD69 induction in IL-2- and IL-15-treated NK cells. Human PBMC from five healthy blood donors were cultured in the presence of different concentrations of IL-2 or IL-15 for 48 h. CD3⁻CD56⁺ NK cells were then analyzed by flow cytometry for the expression of the early activation marker CD69. CD69 MFI is plotted vs IL-2 and IL-15 concentrations in a log scale. Error bars indicate SDs.

![FIGURE 2](http://www.jimmunol.org/)

Expression of CD122 and γc in cytokine-stimulated NK cells. Human PBMC were cultured for 72 h in the presence of either IL-2 (5 nM) or IL-15 (1 nM). CD3⁻CD56⁺ NK cells were analyzed by flow cytometry 12, 24, 48, and 72 h after stimulation. A and C, Results from a representative donor showing the expression of CD122 (A) and γc (C) assayed 12 h after cytokine stimulation. Fluorescence intensity distribution for CD122 and γc are shown as solid gray-filled histograms. The profile obtained with the IgG-PE isotypic control is plotted as a solid line. B and D, The MFI of CD122 (B) and γc (D) from six donors are reported as a function of the duration of cytokine treatment. Bars represent SDs (n = 6).
of CD122-expressing cells remained high (Fig. 2B) and did not vary significantly over 3 days from six healthy blood donors.

The γc (CD132) was not detectable at the surface of quiescent NK cells (Fig. 2C) from 10 healthy blood donors. However, stimulation by IL-2 or IL-15 led to the rapid induction of γc surface expression in the whole NK cell population, with a maximum reached as early as 12 h after stimulation (Fig. 2D). Surface expression of γc then persisted at high levels for up to 3 days. Thus, cytokine stimulation led to persistently high expression of the two chains comprising the intermediate-affinity receptors CD122 and γc.

Expression of immature and mature γc in resting and activated NK lymphocytes

Because we did not detect γc by flow cytometry at the surface of highly purified quiescent NK cells while we expected this chain required for the IL-2/IL-15 NK cell response, we further studied the expression of this chain using several anti-γc analyzed by Western blot. We used the TUGh4 mAb specific for the mature form of γc and the polyclonal Ab Gαγc pAb that detects both the mature and immature forms of the γc as previously described (41). We observed that mature γc was detectable neither by flow cytometry (Fig. 3A) nor by Western blotting from NK cell lysed (Fig. 3B) at the surface of highly purified quiescent NK cells from more than 10 healthy donors (a representative example is shown in Fig. 3).

However, NK cells stimulated for 12 h with IL-2 or IL-15 showed a strong surface expression of mature γc by flow cytometry (Fig. 3A, bottom plots) as well as by Western blotting (Fig. 3B, 62- to 64-kDa band). This confirmed the strong expression of mature γc chain 12 h after cytokine stimulation. We next used a polyclonal Ab (Gαγc pAb) that detects both the mature and immature forms of the γc by Western blotting (Fig. 3C). Interestingly, resting NK cells contained a significant pool of immature γc (50–52 kDa). Stimulation of cultured NK cells by IL-2 or IL-15 for 12 h led to both the maturation of γc (apparition of the 62- to 64-kDa band) and to the accumulation of immature γc (increase in the 50- to 52-kDa band), suggesting that both maturation and neo-synthesis should contribute to the rapid induction of γc expression at the cell surface. The presence of a pre-existing intracellular pool of the γc can explain its rapid kinetics of expression at the cell surface upon NK cell activation, by bypassing the lag phase required for the induced protein neo-synthesis.

Sequential expression of IL-15Rα and IL-2Rα on cytokine-stimulated NK cells

The expression kinetics of IL-2Rα (CD25) and IL-15Rα was assessed on NK cells from six healthy donors. CD25 was undetectable in most of quiescent NK cells, although a few CD25+ cells were detected among the CD56 bright NK cell subset (5% of NK cells; Fig. 4A). NK cells became predominantly CD25+ 48 h after IL-2 and IL-15 stimulation (Fig. 4A). The kinetics of CD25 induction after IL-2 stimulation was slow, with an initial increase detectable after 24 h and a maximum reached after 48 h of culture (Fig. 4B). The kinetics of CD25 expression after IL-15 stimulation was slightly more rapid, with a detectable induction from 24 h and a plateau reached after 48 h. Of note, the CD25 MFI reached at the plateau (48 h) was three times higher after IL-15 than after IL-2 stimulation, suggesting a more potent activation of NK cells by IL-15.

The expression of IL-15Rα showed a strikingly different pattern. IL-15Rα was expressed at low but detectable levels in resting NK cells (Fig. 4C). Both IL-2 and IL-15 stimulation caused a transient induction of IL-15Rα, with maximum expression reached 24 h after stimulation. IL-15Rα was then rapidly down-regulated and became undetectable 36 h after stimulation, while unstimulated NK cells maintained low but detectable IL-15Rα expression. Again, IL-15 induced IL-15Rα expression three times more efficiently than IL-2 in NK cells. It was noteworthy that IL-15Rα down-regulation was as complete after IL-15 than after IL-2 treatment, even though maximal receptor expression was higher in the former case. Taken together, these findings suggested a differential regulation of the high-affinity receptors for IL-15 and IL-2 in activated human NK cells.
Sequential expression of high-affinity IL-2 and IL-15 receptors at the surface of NK cells

It was important to verify that expression of IL-2Rα and IL-15Rα resulted in the formation of high-affinity receptor complexes at the surface of NK cells. To this goal, we measured the binding constants of 125I-labeled IL-2 and IL-15 for their receptors at the surface of purified human NK cells from three healthy donors. The binding of labeled cytokines from picomolar to micromolar concentrations was assayed at 4°C at times 0, 18, and 48 h after preactivation of NK cells with IL-2 (5 nM) or IL-15 (2 nM). On resting cells, high-affinity binding sites were barely detectable (Fig. 5B), while intermediate-affinity sites were present (Fig. 5A). At peak stimulation (18 h after cytokine pretreatment for IL-15R and 48 h for IL-2R), both intermediate- and high-affinity binding sites were detected. The $K_d$ for the intermediate-affinity sites were in the same range for both cytokines, with a value of 0.8 ± 0.2 nM for IL-2 and 0.4 ± 0.2 nM for IL-15 (Fig. 4A). The numbers of intermediate-affinity binding sites for IL-2 and IL-15 were comparable, compatible with these sites consisting in the same moieties (CD122 and $\gamma_5$). Intermediate-affinity binding sites preexisted in quiescent NK cells (700–800 binding sites/cell), likely due to the capacity of CD122 homodimers to bind cytokines (42). The number of intermediate-affinity binding sites increased upon activation, and reached higher levels after IL-15 activation (1700 sites/cell) than IL-2 activation (1100 sites/cell), consistent with the more efficient induction of CD122 and $\gamma_5$ expression by IL-15.

The $K_d$ for the high-affinity binding sites were almost identical for both cytokines, with values of 13 ± 2 pM for IL-2 and 12 ± 2 pM for IL-15 (Fig. 5C). Although the affinities of IL-2 and IL-15 for receptors at the surface of activated NK cells were comparable, the number of high-affinity binding sites differed significantly. Eighteen hours after IL-2 stimulation, we titrated 1.5-fold more high-affinity IL-15 binding sites (90 sites/cell) than high-affinity IL-2 binding sites (60 sites/cell; Fig. 5D). Similarly, 18 h after IL-15 stimulation, the number of high-affinity IL-15 binding sites (160 sites/cell) was ~2.5-fold higher than that of high-affinity IL-2 binding sites (60 sites/cell; Fig. 5D). After 48 h of stimulation by either cytokine, the high-affinity binding sites for IL-15 became undetectable ($n < 20$ sites/cell). In contrast, 48 h of IL-2 or IL-15 stimulation resulted in maximal induction of high-affinity IL-2 binding sites (220 and 480 sites/cell, respectively; Fig. 5, C and D). These results are in agreement with the kinetics of expression of IL-2Rα and IL-15Rα, and support the notion of a sequential induction of high-affinity receptors for IL-15 and IL-2 at the surface of activated NK cells. In addition, comparison of the numbers of high-affinity binding sites induced after IL-15 and IL-2 treatment (Fig. 5D, compare top and bottom graphs) confirmed the greater potency of IL-15 at activating NK cells.

Early phosphorylation events after cytokine stimulation of quiescent NK cells

To investigate the functional responses of quiescent NK cells to cytokines, we assayed the activation of the Jak/STAT pathway after 15 min of stimulation with either IL-2 or IL-15 in six healthy donors. Purified NK cells were stimulated with each cytokine at 150 pM, lysed, and subjected to immunoprecipitation with a Jak3-specific Ab. The coimmunoprecipitated signaling complexes were analyzed by immunoblotting with phospho-specific Abs to p-Jak and p-STAT5. Both cytokines induced the phosphorylation of Jak1 and Jak3 kinases (Fig. 6A), as well as that of the STAT5 transcription factor (Fig. 6B) in quiescent NK cells. However, IL-15 was more efficient at initiating these early phosphorylation events (Fig. 6, A and B). These findings were consistent with the increased

FIGURE 4. Sequential expression of IL-15Rα and IL-2Rα in cytokine-treated NK cells. Human PBMC were cultured for 72 h in the presence of either IL-2 (5 nM) or IL-15 (1 nM). The kinetics of IL-2Rα and IL-15Rα expression was analyzed by flow cytometry. A and C, Fluorescence intensity distribution of IL-2Rα and IL-15Rα in NK cells from a representative blood donor. The expression of IL-2Rα (A) and IL-15Rα (C) are represented at peak stimulation, 48 and 18 h, respectively, after cytokine treatment. Profiles of IL-2Rα and IL-15Rα expression are shown as solid gray-filled histograms, while the profile obtained with the IgG-PE isotypic control is plotted as a solid line. B and D, The MFI of IL-2Rα (B) and IL-15Rα (D) are reported as a function of the duration of cytokine treatment.
efficiency of IL-15 at inducing CD69 and cytokine receptor chain expression at the cell surface in response to low cytokine concentration.

**Time-dependent regulation of IL-2- and IL-15-induced STAT5 phosphorylation in activated NK cells**

Functional responses of activated NK cells from six healthy donors were analyzed by pretreating PBMC with cytokines to reach optimal receptor expression, restimulating the cultures with a range of IL-2 and IL-15 concentrations and measuring the phosphorylation of STAT5 at Y694 in the CD3\(^+\)/H11002 CD56\(^+\)/H11001 population. PBMC were pretreated with 150 pM IL-15, which yielded optimal expression of IL-15R\(^+\)/H9251 at 18 h and of IL-2R\(^+\)/H9251 at 48 h.

IL-2 stimulation (500 pM) induced more efficient STAT5 phosphorylation in NK cells preactivated for 48 h than for 18 h with IL-15 (Fig. 7A), a finding compatible with the higher IL-2R\(\alpha\) expression at 48 h (Fig. 4B). Dose-response curves were then plotted to precisely compare the reactivity of NK cells after 18 and 48 h of pretreatment with IL-15. After 18 h of pretreatment, NK cells responses to IL-2 were of low intensity, with an increase in the p-STAT5 MFI detected at IL-2 doses comprised between 100 and 200 pM (Fig. 7B). In contrast, efficient activation of p-STAT5 was detected after stimulation with as low as 10 pM IL-2 in NK cells preactivated for 48 h (Fig. 4C). NK cells cultured in the absence of IL-15 (open symbols, Fig. 7) did show a detectable response to IL-2 or IL-15 stimulation, even at the 10 pM concentration, consistent with a limited expression of IL-2R\(\alpha\) and IL15R\(\alpha\) chains after 2 days of culture.

Functional responses of preactivated NK cells to IL-15 stimulation clearly differed from those observed with IL-2 stimulation. NK cells preactivated for 18 h with IL-15 responded optimally to a second IL-15 stimulation, while cells preactivated for 48 h showed no detectable STAT5 phosphorylation after a second IL-15 addition (Fig. 7D). Again, these findings were consistent with the undetectable expression of IL-15R\(\alpha\) at 48 h (Fig. 4D). Dose-response curves showed that NK cells responded to picomolar concentrations of IL-15 after an 18-h preactivation, when IL-15R\(\alpha\) expression was maximal (Fig. 7E). Interestingly, NK cells preactivated for 48 h showed an undetectable p-STAT5 response to picomolar IL-15 stimulation, while NK cells that were cultured for 48 h in the absence of cytokine did show a response, likely due to the spontaneous expression of IL-15R\(\alpha\) (Fig. 7F). Thus, prior IL-15 activation inhibited subsequent responses to the same cytokine, defining a negative regulatory loop in the IL-15/IL-15R system.

These experiments were repeated with NK cells preactivated with IL-2. The data confirmed that IL-15 functional responses were optimal 18 h after preactivation and down-regulated afterward, while IL-2 responses peaked from the 48-h time point onward (data not shown). Altogether, the data showed a clear association between the number of high-affinity IL-2R and IL-15R expressed at the surface of NK cells and their signaling responses. Importantly, activated NK cells...
remained responsive to picomolar doses of IL-15 for a limited time period, while responsiveness to IL-2 increased progressively over time.

**IL-15 complexed to IL-15RA retains the capacity to signal in activated NK cells**

IL-15 can act on its target cells as a free soluble molecule or in a trans-presented form while complexed to IL-15RA at the surface of producer cells. Since studies in mouse models have proven the physiological relevance of trans-presented IL-15 (20, 24, 25), we assessed the reactivity of NK cells from six healthy donors to this cytokine is one of the primary mediators of NK cell function.

To determine the physiological relevance of our findings, we tested the reactivity of NK cells from six healthy donors to this cytokine by flow cytometry on the CD3+CD56+ NK cell population. A and D, Representative histograms of p-STAT5 expression after stimulation with 500 pM IL-2 (A) or 500 pM IL-15 (D). In each case, PBMC were analyzed after 18 h of culture in the absence of exogenous cytokines (thin line), 18 h of culture in the presence of IL-15 (thick line), or 48 h of culture in the presence of IL-15 (shaded histogram). B and C, PBMC precultured for 18 h (B) or 48 h (C) were stimulated with different amounts of IL-2 (10, 20, 100, 200, and 500 pM) for 15 min before analyzing p-STAT5 expression in the CD3+CD56+ population. MFI of p-STAT5 are plotted vs cytokine concentration. Thick lines represent responses of cells precultured in the presence of IL-15, while thin lines represent responses of cells precultured in the absence of exogenous cytokine. E and F, PBMC precultured for 18 h (B) or 48 h (C) were stimulated with different amounts of IL-15 (10, 20, 100, 200, and 500 pM) for 15 min before analyzing p-STAT5 expression in the CD3+CD56+ population. Results are represented as above.

**IL-2 and IL-15 functional responses are controlled by high-affinity receptor expression**

To determine the physiological relevance of our findings, we tested effector functions of NK cells at different times following cytokine treatment. We measured the surface expression of CD107a (or LAMP-1), an endosomal marker which is exposed upon degranulation and which has been associated with NK cell cytotoxic activity (43). We also measured intracellular IFNγ production, since this cytokine is one of the primary mediators of NK cell function. NK cell functions were analyzed in cells from six healthy donors at the 18-time point, NK cells responded weakly to IL-2 restimulation (Fig. 9, A and E). In contrast, at the 48-time point, efficient CD107a
and IFN-γ expressions were detected after stimulation with as low as 10 pM IL-2 (Fig. 9, C and G).

Functional responses of IL-15-pretreated NK cells to IL-15 stimulation clearly differed from those observed with IL-2 stimulation. At the 18-h time point, NK cells responded optimally to stimulation clearly differed from those observed with IL-2 stimulation. At the 18-h time point, NK cells responded optimally to stimulation clearly differed from those observed with IL-2 stimulation. At the 18-h time point, NK cells responded optimally to stimulation clearly differed from those observed with IL-2 stimulation.

Persistence of responses to the IL-15/sIL-15Ra complex in activated NK cells. Human PBMC were cultured in the presence of 150 pM IL-15 for 48 h to achieve complete IL-15Rα down-regulation. PBMC were then restimulated with IL-15 or the IL-15/IL-15Ra complex for 15 min and analyzed for p-STAT5 in the CD3+CD56+ NK cell population. Representative histograms of p-STAT5 expression after restimulation with induced by IL-2, IL-15 (thin line), the complex IL-15/IL-15Ra complex (thick line), or IL-2 (shaded histogram) at the 10 pM concentration (left) and the 500 pM concentration (right). B, Dose-response curves of p-STAT5 induction in NK cells precultured in IL-15 for 48 h and restimulated with IL-2 ( ), IL-15 ( ), or the IL-15/IL-15Ra complex ( ). Bars represent the mean values obtained in n = 6 experiments. Error bars represent SDs.

γc family cytokines induce a common cytokine receptor expression program on NK cells

To test the generality of our findings, we evaluated IL-2R and IL-15R expressions in NK cells preactivated by IL-7, another cytokine of the γc family. Phenotyping of NK cells from six healthy blood donors showed that IL-7 treatment did not change significantly the already high expression levels of CD122, but caused a rapid induction of the γc, which reached high levels from 12 h onward (Fig. 10, A and B). CD25 induction was progressive, with not detectable CD107 expression at 24 h of IL-7 treatment and slowly increasing levels during the 3-day culture (Fig. 10C). Once again, IL-15Ra expression was transient, with efficient induction at 12 h and complete down-regulation at 48 h. Thus, the pattern of cytokine receptor expression induced by IL-7 paralleled that observed after IL-2 and IL-15 treatments. This notion was reinforced by the fact...
that all three cytokines caused a progressive down-regulation of the IL-7Ra chain CD127, while untreated NK cells up-regulated CD127 (Fig. 10E). Thus, γc cytokines triggered a common cytokine receptor expression program in human NK cells.

**Discussion**

This study provides evidence for sequential expression of IL-15Ra and IL-2Ra at the surface of cytokine-activated human NK cells. Treatment with IL-2, IL-15, or IL-7 resulted in a similar receptor chain expression pattern, which conferred transient responsiveness to free IL-15, followed by long-lasting responsiveness to IL-2 and trans-presented IL-15. This sequence of events did not depend on the initial stimulus, even though IL-15 proved more efficient at initiating early activation events. Thus, human NK cells show a programmed response to γc cytokine stimulation, which may differentially control NK cell function in the innate and adaptive stages of the immune response.

Detailed analysis of receptor chains expression provides clues to the respective role of IL-2 and IL-15 in human NK cell physiology. NK cells appear primed to respond quickly and efficiently to an initial IL-15 stimulus. 1) Resting NK cells constitutively express low levels of IL-15Ra and high levels of the β-chain CD122. The only chain needed to reconstitute the high-affinity receptor γc shows a rapid kinetics of induction, so that high-affinity receptors are rapidly available to amplify the IL-15 response. In contrast, the IL-2Ra chain is barely detectable in quiescent NK cells and is induced with a slow kinetics, resulting in delayed optimal IL-2 responses. 2) IL-15Ra reaches a maximum of expression between 16 and 20 h after activation, at a time when γc is also optimally induced. As a consequence, the number of high-affinity binding sites for IL-15 is >2-fold higher than that for IL-2 one day after activation (Fig. 5), resulting in more efficient signaling responses (Fig. 7, B and E). We verified that phosphorylation of the early effectors Jak1 and Jak3 paralleled that of STAT5 (data not shown), confirming that differences in IL-15 and IL-2 responses were controlled at the receptor level.

The mechanism initiating cytokine responses in quiescent NK cells is not entirely elucidated, since γc is not expressed at detectable level at the surface of these cells (44). Commercial Abs to γc do not appear sensitive enough to detect low amounts of the protein. Indeed, we did not detect γc expression by flow cytometry (Fig. 2D) nor by immunoradiosay with a 125I-labeled anti-γc Ab with a sensitivity threshold above 60 molecules/cell (data not shown). However, binding of 125I-labeled IL-2 or IL-15 revealed the presence of ~750 intermediate-affinity receptor sites per quiescent NK cells, well above the γc detection threshold (Fig. 4A). We and others have previously shown that CD122 can assemble into homodimers and bind IL-2 with intermediate affinity, which would explain the detection of IL-2 binding sites on quiescent NK cells (42, 45, 46). CD122 homodimers may be able to initiate signaling, which would then be amplified by the rapid induction of γc. Alternatively, trace amounts of γc may build up intermediate-affinity receptors by association with abundant CD122 chains present at the surface of quiescent NK cells and may then trigger signaling. Once signaling is initiated, the rapid induction of γc expression provides an efficient means to increase the number of active receptors. Several mechanisms ensure the induction of γc expression. 1) We have observed that γc mRNA is expressed at significant levels in quiescent NK cells and is quickly induced after activation (data not shown). 2) γc protein synthesis is also increased after NK cell activation, as indicated by the increase in total γc content in activated NK cell protein extracts. 3) γc proteins preexist in an immature form in resting NK cells and are rapidly matured upon activation (Fig. 3). We have previously shown that a similar intracellular pool of immature γc is present in resting CD4+ T cells and that this immature form can be rapidly glycosylated and exported to the cell surface upon activation (41). Thus, the maturation of a starter pool of γc represents a conserved mechanism which confers cytokine responsiveness to recently activated leukocytes. Of note, several receptors that play key roles in immune activation, such as MHC molecules, show a similar pattern of intracellular retention and rapid export to the cell surface upon activation (47, 48). Once signaling is triggered, the inducibility of γc mRNA and protein expression help sustain and amplify the activation, resulting in a positive feedback loop between receptor induction and receptor signaling.

Triggering of signaling through the intermediate-affinity receptor also induces expression of the IL-15Ra and IL-2Ra chains, but with slower and sequential kinetics. Expression of these receptor...
chains ensures high-affinity interactions with their respective cytokine ligands, but also extends the time of cytokine residency on the binding sites (greater kinetic dissociation constant $k_{d}$). As a consequence, early signaling events, such as STAT5 phosphorylation, and late functional responses, such as CD107a and IFN-γ expression, are more efficient. For instance, we found that IL-2 binding on 200 high-affinity receptors resulted in the same induction of p-STAT5 as the binding of 1600 intermediate-affinity receptors (cf Figs. 4 and 7). The sequential expression of IL-15Rα and IL-2Rα clearly controlled both signaling and functional NK cell responses, since receptor expression, STAT5 phosphorylation, CD107a cell surface expression, and IFN-γ production all followed the same pattern. Namely, activated NK cells showed optimal signaling and functional responses to IL-15 stimulation at the 18-h time point, at the peak of IL-15Rα expression, while optimal responses to IL-2 were achieved later, at 48 h, when IL-2Rα expression was maximal. These results point to a tight temporal regulation of NK cell functions.

Although several parameters favored an initial responsiveness of NK cells to IL-15, this phenomenon was transient. Two days after activation, NK cells became refractory to picomolar IL-15 stimulation, while sensitivity to IL-2 stimulation increased. The loss of responsiveness to IL-15 was associated to a rapid down-regulation of IL-15Rα expression as measured by flow cytometry (Fig. 4D). Accordingly, the high-affinity binding sites for IL-15 became undetectable at the surface of NK cells. Since IL-15Rα mRNA levels remained stable at this stage (data not shown), post-transcriptional regulation likely accounts for the loss of IL-15Rα expression. A possible mechanism is the cleavage of IL-15Rα by the TNF-α-converting enzyme TACE/ADAM17 protease at the cell surface, a phenomenon shown to generate a soluble inhibitory form of the receptor (49, 50). Remarkably, IL-15Rα down-regulation was drastic, with expression levels at the cell surface lower in activated NK cells than in resting NK cells. Accordingly, resting NK cells showed a degree of response to picomolar IL-15 concentrations, while activated NK cells remained unresponsive (Fig. 5F). Such a tight negative regulation may serve to avoid deleterious consequences of excessive NK cell activation. Tight control of IL-15Rα may be particularly important in humans who can show detectable levels of circulating IL-15, particularly in cases of inflammation and autoimmune pathologies (51, 52). This contrasts with murine models where IL-15 remains mostly undetectable in the circulation. The presence of free IL-15 in humans emphasizes the need of keeping IL-15 paracrine effects in check through receptor down-regulation.

The fact that IL-15 down-regulates its own receptor has been reported in murine and human T cells (38, 53). A parallel can be drawn with the notion of “altruistic receptor down-regulation” established by Singer and colleagues (54) for IL-7R whereby activated T cells down-regulate IL-7R at the same time as they acquire competency to use other cytokines (such as IL-2), so that competition with resting T cells for limiting IL-7 resources is minimized. However, the notion of an early and transient window of induction of IL-15Rα by γc family cytokines is novel and may be more relevant to the control of NK cell activation than to the maximization of IL-15 availability. IL-15 is produced by cells of the monocyte/macrophage lineage, DC, and stromal cells. Production of this cytokine is markedly increased in APC activated by pathogens, a phenomenon that is thought to play a key role in promoting NK cell activation (25, 55). Activated NK cells, in turn, contribute to DC maturation through contact-dependent mechanisms and abundant secretion of cytokines, such as TNF-α, IFN-γ, and GM-CSF (6, 7). The NK/DC cross-talk has consequences on other immune compartments as well, since NK-mediated DC activation contributes to the initiation of T cell-dependent responses (56). It ensues that excessive NK cell activation through persistent IL-15 signaling may have numerous unwanted consequences, including overproduction of inflammatory cytokines, bystander cell lysis, or perturbed development of adaptive responses.

Loss of IL-15Rα at the surface of activated NK cells may render these cells dependent on stimulation through IL-15/sIL-15Rα complexes trans-presented by APC, resulting in a tighter regulation of the NK activation process. Trans-presentation requires intercellular contact (19, 23), ensuring the coordinated activation of NK cells and APC. In addition, we showed that NK cells progressively acquire a responsiveness to IL-2, which is produced in minute amounts by activated DC (57) and mainly originates from activated T cells. The implications of gaining IL-2 responsiveness at a late stage of NK cell activation are several. 1) Because IL-2 is mainly secreted by activated CD4+ T cells, it will place NK cell activation under the control of adaptive immunity. NK cell cytotoxic activity will be targeted to sites where foreign Ags and responding CD4+ T cells accumulate, avoiding a generalized NK cell activation that could prove deleterious in chronic infections. 2) Since CD4+ T cell activation and IL-2 production take place predominantly within secondary lymphoid organs, activated NK cells may be recruited to and persist in these sites and influence the development of adaptive immune responses. IFN-γ secretion by NK cells may facilitate CD4+ T cell differentiation toward a Th1 phenotype (8). In addition, activated NK cells have the capacity to kill immature DC, which may play a key role in maintaining tolerance and in terminating immune responses (58). 3) Last, high-dose IL-2 has been reported in certain studies to induce NK cell apoptosis (35), which may help terminate NK cell activation through a negative feedback loop. Thus, IL-2 responsiveness of NK cells would contribute to the innate/adaptive immunity cross-talk at multiple levels. Although early IL-15 responsiveness is probably important in the rapid induction of NK cell innate effector functions, late IL-2 responsiveness may confer a regulatory role to NK cells during the adaptive stage of the immune response. Thus, the sequential expression of IL-15Rα and IL-2Rα may play a key role in coordinating the innate and adaptive branches of the immune system.

In conclusion, this study provides evidence for the sequential expression of IL-15 and IL-2 high-affinity receptors at the surface of activated human NK cells. This cytokine receptor expression program can account for the differential effects of IL-2 and IL-15 on NK cells and may contribute to the tight control of NK cell activity in humans. These findings are relevant for the optimization of immunotherapeutic approaches that involve cytokine administration by highlighting the time frame relevance for the efficient concentrations of cytokine to promote NK cell effector functions.

Acknowledgments
We thank Dr. Nicholas Huntington (Institut Pasteur, Paris, France) for reagents and critical reading of this manuscript, Dr. Yannick Jacques and Gregory Bouchaud (Institut de Biologie, Nantes, France), and Dr. Christian Vossenriech (Institut Pasteur) for helpful discussions.

Disclosures
The authors have no financial conflict of interest.

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