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Cutting Edge: A Novel Mechanism Bridging Innate and Adaptive Immunity: IL-12 Induction of CD25 To Form High-Affinity IL-2 Receptors on NK Cells

Seung-Hwan Lee,1,2 Maria F. Fragoso,1 and Christine A. Biron

NK cell expression and use of the IL-2Rα–chain (CD25), required for the high-affinity IL-2R, remain poorly understood. The studies reported in this article demonstrate that infections with murine CMV (MCMV), but not with lymphocytic choriomeningitis virus, induce CD25 on NK cells, along with high levels of IL-12 and IL-18. The cytokines act ex vivo to increase CD25 levels, and IL-12, IL-12R, and STAT4, but not the NK activating receptor Ly49H, are required for peak induction in vivo. All examined NK cell populations are driven into proliferation and incorporate BrdU in response to high ex vivo concentrations of IL-2, but only those from MCMV infection respond to low ex vivo concentrations of IL-2. The numbers of NK cells elicited during MCMV infection are reduced by IL-2 neutralization. Thus, a link between innate and adaptive immunity is established by which composition of innate cytokine responses sets up to promote NK cell use of a factor supporting adaptive responses. The Journal of Immunology, 2012, 189: 000–000.

Natural killer cells of the innate immune system express the IL-2/15Rβ–chain (CD122) and the common γ-chain (CD132) that are used as receptor components for multiple cytokines and sufficient to stimulate proliferation in response to high concentrations of IL-2. Formation of the high-affinity IL-2R, however, also requires expression of the IL-2Rα–chain (p55), identified as CD25. Starting with early studies in humans and mice, the conditions supporting NK cell expression and use of the CD25 chain have remained elusive (1–5). IL-2 is best characterized as an adaptive cytokine produced by T cells to support T cell proliferation, with induction of high-affinity IL-2Rs in response to TCR stimulation and IL-2 exposure being critical in the selection of T cell subsets for expansion (6).

NK cells are stimulated into blastogenesis and proliferation under a variety of conditions in vivo (1, 7, 8). During early infections of mice with either murine CMV (MCMV) or lymphocytic choriomeningitis virus (LCMV), type 1 IFNs, namely IFNα/β, promote NK cell blastogenic responses, at least in part, by inducing IL-15, and polyinosinic-polycytidylic acid, a chemical inducer of type 1 IFNs, as well as administered type 1 IFNs do the same in vivo (1, 7, 9). NK cells elicited through this pathway fail to express CD25 (IL-2Rα), have IL-2–independent in vivo responses, and only respond to high IL-2 concentrations ex vivo (10). Facilitated by expression of an activating receptor with specificity for a virus-induced ligand, Ly49H, NK cell responses to MCMV infection can extend into periods overlapping adaptive immunity (4, 11, 12).

The studies reported in this article investigated MCMV infections to demonstrate that NK cells induced under these conditions express CD25, acquire the ability to respond to low-dose IL-2 concentrations ex vivo, and use IL-2 for in vivo proliferation. The CD25 molecule is uniquely induced to high levels during MCMV, but not LCMV, infection. The response is dependent on IL-12, IL-12R, and the STAT4-sigaling molecule but is Ly49H independent. Thus, the enigma concerning the pathway to expression and use of the high-affinity IL-2R on NK cells is resolved. More importantly, a novel cytokine-dependent mechanism for CD25 induction and a major new link between innate and adaptive immunity are identified.

Materials and Methods

Mice

C57BL/6 (B6) mice were from Taconic Farms, B6 IL-12Rb2−/− mice (13) were originally obtained for breeding, and B6 RAG1-mutated mice with controls were from The Jackson Laboratory. B6 STAT4−/− mice (originally obtained from M. Kaplan, Indiana University School of Medicine, Indianapolis, IN) (14) and B6 Ly49H−/− mice (12) were bred and housed under specific pathogen-free conditions in our facility. Mice were used at 6–14 wk of age. The Institutional Animal Care and Use Committee approved the experiments.

In vivo manipulation

Mice were infected i.p. with either 5,000 or 50,000 PFU salivary gland-derived Smith MCMV from American Type Culture Collection (12) or with 4 × 106 PFU Armstrong LCMV strain clone E350 (15, 16). For IL-2 blocking, 250 μg each of two anti–IL-2 Abs (S4B6 and JES1A12; Bio X Cell) was administered i.p. Mice received injections at days 4 and 2 prior to harvest. For IL-12/23 blocking, 750 μg an Ab directed against the common p40 chain of IFN-γ was given i.p. on day 4 and 2 prior to harvest.

Abbreviations used in this article: B6, C57BL/6; LCMV, lymphocytic choriomeningitis virus; MCMV, murine CMV; rh, recombinant human; rm, recombinant murine; wt, wild-type.

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mouse IL-12/23 (clone C17.8; Bio X Cell) was administered i.p. 4 h before infection. For controls, equal amounts of isotype-matched Abs (Bio X Cell) or rat IgG (Sigma-Aldrich) were used.

Flow cytometric analyses

Splenic leukocytes were incubated for 20 min in 2% FBS-PBS with 2.4G2 Ab. NK cells were identified as NK1.1+TCR− populations. Under these conditions, there are no relevant differences in the NK cell subsets identified as TCR− or CD3− (6). Cell surface staining was performed using Abs directed against PE-CD25 (clone PG61.5), PE-CD122, PE-CD132, PerCP-NK1.1, FITC-TCRβ, allophycocyanin-Ly49H, PerCP-cy5.5-TCRβ, allophycocyanin-CD8α, PE-NK1.1, PE-CD49b, and allophycocyanin-CD49b (BD Biosciences or eBioscience). For BrdU detection, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), treated with DNase I (Sigma-Aldrich), and stained with FITC–anti-BrdU Ab (clone 3D4; BD Bioscience). Samples were acquired using a FACSCalibur (BD Biosciences), and data were analyzed with FlowJo (Tree Star) software.

Ex vivo cell manipulations

Sensitivity to IL-2–induced proliferation was evaluated using total splenic leukocytes at 3 × 105 cells (data not shown) or NK cells, enriched to 50–70% NK1.1+TCR− cells, using DX5 positive selection with magnetic beads (Miltenyi Biotec), at 1 × 105 cells/well in 96-well plates. rIL-2 (0, 0.4, 2, 10, 100, 500, 2500 U/ml) was added. After 40 h, cells were incubated with BrdU at 20 μM for an additional 2 h. Both recombinant human (rh)IL-2 (17 × 10^6 U/mg protein; Chiron) and recombinant murine (rm)IL-2 (10 × 10^6 U/mg protein; eBioscience) (data not shown) were tested with similar effective concentrations. BrdU-incorporating NK cell proportions in total and enriched populations were similar. To evaluate CD25 induction on NK1.1+TCR− cells, 5 × 10^5 total splenic leukocytes isolated from uninfected B6 mice were stimulated in 96-well plates with type I IFN (rIFN-α [1000 U/ml; Biogen], rIFN-β [1000 U/ml; Biogen]), rhIL-2 (10 and 500 U/ml; Chiron), rmIL-6 (100 ng/ml; R&D Systems), rmIL-12 (1 pg/ml–10 ng/ml; eBioscience), rmIL-18 (1 pg/ml–10 ng/ml; eBioscience), rmTNF-α (100 ng/ml; R&D Systems), or rmIFN-γ (100 ng/ml; eBioscience) for 24 h.

Statistical analysis

Results are means ± SE. The statistical significance of differences was determined by the unpaired two-tailed Student t test. A paired test was used for the comparison of results across the IL-2 titration.

Results and Discussion

Expression and function of CD25 on NK cells

Splenic populations from B6 mice that were uninfected (day 0) or that were infected with low (5,000) or high (50,000) PFU doses of MCMV at day 2, day 3.5, and day 5 prior to harvest were prepared for analysis. The NK cells were identified as NK1.1+TCR−. The CD25 chain was induced to detectable levels during MCMV infection, with up to 86% of NK cells having peak expression at day 3.5 (Fig. 1A). All three chains of the high-affinity IL-2R were examined using cells from day 0 or day 3.5 high dose MCMV-infected mice (Fig. 1B). Although MCMV infection was required to induce detectable CD25, cells from day 0 and infected mice expressed CD122 and CD132. The levels of CD122 remained relatively constant, whereas CD132 was elevated on day 3.5. As expected, the proportions of Ly49H+ cells were increased on day 3.5. The effects of LCMV infection were evaluated on day 3.5, day 5, and day 7 after i.p. infection with 4 × 10^5 PFU. Detectable CD25 expression was not induced, and CD122 expression was consistently observed after LCMV infection. On day 3.5 and day 5, CD32 expression was at levels intermediate to day 0 and day 3.5 of MCMV infection (Fig. 1B).

Although cells expressing CD122 and CD132 can proliferate in response to high IL-2 concentrations (e.g., >100 U/ml), the CD25 chain is required to induce proliferation in response to low IL-2 concentrations (e.g., ≤10 U/ml) (17). To determine the functional consequences associated with the various levels of IL-2R chains, NK cells were enriched from day 0, day 3.5 MCMV infection, day 3.5 LCMV infection, and day 5 or day 7 LCMV infection, and responsiveness to various rIL-2 concentrations, for incorporation of the DNA precursor molecule BrdU, was evaluated ex vivo. The cells were exposed to IL-2 for 40 h, with BrdU added for the last 2 h. Approximately 20–50% of NK cells prepared from day 0,
day 3.5, day 5, or day 7 LCMV infection, but >70% of those from day 3.5 of MCMV infection, were BrdU+ in response to 500 U/ml of rIL-2 (Fig. 2A). Only those prepared from the day 3.5 MCMV infection incorporated BrdU in response to 10 U/ml of rIL-2. Titration studies demonstrated that, compared with the other NK cell populations, 100-fold lower concentrations of IL-2 were required to stimulate BrdU incorporation of NK cells from day 3.5 of MCMV infection (Fig. 2A).

A role for IL-2 in supporting endogenous NK cell proliferation during MCMV infection was evaluated by neutralizing the factor in vivo. Combinations of Abs directed against different parts of IL-2 were used. Control Ab-treated mice received equal concentrations of isotype-matched Ab. Splenic NK cell yields were assessed at day 1.5 and 3.5 after low-dose MCMV infection. Compared with day 0, both the control and anti–IL-2-treated mice had decreases in NK cell numbers on day 1.5 (Fig. 2B). These recovered and were elevated on day 3.5 of infection in the control, but not the anti–IL-2–treated, group. The Ly49H proportions within the NK cell subsets were preferentially increasing during this period, and the IL-2–dependent effects were predominantly found in this subset. Thus, during MCMV infection, NK cells are uniquely induced to express the CD25 chain, form the high-affinity receptor for IL-2, and respond to low doses of IL-2 ex vivo, and IL-2 supports their proliferation in vivo.

**Mechanism for CD25 induction**

Because both Ly49H receptors and TCRs use overlapping signaling pathways, and TCR signaling leads to IL-2R induction, experiments were carried out to evaluate the contribution of Ly49H to CD25 induction on NK cells. Wild-type (wt) control B6 mice and B6 mice rendered Ly49H deficient as a result of genetic mutation (Ly49H−/−) were either day 0 uninfected or day 2 infected with 5000 PFU of MCMV and examined for the contribution made by Ly49H to CD25 induction. The proportions of NK cells expressing CD25 reached 80% after infection of Ly49H−/− mice (Fig. 3A); thus, the activating molecule was not required.

The major cytokines during early LCMV infection are type 1 IFNs, and circulating levels of biologically active IL-12p70 heterodimer and processed IL-18 are low to undetectable (15, 18). In contrast, MCMV infection induces IL-12 and IL-18, as well as type 1 IFNs (18–20). In these studies, the mean ± SE serum levels of IL-12p70 were undetectable during LCMV infection but reached 789 ± 92 pg/ml at day 1.5 during high-dose MCMV infection. The role of endogenous IL-12 in CD25 induction was evaluated in B6 mice treated with control Abs or Abs neutralizing IL-12 as a result of binding to the p40 chain, anti-IL-12/23 (Fig. 3B), as well as in B6 wt mice compared with IL-12 unresponsive (i.e., mutated in the IL-12Rβ2 chain specific for the cytokine) mice (Fig. 3C). Both approaches resulted in profound inhibition of CD25 induction on NK cells at day 2 of MCMV infection, with less than half of the populations identified as receptor positive, and these expressing much lower levels of CD25.

STAT4 is the major signaling molecule from IL-12R. Experiments carried out in B6 wt and STAT4-deficient (STAT4−/−) mice (Fig. 3D) demonstrated that STAT4 was critical for maximal CD25 induction, with the proportions of,
and intensities on, expressing cells dramatically reduced in its absence. Thus, IL-12, IL-12R, and STAT4 provide a pathway for CD25 induction in vivo during MCMV infection.

To evaluate the effects ex vivo and to separate induction from any viral effects, splenic leukocytes from uninfected B6 mice were cultured with media control or various cytokines overnight. The populations were harvested and stained for NK1.1, TCR\(\beta\), and CD25. The cytokines tested included the innate cytokines induced at early times after MCMV infection (18–20) and IL-2 (Fig. 4A). IL-12 was the strongest inducer of CD25. Titration studies showed that IL-12 alone induced a plateau 80% of NK cells expressing CD25 at 100 pg/ml to 1 ng/ml, whereas IL-18 alone induced a plateau response reaching 30% expressing cells at 10 ng/ml. When added together, there was a synergistic interaction, with >80% of the NK cells induced to express high levels of CD25 (Fig. 4B). The responses were independent of T cells, because similar results were obtained with NK cells from T and B cell-deficient RAG1-mutant mice (data not shown). Thus, CD25 induction on NK cells can be achieved without viral infection by addition of innate cytokines and is T cell independent.

To evaluate the roles for IL-12R and STAT4 ex vivo, populations were taken from B6 wt, IL-12R\(\beta^2--/\)-, and STAT4\(-/-\)-mice and stimulated with the cytokines (Fig. 4C). All of the CD25 induction mediated by IL-12, including any synergism with IL-18, was ablated using IL-12R\(\beta^2--/\)-populations, and all of the effects mediated by IL-12 alone were ablated in STAT4\(-/-\) populations. There was also a significantly reduced interaction between IL-18 and IL-12. Thus, the IL-12–STAT4 pathway to NK cell CD25 expression can be mechanistically differentiated in culture.

In summary, these studies conclusively demonstrate that, under the appropriate conditions, NK cells express CD25 and a high-affinity IL-2R and define a new link between innate and adaptive immunity. The pathway to induction is shown to be through IL-12 but independent of Ly49H. A lack of requirement for the activating receptor separates the response from that elicited through TCRs on T cells; however, previous work from our group has shown that NK cells are unique in their basal readiness for IL-2 responsiveness and high STAT4 expression (16, 21, 22). Thus, they are equipped prior to infection to respond to the innate cytokine with responsiveness to an adaptive cytokine. The results help to explain a number of observations about NK cells, including the importance of IL-12 and IL-18 in supporting expansion of Ly49H NK cells during MCMV infection (23), the ability to use IL-12 and IL-18 in culture to derive populations promoting long-term mouse NK cells (24), the role of IL-12 and STAT4 in supporting the development of long-lasting “memory” NK cells during MCMV infection (25), and the responsiveness of human NK cell subsets to IL-2 for IFN-\(\gamma\) secretion (26). They also help to bring understanding of the human and mouse systems closer together by demonstrating how the reported response of a human NK cell subset to IL-12 exposure with CD25 expression (5) works in the mouse during endogenous infections. Contrasting the two species suggests interesting avenues for further investigations to reconcile apparent differences, including evaluating STAT4 levels in human NK cell subsets relative to their IL-12 responsiveness for CD25 expression, as well as the role of IL-2 in mouse NK cell IFN-\(\gamma\) production at the later times associated with extended MCMV infection (12).

There are likely to be other factors in place with overlapping, alternative, or augmenting functions for supporting NK cell proliferation. Although the induction of CD25 was seen in response to both low- and high-dose MCMV infection in these studies, the requirement for IL-12 in vivo was best demon-
strated during low-dose infection. Type I IFNs have the potential to alternatively elicit CD25 because the receptors for these factors are also expressed on NK cells and because they can signal basally through STAT4. This can explain the low-level CD25 induction on NK cells observed when IFN-α or IFN-β was evaluated in culture with populations from uninfected mice (Fig. 4A). However, the pathway from type I IFNs to STAT4 is tightly regulated because the cytokines concurrently induce STAT1 and once elevated, this molecule suppresses the proliferation of murine natural killer cells in vivo. The Journal of Immunology 5 165: 4787–4791.


