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Proliferation Conditions Promote Intrinsic Changes in NK Cells for an IL-10 Response

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Constitutively found at high frequencies, the role for NK cell proliferation remains unclear. In this study, a shift in NK cell function from predominantly producing IFN-γ, a cytokine with proinflammatory and antimicrobial functions, to producing the immunoregulatory cytokine IL-10 was defined during extended murine CMV infection. The response occurred at times subsequent to IL-12 production, but the NK cells elicited acquired responsiveness to IL-12 and IL-21 for IL-10 production. Because neither IL-12 nor IL-21 was required in vivo, however, additional pathways appeared to be available to promote NK cell IL-10 expression. In vitro studies with IL-2 to support proliferation and in vivo adoptive transfers into murine CMV-infected mice demonstrated that NK cell proliferation and further division enhanced the change. In contrast to the sustained open profile of the IFN-γ gene, NK cells responding to infection acquired histone modifications in the IL-10 gene indicative of changing from a closed to an open state. The IL-10 response to IL-12 was proliferation dependent ex vivo if the NK cells had not yet expanded in vivo but independent if they had. Thus, a novel role for proliferation in supporting changing innate cell function is reported. The Journal of Immunology, 2014, 193: 354–363.

Natural killer cells of the innate immune system are important in early defense against viral infections of mice and humans. In addition to being induced to mediate direct antiviral effects, they are stimulated by cytokines and through activating receptors to undergo proliferation (1–5). The biological importance of this response has remained elusive. In contrast to the low numbers of Ag-specific T cell subsets needing expansion for defense, NK cells are basally found at high frequencies, and large proportions express particular activating receptors (5). During early murine CMV (MCMV) infection, induced IL-12 stimulates NK cell production of IFN-γ to support proinflammatory effects and consequently enhance innate antiviral defense (3, 6). NK cells can sometimes make the immunoregulatory cytokine IL-10 (4, 7–12), and they do so at later times during sustained MCMV replication as a result of deficiency in cytotoxic function (4). Under these conditions, NK cell IL-10 acts to limit adaptive responses for protection against immune-mediated disease.

So then, how are NK cells regulated to balance their proinflammatory and immunoregulatory effects? Extrinsic factors such as the cytokines available to stimulate IL-10 expression might be differentially induced to support these responses. IL-12 can sometimes elicit, and other cytokines can conditionally induce, IL-10 (7, 9, 13–17), but their roles in eliciting NK cell IL-10 during viral infection have not been examined. Alternatively, there could be intrinsic changes in cellular “preparedness” to make IL-10 (18, 19). Remarkably, the NK cell IL-10 response during sustained MCMV replication in cytotoxic-deficient mice is observed subsequent to their proliferation (4). Because these observations suggest that the events may be linked, experiments were carried out characterizing conditions of MCMV infections in immunocompetent wild-type (Wt) mice associated with an NK cell IL-10 response. The results show that extrinsic factors can play a role. During extended infection, however, NK cells are intrinsically changed to access an IL-10 response with histone modifications for opening up the IL-10 gene. The altered function is dependent on proliferation competence and/or previous division. Thus, a new role for proliferation in supporting changing function of an innate cell response is reported, and one answer to the question of why NK cells proliferate is provided.

Materials and Methods

Mice

Mice were on a C57BL/6 (B6) background. The CD45.2 C57BL/6 and CD45.1 allogenic mice were purchased from Taconic Farms (Germantown, NY). Breeding pairs of B6 mice with the “knockin” insertion of a genetic construct having an internal ribosomal entry site and the GFP gene under the transcriptional control of the IL-10 gene (interleukin-ten inters gfp-enhanced reporter, Tiger) (20), that is, IL-10-GFP-reporter, and IL-12p35-deficient (21) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of IL-21– and IL-21R-deficient mice (22) were obtained from M. Rincon (University of Vermont, Burlington, VT). The CD45.1 Ly-5H-deficient (4, 23) mice were bred. Only IL-10-reporter mice heterozygous for the GFP containing knockin allele were used for studies. The IL-10-GFP reporter allele was introduced into IL-21–deficient and IL-21R-deficient by crossing with IL-10-GFP reporter mice. Mice were used at 6–14 wk of age. Animals obtained from sources outside of Brown University were housed in the animal care facility for at least 1 wk before use. Handling of mice and experimental procedures were conducted in accordance with institutional guidelines for animal care and use, and protocols were approved by the Brown University Institutional Animal Care and Use Committee.

In vivo manipulation

Except where indicated, infections were i.p. with 4,000/5,000 (low dose) or 15,000/70,000 (high dose) PFUs of salivary gland–derived Smith MCMV originally obtained from American Type Culture Collection as described.
Previously (4). For IL-12/23 blocking, 50 μg of an anti-IL-12p40 (the clone C17.8 anti-mouse IL-12/23; BioXCell) was administered by i.p. injection at 4 h prior to infection. For controls, an equal amount of rat IgG2a (BioXCell) was given.

**Sample preparations and assays**

Mice were anesthetized using isoflurane (Aerrane, Baxter Healthcare), and blood was collected via the retro-orbital route into tubes containing heparin. Samples were centrifuged at 10,000 rpm for 10 min. Supernatants were identified as sera because there was always some clotting. The viral titers in homogenized organ samples were quantified by standard plaque assays using mouse embryonic fibroblasts (4). Cytokine levels were measured and are presented as picograms per milliliter for serum or picograms per 10⁶ cells for conditioned media. IFN-α and IFN-β by ELISA (R&D Systems) and IL-21, IFN-γ, IL-12p70, IL-6, and IL-10 using cytometric bead assays (BD Biosciences). For RT-PCR, primers synthesized by Operon were as follows: IL-10 (forward, 5'-CTGTATGCTGCTCTCTTATCTA-3', reverse, 5'-CCTGCTCCACTGCTCTGTATTA-3') (14), IL-21 (forward, 5'-CCCTGCTGCTCTGTATGATC-3', reverse, 5'-ATACACGGAAGGGCATTTAGC-3') (24), and Gapdh (forward, 5'-ACCACAGGTCTCCTGATCACC-3', reverse, 5'-TCCACACCCCTGTGGTCTGA-3'). Cell and mRNA samples were from spleens.

**Flow cytometric analyses**

Splenic leukocytes were incubated for 20 min with 2.4G2 Ab to reduce nonspecific staining. Cell surface markers were identified using labeled Abs (BD Biosciences and eBioscience): NKp46-PE, TCR-β-FITC, TCR-β-PE, TCR-β-PerCP5.5, TCR-β-allophycocyanin, TCR-β-allophycocyanin-Cy7, TCR-β-PE-Cy7 (PE-Cy7), Ly49H-allophycocyanin, NK1.1-PE, NK1.1-PerCP, NK1.1-allophycocyanin, CD45.2-PerCP5.5, CD45.2-FITC, CD49B-PE-Cy7, CD49B-allophycocyanin, and CD49B-PE. For intracellular IFN-γ, cells were brefeldin A treated (5 μg/ml) for 4 h, surface stained, fixed/permeabilized (in CytoFix/CytoPerm buffer; BD Biosciences), and labeled with anti–IFN-γ-PE. For increased GFP detection, a recombinant rabbit monoclonal anti-GFP Ab (Invitrogen), followed with an Alexa and labeled with anti–IFN-γ-FITC (BD Biosciences), and stained, fixed/permeabilized (in CytoFix/CytoPerm buffer; BD Biosciences), and labeled with anti–IFN-γ-Cy5 (eBioscience). Cell surface markers were identified using labeled Abs (BD Biosciences and eBioscience): NKp46-PE, TCR-β-allophycocyanin-Cy7, TCR-β-PE-Cy7, Ly49H-allophycocyanin, NK1.1-PE, NK1.1-PerCP, NK1.1-allophycocyanin, CD45.2-PerCP5.5, CD45.2-FITC, CD49B-PE-Cy7, CD49B-allophycocyanin, and CD49B-PE. For intracellular IFN-γ, cells were brefeldin A treated (5 μg/ml) for 4 h, surface stained, fixed/permeabilized (in CytoFix/CytoPerm buffer; BD Biosciences), and labeled with anti–IFN-γ-PE. For increased GFP detection, a recombinant rabbit monoclonal anti-GFP Ab (Invitrogen), followed with an Alexa Fluor 488–labeled goat anti-rabbit IgG (Invitrogen), were added to fixed/permeabilized cells. Samples were acquired using either a FACS-Calibur or FACS-Aria (BD Bioscience), and data were analyzed with either the CellQuest Pro (BD Biosciences) or FlowJo (Tree Star) software. The FlowJo Proliferation platform was used for analysis of cell division.

**NK cell preparations and ex vivo manipulations**

For conditioned media, 5 × 10⁶ total or 1 × 10⁶ purified cells were incubated in the presence or absence of mouse recombinant cytokines, IL-12 (10 or 50 ng/ml; eBioscience), IL-21 (100 ng/ml; eBioscience), IL-22 (100 ng/ml; PeproTech), IL-23 (100 ng/ml; R&D Systems), or IL-27 (100 ng/ml; R&D Systems). Supernatants were harvested after 24 h. Splenic NK and T cell subsets were FACS sorted (FACS Aria; Flow Cytometry Facility at Brown University) based on the expression of CD40b and TCR-β using leukocytes from day 3.5–infected mice stained with PE-CD49b and allophycocyanin-TCR-β. Purity was >80–92%. Where indicated, NK cells were prepared, using immune-magnetic bead selection (Stem Cell Technologies Negative Selection Mouse NK Cell Enrichment kit), to a purity of ~80%, that is, 76–85% on day 0, and 85–87% on day 3.5, with 40% as compared with 60% of the NK cells expressing Ly49H in day 0 as compared with day 3.5 preparations. Thus, the Ly49HI⁺ cell frequencies in the populations were increased from 4 to 6 out of 10 cells. The cells were labeled with 5 μM Cell Proliferation Dye eFluor670 (eBioscience), washed, and used in culture or in adoptive transfers. In some experiments, cells were pretreated with 50 μg/ml mitomycin C (MMC) (Sigma-Aldrich) to block proliferation. Splenic leukocytes (1 × 10⁶) or purified NK cells (1 × 10⁶) were cultured for 48 or 72 h in the presence of 25 or 1000 U/ml IL-2 (sp. act. ≥ 5.7 × 10⁶ U/mg; eBioscience), with or without the addition of 50 ng/ml IL-12 (≥ 5.7 × 10⁶ U/mg; eBioscience) 24 h prior to harvest.

**Adoptive transfer**

For adoptive transfer experiments, 2 × 10⁶ splenic leukocytes or 4 × 10⁶ purified NK cells from CD45.2 IL-10-GFP reporter mice, that were uninfected or infected with 70,000 PFU MCMV for the indicated times, were adoptively transferred by i.v. injection to either Wt or Ly49HI⁺ B6 mice with the CD45.1 allotypic marker. Where indicated, the cells were labeled with eFluor670 Proliferation Dye (eBioscience) before transfer. The Wt recipient mice were infected with 5,000 PFU, and the Ly49HI⁺ mice were infected with 4000 PFU MCMV as described previously. Approximately

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

**FIGURE 1.** Viral replication and cytokine production during MCMV infection at low and high doses. Mice, Wt B6, were infected with 5,000 (low dose) or 70,000 (high dose) PFU MCMV for indicated times. (A) Spleen and liver virus was determined by plaque assay. (B) Serum levels of IFN-α and IFN-β were measured by ELISA and of IL-12p70, IL-6, IFN-γ, and IL-10 by Cytometric Bead Assays. For (A) and (B), 5000 PFU data (mean ± SD of three mice) are representative of two, and 70,000 PFU data (mean ± SD of three mice) of three experiments. (C) Splenic IL-10 mRNA was measured by semiquantitative RT-PCR with Gapdh mRNA used as input control. Results are representative of three experiments. (D) Serum IL-21 levels were measured using CBA. Each symbol represents an individual mouse. Bars are averages. (E) Splenic IL-21 mRNA was measured by semiquantitative RT-PCR with Gapdh as control. Results represent three experiments. When shown, gray horizontal lines are limit of detection for assay. (F) IL-10 and IFN-γ production in media conditioned, without stimulation, for 24 h with total splenic leukocytes from day 0 or 3.5 or FACS-purified NK (CD49b⁺ TCR-β⁺) and T (CD49b⁻ TCR-β⁻) cells from day 3.5 high-dose–infected mice. Results represent two experiments. Values below detection are indicated by ϕ.
as a result of genetic mutation in gene for the p35 subunit (IL-12p35).

NK cell IL-10 response was evaluated in B6 mice rendered IL-12 deficient indicated by cytokines. Data represent two experiments. Values below detection are infected B6 mice was determined after 24 h stimulation with indicated production by total leukocytes or FACS-purified subsets from day 3.5–6 stimulation in culture with IL-12, IL-21, IL-22, IL-23, or IL-27. Data in supplemen-
tional Fig. 1A–D). The splenic populations were increasing during infection (Supplemental Fig. 1A–D). The splenic cytokines (A) and IL-10-GFP expression in NK and T cells (B) after 24 h stimulation in culture with IL-12, IL-21, IL-22, IL-23, or IL-27. Data in (A) and (B) are representative of three experiments. (C) IL-10 and IFN-γ production by total leukocytes or FACS-purified subsets from day 3.5–infected B6 mice was determined after 24 h stimulation with indicated cytokines. Data represent two experiments. Values below detection are indicated by *. (D) The requirement for endogenous IL-12 in the 3.5-day NK cell IL-10 response was evaluated in B6 mice rendered IL-12 deficient as a result of genetic mutation in gene for the p35 subunit (IL-12p35/–) and in IL-10-GFP reporter mice rendered IL-12 deficient as a result of treatment with anti–IL-12p40. (E) Requirement for endogenous IL-21 in the NK cell IL-10 response was evaluated in mice rendered IL-21 deficient as a result of genetic mutation of the cytokine gene (IL-21/–) or of the gene for the cytokine receptor (IL-21R/–) in comparison with hemizygous littermates bred to also carry a IL-10-GFP reporter gene. The numbers in histograms indicate proportions of IL-10-GFP–expressing cells. Gray vertical lines indicate peak of basal GFP expression. Symbols in summary data are results with samples from individual mice. Mean ± 1.5–3.5 millions of cells were acquired per sample and CD45.2 donor NK and T cells were analyzed for IL-10-GFP and IFN-γ expression.

Chromatin immunoprecipitation assays

NK cells were purified from uninfected mice and MCMV-infected mice at days 1.5 and 3.5 by negative selection. Chromatin immunoprecipitation was carried out using Abs against H3K4 trimethylation (Millipore number 04-745), H3K27 trimethylation (Millipore number 07-449), and H3K36 trimethylation (Abcam number 9050). Recovered DNA samples of ~10–50 ng were made into libraries with illumine adapters and sequenced for single-read 50 cycles using Hiseq2000 (Illumina, San Diego, CA). Uniquely matching reads were mapped to mouse mm9 reference genome, and significant islands were called with special clustering approach for identification of ChIP-enriched regions (25). Integrative Genomics Viewer (Broad Institute) was used to generate histone mark distribution around the IL-10 locus. The data have been added to the Gene Expression Omnibus repository (GSE55834).

Statistical analysis

Statistical significance of differences was determined by the unpaired two-tailed Student t test. Where indicated, significance of differences between multiple groups was also evaluated by ANOVA.

Results

IL-10 production by NK cells during sustained viral infection

Following low-dose MCMV infection of Wt B6 mice with 5000 PFU, the virus was controlled in spleens by day 2 but prolonged in livers (Fig. 1A). The expected (3, 6, 26–28) systemic innate cytokine IFN-α, IL-12p70, IL-6, and IFN-γ responses peaked on day 1.5 of infection with IFN-β levels below detection (Fig. 1B). Serum IL-10 was marginally detectable in a subset of samples at later times. High-dose infection with 70,000 PFU resulted in sustained and elevated viral replication in spleens and livers, with similar kinetics of early serum cytokine responses now having higher magnitudes and detectable IFN-β. Notably, IL-10 was elicited but temporally distinct from the other cytokine responses, with increases on day 3.5. Splenic IL-10 mRNA elevation started earlier and reached higher levels during high-dose infection (Fig. 1C). Serum IL-21, another cytokine reported to induce IL-10, was only found in a few samples from high-dose infected mice (Fig. 1D), but either dose stimulated IL-21 mRNA expression (Fig. 1E).

NK cells responding in spleens on day 3.5 after high-dose MCMV challenge were characterized for their expression of known NK cell markers (29). They had diminished expression of CD49b NK cell markers but not the TCR for Ag (i.e., TCR-β) (Supplemental Fig. 1A–D). The splenic proportions of CD49b+ TCR-β– NK cells and Ly49H+ subsets in these populations were increasing during infection (Supplemental Fig. 1D, 1E). The NK (CD49b+ TCR-β–) and T (CD49b+ TCR-β+) cells were FACS purified from high-dose infected mice on 3.5 days, and their cytokine production in culture was compared with that of uninfected (day 0) or day 3.5 total leukocytes. Detectable IL-10 was only, and residual IFN-γ was predominantly, produced by the NK cells (Fig. 1F). To overcome difficulty in detecting IL-10 by flow cytometry, B6 mice reporting IL-10 with expression of GFP (IL-10-GFP) were analyzed. In comparison with T cell subsets, NK cells had high IL-10-GFP by day 3.5 of infection (Supplemental Fig. 1F). Analysis of all splenic leukocytes demonstrated that under these conditions of infection, the cells expressing IL-10-GFP were localized within subsets expressing NK cell markers (Supplemental Fig. 1G). Taken together, these results show that Wt mice with elevated and extended viral replication have systemic IL-10 and that NK cells are the major IL-10 producers.

SD, of totals of three to six mice, are shown with bars. Studies are representative of, or pooled from, two to three independent experiments. *p < 0.05, **p < 0.01.
and IFN-γ for eFluor670 dilution, along with intracellular expression of IL-10-GFP 72 h after culture with IL-2 with IL-12 added for the last 24 h and analyzed added at 24 h prior to harvest. **(B)** IL-10-GFP or IFN-γ expression at the 24-, 48-, or 72-h harvest. In contrast to IL-10, IFN-γ expression was discernible under the 72 h harvest conditions. With the higher IL-2 concentrations to be driven into expansion (31–33), IL-2 was added at 25 U/ml for control and at 1000 U/ml to induce proliferation. IL-12 (50 ng/ml) was added at the initiation of culture (24-h harvest), at 24 h (48-h harvest), or at 48 h (72-h harvest). Brefeldin A was added at 4 h prior to harvest. The cells were prepared and examined for intracellular expression of IL-10-GFP and IFN-γ. Because NK cells from uninfected mice require high IL-2 concentrations to be driven into expansion, purification of naive NK cells, prepared from uninfected IL-10-GFP mice by negative selection, were examined. Because NK cells from uninfected mice require high IL-2 concentrations to be driven into expansion (31–33), IL-2 was added at 25 U/ml for control and at 1000 U/ml to induce proliferation. IL-12 (50 ng/ml) was added at the initiation of culture (24-h harvest), at 24 h (48-h harvest), or at 48 h (72-h harvest). Brefeldin A was added at 4 h prior to harvest. The cells were prepared and examined for intracellular expression of IL-10-GFP and IFN-γ. With low-level IL-2, there was no discernible induction of IL-10-GFP but good induction of IFN-γ expression at the 24-, 48-, or 72-h harvests. In the presence of high IL-2 concentrations, induction of IFN-γ was higher and the IL-10-GFP expression increased with time in culture (Fig. 3A). Purified naive NK cells, again prepared from uninfected IL-10-GFP mice by negative selection, were labeled with the proliferation dye eFluor670 and cultured to examine the induction of IL-10 production using purified subsets in culture were consistent: only the day 3.5 NK cells responded to IL-12 or IL-21 with increased IL-10, and a distinct IFN-γ enhancing effect was mediated by IL-12 (Fig. 2C). High-dose infections of reporter mice with selective defects in responsiveness to IL-12 (Fig. 2D) or IL-21 (Fig. 2E) demonstrated IL-10 induction and NK cell IL-10-GFP expression in all infected mice having single defects. Therefore, NK cells conditioned during MCMV infection have an induced responsiveness to IL-12 and IL-21 for IL-10 expression, but these cytokines are not individually required for infection-induced NK cell IL-10 in vivo.

### Proliferation accompanies acquisition of the NK cell IL-10 response

The correlation of the IL-10 response with proliferation was examined independently of viral infection in culture studies using IL-2-driven expansion. Purified naive NK cells, prepared from uninfected IL-10-GFP mice by negative selection, were examined. Because NK cells from uninfected mice require high IL-2 concentrations to be driven into expansion (31–33), IL-2 was added at 25 U/ml for control and at 1000 U/ml to induce proliferation. IL-12 (50 ng/ml) was added at the initiation of culture (24-h harvest), at 24 h (48-h harvest), or at 48 h (72-h harvest). Brefeldin A was added at 4 h prior to harvest. The cells were prepared and examined for intracellular expression of IL-10-GFP and IFN-γ. With low-level IL-2, there was no discernible induction of IL-10-GFP but good induction of IFN-γ expression at the 24-, 48-, or 72-h harvests. In the presence of high IL-2 concentrations, induction of IFN-γ was higher and the IL-10-GFP expression increased with time in culture (Fig. 3A). Purified naive NK cells, again prepared from uninfected IL-10-GFP mice by negative selection, were labeled with the proliferation dye eFluor670 and cultured to examine the induction of IL-10-GFP and IFN-γ under the 72 h harvest conditions. With the high IL-2 concentrations, NK cell yields were ~4-fold higher, and the cells had undergone multiple divisions, with more than seven cell subsets of eFluor670 dilution identifiable (Fig. 3B). In comparison with cells in low IL-2, higher IL-10-GFP expression was discernible with levels increasing upon further divisions to an apparent plateau at division 4 or 5. In contrast to IL-10, IFN-γ was dramatically in-

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**FIGURE 3.** Cell division and changing NK cell responses ex vivo. Purified NK cells from uninfected IL-10-GFP mice, prepared by negative selection and labeled with eFluor670 proliferation dye, were cultured in the presence of 25 or 1000 U/ml IL-2 for 24, 48, or 72 h, with 50 ng/ml IL-12p70 added at 24 h prior to harvest. Cells were collected and analyzed for intracellular expression of IL-10-GFP or IFN-γ with or without eFluor670 dilution. Representative flow cytometry plots are shown. (A) Expression of IL-10-GFP or IFN-γ after culture in IL-2 for 24, 48, or 72 h with IL-12 added at 24 h prior to harvest. (B) Cells were collected from samples at 72 h after culture with IL-2 with IL-12 added for the last 24 h and analyzed for eFluor670 dilution, along with intracellular expression of IL-10-GFP and IFN-γ. Representative flow cytometry plots of divisions and cytokine expression are shown. Summary data are replicate samples from a single experiment using pooled cells (mean ± SD) and are representative of three independent experiments. Statistical analyses evaluated the differences in proportions of cells expressing each cytokine after one as compared with seven divisions. *p < 0.05. (C) As in (B) but IL-10-GFP expression was characterized in the NK cell subsets either positive or negative for the Ly49H activating receptor. Representative flow cytometry plots of divisions and IL-10-GFP expression are shown. Summary data are replicate samples from a single experiment using pooled cells (mean ± SD) and are representative of two independent experiments.
duced following IL-12 exposure even in cells maintained under low IL-2 concentrations. The proportion and intensities of IL-10-GFP–expressing cells were higher in the first division group from the high-dose IL-2 condition, stayed high through four divisions, and then declined. The changing responsiveness resulted in NK cell populations with fewer divisions largely expressing IFN-γ and those with the most divisions largely expressing IL-10. The modification was independent of the Ly49H activating receptor because the shift to IL-10-GFP was observed in both Ly49H+ and Ly49H− subsets (Fig. 3C).

To evaluate the association of proliferation with cytokine responses in vivo, mature naive NK cells, purified from uninfected CD45.2 IL-10-GFP-reporter mice by negative selection, were labeled with the eFluor670 proliferation dye, and transferred to uninfected Ly49H-deficient B6 CD45.1. The recipient mice were then MCMV infected. Examination of the levels of eFluor670 expression demonstrated that although the transferred cells readily expressed IFN-γ at day 1.5 of infection, most of the cells had not yet undergone proliferation and expressed only low levels of IL-10-GFP (Fig. 4A). Proliferation was enhanced by day 2.5 and dramatically extended by day 3.5 with many of the transferred NK cells having undergone extensive numbers of divisions. Although they expressed less IFN-γ, the NK cells expressed higher levels of IL-10-GFP at days 2.5 and 3.5 of infection (results summarized in Fig. 4B). Taken together, these studies show that the NK cell IL-10 response is associated with extensive proliferation either under in vitro conditions independent of infection or in vivo during infection.

**Intrinsic changes in NK cells to allow IL-10 response**

To define intrinsic as compared with extrinsic influences, adoptive transfer experiments were carried out with cells isolated from day 0, day 1–infected, or day 3–infected IL-10-GFP-reporter CD45.2 B6 mice and then delivered to Wt CD45.1 B6 mice that were day 0 or MCMV infected at day 1 or 3 prior to transfer (Fig. 5A). The donor mice were infected with 70,000 PFU, and recipient mice were challenged with 5,000 PFU. Transferred cells were allowed to experience endogenous stimuli in recipient mice for 1 d, harvested, and analyzed for IL-10-GFP expression in gated NK cells among CD45.2 donor populations. When transferred to day 0 environments, NK cells from day 3–infected donors (noted by red lines in histograms) showed modest residual IL-10-GFP expression. Dramatic IL-10-GFP induction was observed when NK cells from day 3–infected donors were transferred into day 1 recipients, whereas NK cells from day 0 or 1 donors showed minimal expression. The IL-10-GFP levels were intensified in Ly49H+TCR-β− as compared with CD49b+TCR-β− cells (Fig. 5A). In day 3 recipients, expression was detectable but reduced as compared with day 1 recipients. Parallel experiments were carried out examining IFN-γ. The NK cell populations from day 0, 1–, or 3–infected mice all had IFN-γ induction when transferred into day 1–infected mice (Fig. 5B). To test the role for endogenous IL-12 in supporting the responses, the effects of anti-IL-12p40 treatment were evaluated. Blocking IL-12 abolished induction of IL-10-GFP and IFN-γ in NK cells prepared from day 3 and transferred into day 1 MCMV–infected mice (Fig. 5C). Hence, NK cells are intrinsically pre-equipped for IFN-γ induction in response to IL-12 but acquire the ability to respond with IL-10 as infection progresses. When they experience IL-12 in an in vivo environment, they now produce both cytokines.

**Chromosomal changes in the NK cell IL-10 gene during infection**

One mechanism with the potential for regulating access to the IL-10 gene is epigenetic modification of associated histones to control access of transcriptional machinery (25, 34). In particular, the...
Histone 3 modifications K4 trimethylation (m3) or K36 trimethylation (m3) are indicative of euchromatin with an "open" structure, whereas K27 trimethylation (m3) is indicative of a "closed" structure. To evaluate the possible involvement of epigenetic modification in the control of NK cell IL-10 expression during MCMV infection, chromatin immunoprecipitation was performed. Splenic leukocytes were harvested from day 0, 1.5, or 3.5 MCMV–infected mice and subjected to differential centrifugation and magnetic bead negative selection to enrich for NK cells, yielding high purity and sufficient numbers for study. Cell pellets were prepared, cross-linking was carried out, and Abs specific for the histone 3 activation marks K4m3 and K36m3 and the inhibitory mark K27m3 were used. The two activating methylations, K4m3 and K36m3, were largely absent from the IL-10 gene at day 0 in NK cells and increased dramatically as infection progressed. The K27m3 inhibitory methylation was present in days 0 and 1.5 NK cells but absent in day 3.5 NK cells (Fig. 6A). In contrast, the IFN-γ gene has been reported to be opened in NK cells from uninfected mice (35), and it had an opened profile in the NK cells purified from day 0 as well as days 1.5 and 3.5 MCMV–

**FIGURE 5.** Intrinsic and extrinsic influences on NK cell responsiveness for IL-10 and IFN-γ expression. Donor splenic leukocytes from B6 IL-10-GFP (CD45.2) mice, day 0 or infected with high-dose MCMV for 1 or 3 d, were transferred into B6 (CD45.1) recipients that were either day 0 or infected with low-dose virus for 1 or 3 d. Recipient mice were left for 1 d before harvesting and analyzing IL-10-GFP (A) or IFN-γ (B) expression within gated donor (CD45.2) NK cells. Filled gray histograms indicate basal cytokine expression in transferred day 0 cells into day 0 mice. Blue, green, and red histograms indicate expression in donor NK cells prepared from day 0, day 1–infected, and day 3–infected IL-10-GFP mice, respectively. Summary data are from three experiments, with four to six mice per group total. (C) As in (A) and (B) with the modification that donor leukocytes from day 3–infected IL-10-GFP mice were adoptively transferred into day 1 B6 recipients that had received 750 μg anti–IL-12p40 or control Ab 4 h before infection. Results are from two experiments, five mice per group total. (A and B) Numbers on histograms are proportion of cells within gate. Representative data are shown. Results are summarized with symbols representing individual mice. Statistical significances are shown: **p < 0.01, ***p < 0.0001. For the summary data for (A), the results are also significant by one-way ANOVA with ***p < 0.0001.
NK cells prepared from leukocytes isolated from 20 (day 0), 40 (day 1.5), and 40 (day 3.5) mice were purified to immunoprecipitation and massive parallel sequencing of purified NK cells from Wt B6 mice uninfected (day 0) or MCMV infected for 1.5 and 3.5 d. FIGURE 6. Increased accessibility of the IL-10 locus during the course of MCMV infection. Distribution of histone marks was assessed by chromatin immunoprecipitation and massive parallel sequencing of purified NK cells from day 0 or 1.5–infected mice but insignificant using the populations prepared on day 2.5 or 3.5 of infection (results summarized in Fig. 7B). Thus, NK cells acquire an IL-10 response to IL-12 after division, and the shift requires the ability to proliferate but is proliferation independent if the cells have already expanded during infection.

**Discussion**

This paper conclusively demonstrates proliferation-dependent conditioning of NK cells. During sustained viral infection, NK cells acquire the ability to produce IL-10 and shift the response induced by IL-12 from IFN-γ only to IFN-γ and IL-10. The shift to an ability to produce IL-10 is important in contributing to the regulation of CD8 T cell responses to infection (4). It is accompanied by epigenetic changes in the IL-10 gene supporting its expression. Thus, one answer to the question of why NK cells proliferate when their basal frequencies are high is provided, and a previously unappreciated role for proliferation in shaping innate cell “preparedness” for particular functions is reported.

Linking proliferation to switching NK cells from inflammatory to regulatory provides a pathway for taking advantage of these high-frequency innate cells to apply negative pressure on adaptive immunity. The results suggest that the previously reported NK cell IL-10 responses observed in MCMV-infected mice (4, 12) and in hepatitis C virus–infected humans (8) as well as during mouse infections with the parasitic agent *Toxoplasma gondii* (11, 38) are delivered by cells that have proliferated. The intrinsic changes reported in this paper result in the ability of NK cells to express IL-10 in response to different stimuli [i.e., IL-21 as well as IL-12 (Figs. 4, 7)]. Endogenous IL-12 does contribute to the NK cell IL-10 response to the parasitic agent (11) but is not required for the NK cell IL-10 elicited during high-dose MCMV infection (Fig. 2D). IL-21 also is not required (Fig. 2E), and the IL-2–supported proliferation-dependent induction of NK cell responsiveness to IL-12 for IL-10 expression shows that stimulation through particular NK activating receptors is not a prerequisite (Fig. 3). Thus, once NK cells are prepared to express IL-10, they may do so in response to a variety of stimuli if any are present in the environment.

In addition to demonstrating a novel role for proliferation, the studies help explain a complicated literature on IL-12 induction of IL-10 (30, 39). The earlier work noted an IL-10 NK cell response to IL-12 in longer term cultures and/or under conditions of IL-2 exposure that support NK cell proliferation. These experiments show that low IL-2 concentrations, insufficient to support NK cell proliferation, fail to result in the development of an IL-10 response to IL-12 (Fig. 3). Furthermore, they demonstrate that unless the populations have previously divided in vivo, NK cells blocked in their ability to proliferate ex vivo fail to develop an IL-10 response to IL-12 in the presence of high IL-2 concentrations (Fig. 7). Thus, these studies establish the role of proliferation in the changing response. In addition, they provide insights into the biological role for the changing response (i.e., to use NK cells for regulatory functions under conditions of their sustained stimulation for proliferation).
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The results suggest consequences of proliferation that have been overlooked in the shaping of other cell functions. Similar to NK cells, innate monocyte/macrophage populations are at high frequencies, and macrophage proliferation has been observed in situ (40). Thus, macrophage proliferation also might promote conditioning of these cells to deliver differential effects. Dendritic cells have been reported to have epigenetic changes consistent with limiting IL-12 expression during their repopulation following sepsis (41), but the role for proliferation in the development of the cells has not been examined.

The high frequencies of NK cells constitutively found in uninfected mice are in contrast to the low frequencies of Ag-specific T cells under naive conditions. Stimulation through the TCR is required for CD4 or CD8 T cell expansion to numbers sufficient for defense. In the context of infections resulting in extensive proliferation, CD8 T cell expression of complex immune enhancing factors is narrowed, and an IL-10 response can be acquired (42–45). These shifts do parallel the NK cell changes, but the suggested roles for proliferation in regulating CD4 or CD8 T cell cytokine responses have been based on correlations of cytokine expression with dilution of a proliferation dye after stimulation through the TCR (45, 46). Because the shift to NK cell IL-12 expression is shown in this study using IL-2 to support proliferation ex vivo (Fig. 3) as well as MCMV infection to support proliferation in vivo (Fig. 4), the results document changing function in the absence of viral ligands for activating receptors. Moreover, the experiments using MMC to block division (Fig. 7) establish that the change is dependent on the proliferation event.

Cell division and differentiation are complex processes that depend on metabolic changes and accumulation of materials to complete cell division (47–49). The experiments carried out to date are only beginning to define the specific steps in the processes uniquely linked to changing function (50). In addition to demonstrating the importance for proliferation in the evolving function, however, our results document epigenetic changes in the NK cells responding to infection such that the IL-10 gene acquires histone modifications indicative of an open state for transcription and expression (Fig. 6). These data indicate that the intrinsic NK cell changes supported by the conditions of proliferation include those required for epigenetic modification of the IL-10 gene. The magnitude of the changes captured in this study is somewhat surprising and reveals previously unsuspected dynamics in genetic modification. Given the demonstration of NK cell responses through periods overlapping adaptive immunity and under “memory” conditions (51–53) and the discussion of inheritance of epigenic modifications (54), important questions about the short- and long-term consequences of the effects are suggested by the data. The fact that the shift to IL-12 responsiveness for IL-10 expression is induced in culture conditions without purposeful addition of other proinflammatory cytokines indicates that these are not required for the conditioning of intrinsic NK cell responses to induce IL-10 expression. Their roles in promoting histone modification for stable responses, however, remain to be tested. Likewise, the dissection of possible different paths to proliferation, including stimulation through the activating receptors and/or with growth factors such as IL-2, for stable genetic modification remains to be elucidated. Thus, the results indicate avenues of investigation beyond the scope of this paper.

When IL-2 was used to support proliferation in culture (Fig. 3), the proportions of Ly49H+ cells remained constant, and both Ly49H+ and Ly49H− populations acquired the ability to express IL-10. During the MCMV infection, NK cell proliferation is induced through cytokine-dependent and Ly49H-independent pathways at early times postinfection, but the proportions of Ly49H+ cells increase at later times because of stimulation resulting from expression of a viral ligand to the Ly49H receptor (2). In the experiments reported in this paper, the NK cell subsets went from 40 to 80% Ly49H+ cells during infection (Supplemental Fig. 1). This subset responded more dramatically to IL-12 stimulation for IL-10 expression (Fig. 2), but both Ly49H+ and Ly49H− populations expressing IL-10 could be identified during infection (Supplemental Fig. 1). It is not clear whether stimulation through the Ly49H receptor provided additional signals for responsiveness or simply additional support for extended proliferation. Taken together, however, the results demonstrate that an Ly49H signal is not required to drive NK cells to IL-10 expression.

In conclusion, these studies define a novel role for proliferation in shaping innate cell “preparedness” for responses and demonstrate intrinsic molecular genetic modifications accompanying the alterations in function supported by cell division. They provide
novel insights into the dynamic events controlling NK cell function as needed and suggest a more general role for proliferation in regulating functions of multiple immune cell subsets.

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Disclosures
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References


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SUPPLEMENTAL FIGURE 1. Characterization of responding NK cells and their IL-10 expression. (A-G) Mice, Wt B6, were infected with 5,000 or 70,000 PFU for the indicated times. Isolated splenic cells were evaluated by flow cytometry analysis. Subsets expressing NKp46 and Ly49H (A), NK1.1 and Ly49H (B), and CD49b and Ly49H (C) were characterized. (D) NK cells defined as CD49b+TCR-β- were examined for Ly49H expression. Data are representative of two low-dose and three high-dose infection experiments with 2-3 mice per time point (A-D). (E) Percentages of CD49b+TCR-β- NK cells and of NK cells expressing Ly49H were measured at indicated times after infection. Summary data were derived from 3 mice per time point per dose of infection (mean ± s.d.). (F) Characterization of IL-10 expressing cells after high-dose MCMV infection in B6 IL-10-GFP reporter mice. The IL-10-GFP fluorescence in NK and T cells from d 3.5 splenic leukocytes was analyzed by flow cytometry using NK and T cell markers with gating strategy shown. Results are representative of four independent experiments. Numbers in histograms are proportions of cells within indicated gate. (G) Presentation of IL-10-GFP in total viable leukocytes isolated on d 0 and d 3.5 of MCMV infection relative to indicated cell surface molecules. Representation of results in at least 3 independent experiments.