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Coordinated and Distinct Roles for IFN-$\alpha\beta$, IL-12, and IL-15 Regulation of NK Cell Responses to Viral Infection[1]

Khuong B. Nguyen,* Thais P. Salazar-Mather,* Marc Y. Dalod,* Jeffrey B. Van Deusen,† Xiao-qing Wei,‡ Foo Y. Liew,‡ Michael A. Caligiuri,† Joan E. Durbin,§ and Christine A. Biron[2]*

NK cell cytotoxicity, IFN-γ expression, proliferation, and accumulation are rapidly induced after murine CMV infections. Under these conditions, the responses were shown to be elicited in overlapping populations. Nevertheless, there were distinct signaling molecule requirements for induction of functions within the subsets. IL-12/STAT4 was critical for NK cell IFN-γ expression, whereas IFN-$\alpha\beta$/STAT1 were required for induction of cytotoxicity. The accumulation/survival of proliferating NK cells was STAT4-independent but required IFN-$\alpha\beta$/STAT1 induction of IL-15. Taken together, the results define the coordinated interactions between the cytokines IFN-$\alpha\beta$, IL-12, and IL-15 for activation of protective NK cell responses during viral infections, and emphasize these factors' nonredundant functions under in vivo physiological conditions. ***The Journal of Immunology,*** **2002, 169: 4279–4287.**

Natural killer cells are activated and have critical roles in defense during viral infections (1). In the case of challenges with murine CMV (MCMV),[3] NK cells respond within the first 2 days of infection to mediate cytotoxicity, produce IFN-γ, proliferate, and accumulate (1–4). Under these conditions, the innate cytokines IFN-$\alpha\beta$, i.e., type 1 IFNs, and IL-12 are induced early after infection and are critical for stimulation of particular NK cell functions, with IFN-$\alpha\beta$ promoting cytotoxicity and proliferation, and IL-12 eliciting IFN-γ production (1–3). Hence, at early times after MCMV infection, NK cell responses are controlled by innate cytokines and the factors IFN-$\alpha\beta$ and IL-12 are nonredundant endogenous regulators of these.

Although the critical roles for the cytokines in activating NK cell responses have been defined, several important questions remain unanswered. First, the lack of functional overlap between these factors for activation of NK cells raises questions about the endogenous regulation pathways. IFN-$\alpha\beta$ and IL-12 may be accessing different NK cell subsets, or activating the same subset of cells through nonoverlapping intracellular signals. Available biochemical data suggest potential overlapping spectra of transcription factors. IFN-$\alpha\beta$ and IL-12 both can activate STAT1 (5–9). STAT4 is activated by IL-12 functions in both humans and mice (5–8), and by IFN-$\alpha\beta$ in humans (5, 10, 11). Hence, under certain conditions, both STAT1 and STAT4 can be stimulated by type 1 IFNs and IL-12. In addition, recent data indicated that both cytokine classes may have STAT-independent signaling pathways (12, 13). Thus, while it is clear that IFN-$\alpha\beta$ and IL-12 are crucial for induction of NK cell responses during MCMV infection, mechanistic details regarding: 1) whether the factors act on different or the same cell NK cell populations, and 2) the intracellular signaling mechanisms by which these cytokines exert their effects in vivo, remain poorly characterized.

In addition to the undefined signaling mechanisms for cytokine-mediated effects, it is uncertain how IFN-$\alpha\beta$ promote cell proliferation in vivo. These cytokines are not likely to stimulate NK cell proliferation directly, as they fail to elicit NK cell proliferation in vitro and exert anti-proliferative effects at high concentrations (14, 15). Thus, IFN-$\alpha\beta$-mediated promotion of this response may depend on stimulation of secondary cytokine(s) in vivo. One candidate is IL-15 (16, 17). This cytokine can be a potent regulator of NK cell differentiation, proliferation, and survival, and an accessory factor for NK cell IFN-γ (16–23). However, little is known about the expression and function of IL-15 in viral infections. The nonlymphoid cell-derived factor shares many characteristics with the T cell-produced IL-2, and type 1 IFNs can induce IL-15 mRNA expression in vitro (24). IL-15 binds to a receptor complex consisting of the common γ-chain (CD132), the IL-2/IL-15Rβ subunit (CD122), and a unique high affinity chain designated IL-15Rα (16, 17). The IFN-$\alpha\beta$ to IL-15 axis for induction of cell proliferation has been hypothesized as the mechanism for IFN-dependent stimulation of memory CD4 and CD8 T cell proliferation (24). However, definitive demonstration for such a pathway remains elusive. Thus, it is unclear how IFN-$\alpha\beta$ can elicit NK cell proliferation in vivo but not in vitro, and although IL-15 is a possible intermediary, its expression and function during viral infections are largely uncharacterized.

These studies defined the IFN-$\alpha\beta$ and IL-12-mediated immunoregulatory effects on NK cells during viral infections, and delineated the signaling molecules requisite for transduction of these responses. In addition, the role of IL-15 in promoting NK cell responses during viral infections was defined. STAT1, but not STAT4, was required for IFN-$\alpha\beta$-mediated activation of NK cell cytotoxicity. In contrast, STAT4, but not STAT1, was essential for IL-12-dependent NK cell IFN-γ production. STAT1, but not STAT4, was required for the accumulation of proliferating NK

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[3] Abbreviations used in this paper: MCMV, murine CMV; BrdU, 5-bromo-2′-deoxyuridine; LU, lytic unit.

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cells. IL-15 mRNA expression was induced under conditions of IFN-$\alpha\beta$ exposure in vivo, and the lack of NK cell accumulation in the absence of STAT1 was associated with failure to induce IL-15. Finally, NK cell accumulation in response to type 1 IFNs was dramatically reduced in the absence of endogenous IL-15 function. Taken together, these results delineate the in vivo STAT-mediated pathways requisite for activation of specific NK cell functions and reveal unexpected mechanisms for IFN-$\alpha\beta$-induced functions in vivo. In addition, these studies definitively establish for the first time a distinct mechanism for IFN-$\alpha\beta$ support of NK cell proliferation in vivo.

Materials and Methods

Mice

Specific pathogen-free male immunocompetent C57BL/6 and 129SvEv mice were purchased from Taconic Farms (Germantown, NY). Male C57BL/6J, 129 × C57BL/6 F_2 (129B6 F_2), endotoxin-resistant C3H/HeJ, and IL-12 p40$^-$ (25) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). STAT1$^-$ (26) and IL-12 p35$^-$ (27) animals were backcrossed for at least five generations onto the C57BL/6 background before use in experiments. STAT4$^-$ breeding pairs (28), maintained on a mixed 129 × C57BL/6 background, were from Dr. J. Ihle (St. Jude's Children's Hospital, Memphis, TN). 129Sv-IFN-$\alpha\beta$R$^-$ (29) breeding pairs were purchased from B&K Universal (North Humberside, U.K.). All mice used in experiments were 5–12 wk in age. Animals obtained outside of Brown University were housed for at least 1 wk before use. Handling of mice and experimental procedures were in accordance with institutional guidelines for animal care and use.

Viral infections and in vivo cytokine treatments

All experiments were initiated on day 0. For viral infections, mice were either not infected or infected i.p. as described (3, 4, 27, 30–32) with 1.0×10^4 PFU MCMV Smith strain. There were no appreciable effects on the characteristics of NK cell responses induced following infection with different batches of virus, but there were differences in the levels of serum cytokines induced. IL-12 and IFN-$\alpha\beta$ effects on MCMV replication in the spleen have been reported previously (3). After 1.5 days of MCMV infection, STAT1$^-$ spleens had 4.8 ± 0.0 log PFU/g virus, compared with 4.1 ± 0.1 log PFU/g in STAT1$^+$ mice. There were, respectively, 4.7 ± 0.3 and 4.9 ± 0.1 log PFU/g virus in STAT4$^+$ and STAT4$^-$ mice. For in vivo cytokine stimulation, mice were i.p. administered recombinant murine IL-12 (Genetics Institute, Andover, MA), recombinant simian IL-15 (Immunex, Seattle, WA), or recombinant universal IFN-α (Hoffman-LaRoche, Nutley, NJ). Mice received a total of 2 μg of rIL-12 (sp. act. 4.6×10^6 U/mg; 1 μg/day for 2 days), 12 μg of rIL-15 (sp. act. 3.5×10^8 U/mg; 4 μg/day for 3 days), or 3×105 U of rIFN-α (sp. act. 6×10^6 U/mg; 10^5/day for 3 days). Control vehicle treatments were conducted similarly. All experiments were evaluated at 1.5 days after MCMV infection, 2 days following IL-12, and 3 days following rIFN-α or IL-15 administration.

In vivo neutralization of IL-15 functions

IL-15 functions were neutralized in vivo by either blocking the IL-2/IL-15Rβ subunit or by treatment with soluble IL-15 (sIL-15)Rα (33, 34). F(ab)′ of anti-IL-2/IL-15Rβ mAb clone TM-β1 were used and F(ab)′ of clone CRL-8164 were used as controls. F(ab)′ preparation from hybridoma-purified mAb was performed as previously described (35, 36). Briefly, Abs were enzymatically cleaved with mercuripapain (Sigma-Aldrich, St. Louis, MO) in a buffered solution containing 20 mM sodium phosphate, 10 mM cysteine, and 2 mM EDTA. Reactions were stopped by addition of iodoacetamide (Sigma-Aldrich), followed by dialysis, and sterile filtration. For MCMV infection, mice were given 0.5-mg doses F(ab)′ at 2 h before, and 18 h after, viral inoculation. For experiments with rIFN-α administration, F(ab)′ was given to mice on days 0 (1.0 mg), 1 (0.5 mg), and 2 (0.5 mg) relative to the initial injection of recombinant cytokine. Under conditions of treatment, NK cells were still present in spleens and livers of anti-IL-2/IL-15Rβ-treated mice, and anti-receptor F(ab)′ appeared to still bind to NK cells (data not shown). Recombinant sIL-15Rα (34), binding with high affinity to IL-15 but not to IL-2, and a mutated recombinant sIL-15R, designated M4 (33), no longer binding to IL-15, were purified and analyzed by SDS-PAGE and Western blot as described. LPS was not detectable by the Amebocyte *Limulus* test. These reagents were administered with schedules similar to the F(ab)′ treatments but at 400 μg per injection. This dose was established in preliminary experiments as the minimum necessary to observe IL-15 blockade effects following MCMV infection.

Serum preparation and organ collection

Animals were anesthetized at the indicated times, and blood was collected via the retroorbital route into low amounts of heparin. Following centrifugation, supernatants were collected and identified as sera. Mice were sacrificed, and spleens and livers were harvested and maintained at 4°C.

Preparation of cells

Splenic and liver leukocytes were obtained using previously described protocols (3, 4, 27, 30–32, 37, 38). Viable cell yields were determined by trypan blue exclusion. In certain studies, NK cells were enriched by negative magnetic selection as per manufacturer protocol (StemCell Technologies, Vancouver, British Columbia, Canada). Enrichments yielded preparations containing ~50% NK cells, as determined by flow cytometric analyses.

Flow cytometric analyses

Studies evaluating spontaneous ex vivo intracellular expression of IFN-γ were performed using previously described protocols (4, 27, 30, 38). Briefly, cells were fluorescently labeled with the surface markers NK1.1 or DX5 and CyChrome-conjugated anti-CD3ϵ, washed, fixed, permeabilized, and incubated with allophycocyanin-conjugated anti-IFN-γ mAb clone XMG1.2. Specificity for IFN-γ staining was demonstrated by lack of labeling with isotype-matched Ab or by coincubating samples with ascite-purified unconjugated XMG1.2. All directly conjugated mAb and streptavidin-allophycocyanin were purchased from BD PharMingen (San Diego, CA). Intracellular perforin expression was evaluated using protocols similar to those for IFN-γ, using purified anti-perforin mAb clone KM585 (Kamiya Biomedical, Seattle, WA), followed by PE-conjugated polyclonal rabbit anti-rat Abs (BD PharMingen). A rat mAb with the same isotype as the perforin mAb was used for controls. To determine levels of perforin expression, NK cells were gated and evaluated for proportions of cells expressing the molecule. Under these conditions, there was no staining with isotype-control mAbs. Staining conditions were established based upon published protocols (39). Studies evaluating 5-bromo-2′-deoxyuridine (BrdU) incorporation by NK cells after in vivo labeling were adapted from previously described methods (24). Briefly, mice were given drinking water containing BrdU (Sigma-Aldrich) at 0.8 mg/ml starting on day 0 relative to viral infection or cytokine treatment and sacrificed on the indicated days thereafter for preparation of splenic leukocytes. Isolated cells were labeled with NK1.1 or DX5 and CD3, washed, fixed with ethyl alcohol and paraformaldehyde containing Tween 20, and stained with anti-BrdU mAb clone 3D4 or isotype-matched control Abs (BD PharMingen). Samples were acquired using a FACSCalibur (BD Biosciences, Mountain View, CA), with the CellQuest version 3.1 software package. Laser outputs were 15 mW at 488- and 635-nm wavelengths. At least 100,000 events were collected for analyses. In experiments where NK cells were quantified, proportions of NK cells, which were identified as NK1.1$^+$CD3$^-$ in C57BL/6 strains and DX5$^+$CD3$^-$ in 129 and C3H strains of mice, were first calculated. This proportion was then used to calculate the total number of NK cells in the spleen based on total cell yields. Numbers of live spleen cells were quantitated after trypan blue exclusion of dead cells.

Serum cytokine measurements

Serum IFN-γ and IL-12 p40 levels were determined by standard sandwich ELISA as was previously described (3, 4, 27, 30–32, 37, 38). IL-20 p70 ELISA was done according to manufacturer protocol (R&D Systems, Minneapolis, MN). Sandwich ELISA for IL-15 was conducted using recombinant sIL-15Rα as the primary and polyclonal rabbit anti-murine IL-15 as the secondary Ab. Recombinant murine IL-15 was used for cytokine standards. IL-15 and polyclonal anti-IL-15 were provided by Immunex. For ELISAs, limits of detection varied between experiments but always were <20 pg/ml. Colorimetric changes of enzyme substrates were detected at 405- or 450-nm wavelengths using a SpectraMax 250 reader (Molecular Devices, Sunnyvale, CA). IFN-$\alpha\beta$ bioassays were conducted as described (27).

Cytotoxicity assays

Cytotoxicity was measured as release of radioactive isotope from YAC-1 target cells labeled with sodium chromate (^{51}Cr) following incubation with unlabeled effector splenic leukocytes, as described (3, 14). Spontaneous release was <15% of maximum release. Percent specific lysis was calculated as 100 × (cpm test sample − cpm spontaneous release)/(cpm total release − cpm spontaneous release). Consistent with previous studies demonstrating the role of IL-15 for NK cell development (22, 23), treatments to block endogenous IL-15 resulted in decreases in NK cell proportions, both in infected and uninfected mice. Hence, cytotoxicity was evaluated after specific lysis was normalized to the proportions of NK cells. Under

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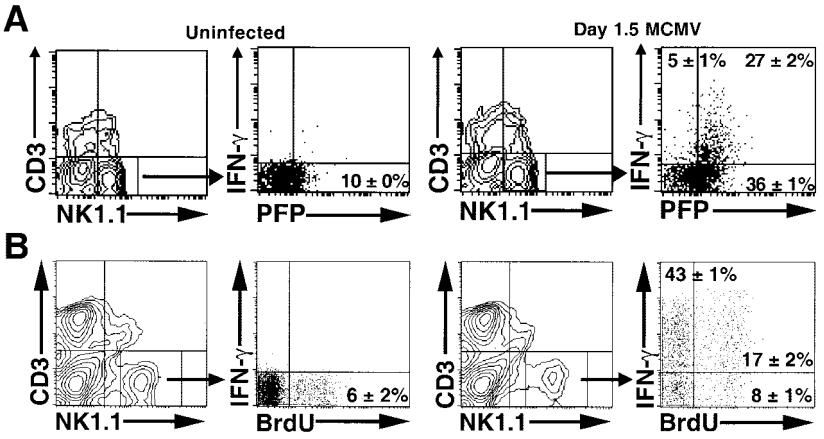

FIGURE 1. Detection of perforin, IFN-γ, and BrdU within NK cells. C57BL/6 mice were uninfected or infected i.p. for 1.5 days with 1×10^4 PFU MCMV, and given BrdU-containing drinking water. Splenic NK cell responses were evaluated. *A*, Enriched NK cells from uninfected and MCMV-infected mice were examined for perforin and IFN-γ expression by flow cytometry. Similar results were obtained with unenriched NK cells (data not shown). *B*, Unenriched NK cells from uninfected or MCMV-infected mice were examined for intracellular IFN-γ expression and BrdU incorporation by flow cytometry. In *A* and *B*, NK cells are defined as $NK1.1^+CD3^-$. In *A*, perforin (log *x*-axes) and IFN-γ (log *y*-axes) were evaluated after gating on NK cells. Plots present events from enriched populations. As these were pooled samples, numbers given within plots are percentages of cells from unenriched cells of three replicate individual mice. In *B*, IFN-γ (log *y*-axes) and BrdU (log *x*-axes) were evaluated after gating on NK cells. Numbers within plots are averages of proportions of cells from three replicate mice. Results are representative of at least two independent experiments.

these conditions, the 100:1 E:T ratio data were used to calculate lytic units, and the lytic units per spleen were compared between different treatment groups.

Semiquantitative RT-PCR analysis for IL-15 expression

Total spleen and bone marrow RNA samples were prepared using the RNAWiz isolation kit (Ambion, Austin, TX). Relative quantitative RT-PCR was performed as reported (32). Briefly, 1 μg samples of total RNA were reversed-transcribed into cDNA, and cDNA samples were then used as templates for PCR amplification using primers specific for IL-15 (24). To control for sample preparation, gel loading, and random variations in RT-PCR, 18S rRNA primers, and 18S rRNA Competimers (Ambion), used to modify 18S cDNA amplification efficiency, were also included in each PCR. Amplifications were conducted in the programmable thermocycler PTC-100 (MJ Research, Waltham, MA) using previously reported parameters (24). IL-15 primer sequences were synthesized by Operon Technologies (Alameda, CA). Products were separated on 1.8% agarose gels and visualized by ethidium bromide staining. IL-15 sequence specificity was confirmed following RT-PCR amplification by hybridizing with an internal oligonucleotide probe.

Results

Induction of NK cell cytotoxicity, IFN-γ expression, and proliferation

After 1.5 days of MCMV infection, NK cells were induced to mediate cytotoxicity, synthesize IFN-γ, and proliferate (Fig. 1, Tables 1 and 2) (1–3). To examine whether the functions could occur within the same NK cell, intracellular flow cytometric analyses were performed for expression of perforin, a cytotoxic effector molecule, IFN-γ, and incorporation of BrdU, a synthetic nucleic analog used to evaluate DNA synthesis, in uninfected mice or those infected for 1.5 days with MCMV. BrdU was supplied continuously in the drinking water during the experimental period. As shown in Fig. 1, very low proportions of splenic NK cells from uninfected mice were expressing perforin or incorporating BrdU, and IFN-γ was undetectable. After 1.5 days of infection, ~60% of NK cells were induced to express perforin, 30–60% to express IFN-γ, and 25% had incorporated BrdU. Simultaneous perforin vs IFN-γ detection revealed that virtually all IFN-γ-expressing cells also were positive for perforin (Fig. 1*A*). Similarly, ~75% of BrdU incorporating NK cells were synthesizing IFN-γ (Fig. 1*B*). Hence, there was large overlap for induction of responses within NK cells following MCMV infection, and at least 20% of NK cells are estimated to have all three functions after infection. Thus, individual NK cells were responding in multiple ways.

Cytokine and STAT requirements for induction of NK cell cytotoxicity vs IFN-γ expression

Confirming earlier studies with Ab neutralization (3), mice blocked in IFN-αβ function by genetic mutation of IFN-αβR and infected for 1.5 days with MCMV were profoundly inhibited in NK cell cytotoxicity (Table I) but not IFN-γ production (Table II). In contrast, infected mice blocked in IL-12 function as a result of

Table I. *Cytokine and STAT requirements for induction of NK cell cytotoxicity following MCMV infection*[a]

Strain	Treatment	% Specific Lysis (± SEM)[b]
129	Uninfected	1 ± 0
129	MCMV	30 ± 5
129 IFN-αβR⁻	Uninfected	1 ± 1
129 IFN-αβR⁻	MCMV	3 ± 0*
C57BL/6	Uninfected	3 ± 1
C57BL/6	MCMV	13 ± 5
C57BL/6 IL-12 p35⁻	Uninfected	1 ± 1
C57BL/6 IL-12 p35⁻	MCMV	17 ± 7
C57BL/6	Uninfected	7 ± 0
C57BL/6	MCMV	28 ± 8
C57BL/6 STAT1⁻	Uninfected	1 ± 1
C57BL/6 STAT1⁻	MCMV	8 ± 3*
129B6 F_2	Uninfected	4 ± 1
129B6 F_2	MCMV	15 ± 3
129B6 STAT4⁻	Uninfected	4 ± 1
129B6 STAT4⁻	MCMV	27 ± 3

[a] Mouse strains indicated above were infected with MCMV for 1.5 days or left uninfected; splenic leukocytes were obtained and NK cell-dependent cytotoxicities were assayed as indicated in *Materials and Methods.* Three to four mice per group were used in experiments.

[b] Specific lysis was determined as described in *Materials and Methods.* The 100:1 E:T cell ratios (± SEM) are shown above, although ratios of 33:1, 11:1, 3:1, and 1:1 also were performed in experiments.

*, $p < 0.02$ compared to infected immunocompetent mice.

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Table II. *Cytokine and STAT requirements for induction of NK cell IFN-γ expression following MCMV infection*[a]

Strain	Treatment	Serum IFN-γ (pg/ml)	% IFN-γ + NK Cells	No. IFN-γ + NK Cells (× 10^3)
129	Uninfected	BLD	BLD	BLD
129	MCMV	2249 ± 353	17 ± 1	200 ± 30
129 IFN-$\alpha\beta$R$^-$	Uninfected	BLD	BLD	BLD
129 IFN-$\alpha\beta$R$^-$	MCMV	1895 ± 176	14 ± 3	220 ± 50
C57BL/6	Uninfected	BLD	BLD	BLD
C57BL/6	MCMV	4086 ± 188	24 ± 5	220 ± 40
C57BL/6 IL-12 p35$^-$	Uninfected	BLD	BLD	BLD
C57BL/6 IL-12 p35$^-$	MCMV	251 ± 28**	5 ± 1**	50 ± 20**
C57BL/6	Uninfected	BLD	BLD	BLD
C57BL/6	MCMV	576 ± 330	30 ± 2	230 ± 10
C57BL/6 STAT1$^-$	Uninfected	BLD	BLD	BLD
C57BL/6 STAT1$^-$	MCMV	5661 ± 501	52 ± 3	270 ± 60
129B6 F_2	Uninfected	BLD	BLD	BLD
129B6 F_2	MCMV	2578 ± 795	48 ± 6	200 ± 30
129B6 STAT4$^-$	Uninfected	BLD	BLD	BLD
129B6 STAT4$^-$	MCMV	BLD**	17 ± 3**	60 ± 50*

[a] Mouse strains were infected with MCMV for 1.5 days or left uninfected, and serum IFN-γ measured by ELISA. Splenic leukocytes were obtained, and NK cell IFN-γ expression was assayed as indicated in *Materials and Methods*. Three to four mice per group were used in experiments. Numbers shown are means ± SEM. ELISA limits of detection varied from experiment to experiment but were between 10 and 20 pg/ml. Flow cytometric limit of detection for specific IFN-γ expression within NK cells was 2%. BLD, Below limit of deletion.

*, $p < 0.05$ compared to infected immunocompetent animals.

**, $p < 0.02$ compared to infected immunocompetent animals.

genetic mutation of the p35 (Table II) or the p40 (data not shown) subunit of IL-12 were inhibited in IFN-γ production (Table II) but not cytotoxicity (Table I). Differences in responses could not be accounted for by changes in proportions or total numbers of NK cells.

To further define the molecular bases for IFN-$\alpha\beta$- and IL-12-mediated immunoregulatory functions, STAT requirements for IFN-$\alpha\beta$- or IL-12-dependent induction of specific NK cell responses were evaluated. NK cell cytotoxicity was comparable in MCMV-infected, STAT4$^-$, compared with immunocompetent, mice after 1.5 days of MCMV infection (Table I). In contrast, cytotoxicity from infected STAT1$^-$ mice was >70% reduced (Table I). The reductions could not be accounted for by differences in the proportions of NK cells in STAT1$^-$ mice (data not shown). Likewise, decreased cytotoxicity was not due to reduced IFN-$\alpha\beta$ expression; both mouse strains had similar levels of serum IFN-$\alpha\beta$ at this time after infection (data not shown). However, IL-12 induction of NK cell IFN-γ required STAT4 but not STAT1. Systemic IFN-γ levels in STAT4$^+$ mice were ~3000 pg/ml, whereas the factor was undetectable in sera of infected STAT4$^-$ animals (Table II). Proportions and numbers of spleen NK cells expressing intracellular IFN-γ in infected STAT4$^-$ mice decreased >60% compared with infected STAT4$^+$ animals (Table II). Under these conditions, STAT4$^-$ had equivalent levels of IL-12 p40 and p70 to STAT4$^+$ mice (data not shown). In contrast, systemic IFN-γ levels were dramatically elevated in infected STAT1$^-$ mice; infected STAT1$^-$ mice had at least 6-fold higher serum IFN-γ compared with STAT1$^+$ animals (Table II). After infection, >50% of NK cells from STAT1$^-$ animals expressed intracellular IFN-γ, compared with 30% of those from STAT1$^+$ mice (Table II). The elevated IFN-γ production under these conditions was associated with increased IL-12 production; STAT1$^-$ animals had more than five times more systemic IL-12 p40 levels compared with STAT1$^+$ mice (data not shown). Taken together, the results demonstrate that NK cell cytotoxicity is dependent on the IFN-$\alpha\beta$/STAT1 pathway, whereas IFN-γ expression requires IL-12/STAT4.

Cytokine and STAT requirements for NK cell proliferation and accumulation

Parameters examined were extended to include requirements for induction of NK cell proliferation by incorporation of BrdU during in vivo labeling. MCMV infection induced dramatic accumulation of NK cells labeled with BrdU (Fig. 2). Under these conditions, biologically active IL-12 was not required for BrdU incorporation (Fig. 2*A*). By comparison, MCMV-infected IFN-$\alpha\beta$R$^-$ animals were significantly reduced in the proportions of BrdU$^+$ cells (Fig. 2*B*). Although percentages and yields of total NK cells were either not reduced or reduced by no more than 50% (yields of 410 × 10^3 (±50) vs 240 × 10^3 (±20) per spleen), numbers of BrdU$^+$ NK cells were decreased by 60–70% as a result of blocking IFN-$\alpha\beta$ functions during infection. There were 90 × 10^3 (±10) NK cells incorporating BrdU in IFN-$\alpha\beta$R$^+$ but only 30 × 10^3 (±10) in IFN-$\alpha\beta$R$^-$ mice. Because activated NK cells are induced to migrate to livers (31, 32), yields from this tissue also were examined. Similar to spleen, liver NK cell numbers were higher in infected IFN-$\alpha\beta$R$^+$ as compared with IFN-$\alpha\beta$R$^-$ mice (230 × 10^3 vs 154 × 10^3 per liver). Thus, during MCMV infections, biologically active IL-12 is dispensable, but IFN-$\alpha\beta$R functions are necessary for accumulation and induction of NK cell proliferation.

STAT4 was not required for the IFN-$\alpha\beta$-dependent stimulation of proliferating NK cell accumulation. STAT4$^+$ and STAT4$^-$ mice had similar proportions and numbers of BrdU$^+$ splenic NK cells (Fig. 2*C*). In contrast, BrdU$^+$ NK cell accumulation was severely impaired in STAT1-deficient mice (Fig. 2*D*). In several experiments totaling >12 mice per group, proportions of BrdU$^+$ NK cells in MCMV-infected STAT1$^-$ mice consistently did not rise above the basal accumulation seen in uninfected mice, in contrast to the >300% increase in the proportions and numbers of BrdU$^+$ NK cells seen in infected STAT1$^+$ animals (Fig. 2*D* and data not shown). Thus, IL-12- and STAT4-independent accumulation of BrdU$^+$, i.e., proliferating NK cells after MCMV infection, requires

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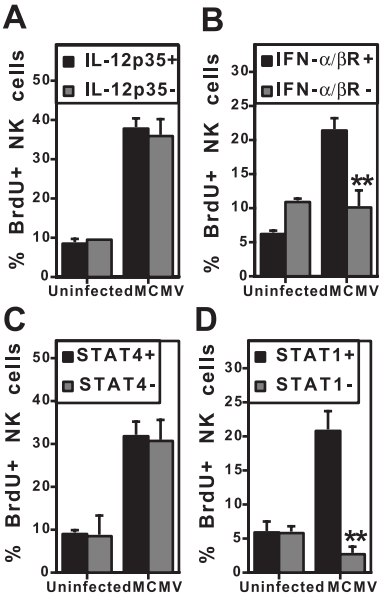

FIGURE 2. Cytokine and STAT requirements for induction of NK cell proliferation. 129SvEv, 129SvEV IFN-$\alpha\beta$R$^-$, C57BL/6 IL-12 p35$^-$, 129B6 F_2, 129B6 STAT4$^-$, C57BL/6, and C57BL/6 STAT1$^-$ mice were treated as described in Fig. 1. Accumulation of proliferating (BrdU$^+$) NK cells after continuous labeling during periods of or equivalent to infection within gated DX5$^+$CD3$^-$ (*B*) or NK1.1$^+$CD3$^-$ (*A*, *C*, and *D*) populations. C57BL/6 vs C57BL/6 IL-12 p35$^-$ are shown in *A*; 129SvEV vs 129SvEV IFN-$\alpha\beta$R$^-$ are shown in *B*; 129B6 F_2 vs 129B6 STAT4$^-$ in *C*; and C57BL/6 vs C57BL/6 STAT1$^-$ in *D*. In *A–D*, shown are averages ± SEM of three to five mice per group. **, $p < 0.02$ compared with immunocompetent mice.

IFN-$\alpha\beta$ and STAT1 functions. Taken together, the experiments showed that under conditions of a mixed cytokine milieu, IFN-$\alpha\beta$-dependent accumulation of proliferating NK cells requires STAT1 but not STAT4.

STAT requirements for induction of NK cell responses after IL-12 or type 1 IFN administration

To directly examine the in vivo pathways activated by individual cytokines, as well as to move to nonviral conditions and thus limit potential secondary effects resulting from changes in inducing cytokine levels or viral burdens, studies were conducted examining in vivo responses to administered recombinant cytokine. The IL-12 induction of NK cell IFN-γ expression also required only STAT4 and not STAT1, and rIFN-α effects on NK cells in vivo did not require STAT4 (data not shown). However, similar to MCMV infection, STAT1 was necessary for rIFN-α-mediated induction of NK cell cytotoxicity (Fig. 3*A*). There were also reductions in the accumulation of BrdU$^+$ NK cells following rIFN-α administration in STAT1$^-$ mice (Fig. 3*B*). Total spleen and liver NK cell yields in IFN-treated STAT1$^-$ mice reflected the impaired ability of the proliferating NK cells to accumulate; there were 2- and 6-fold increases in NK cells, respectively, in spleens and livers after rIFN-α treatment of immunocompetent mice, contrasted to more modest increases in spleens and no dramatic increases in livers of STAT1$^-$ mice (Fig. 3, *C* and *D*). Thus, STAT1 is requisite for IFN-$\alpha\beta$-mediated induction of NK cell cytotoxicity and accumulation of proliferating cells.

IFN-$\alpha\beta$-mediated, STAT1-dependent, induction of IL-15 expression in vivo

The impaired ability of proliferating NK cells from STAT1$^-$ mice to accumulate in vivo may be due to absence of factors that can promote NK cell survival, such as IL-15. To evaluate whether IL-15 was induced during MCMV infection or after rIFN-α exposure and to define requirements for expression, sera as well as spleen and bone marrow leukocyte-conditioned media were evaluated for IL-15 expression by ELISA. IL-15 protein was not detectable in any of the samples. Total RNA samples also were prepared from spleen and bone marrow cells and evaluated in semiquantitative RT-PCRs for IL-15 induction. The conditions of infection as well as rIFN-α treatment both induced elevated levels of IL-15 mRNA (Fig. 4). Increases were more readily detectable in spleen samples from infected, and in bone marrow samples from rIFN-α-treated, mice. Endogenous IFN-$\alpha\beta$ function was critical for induction of IL-15 mRNA during MCMV infection because it was not detected in samples from IFN-$\alpha\beta$R$^-$ mice (Fig. 4*A*). Samples from STAT1$^-$ mice demonstrated that STAT1 also was required for induced IL-15 expression after viral infection (Fig. 4*B*) and after rIFN-α treatment (Fig. 4*C*). Thus, IFN-$\alpha\beta$, through

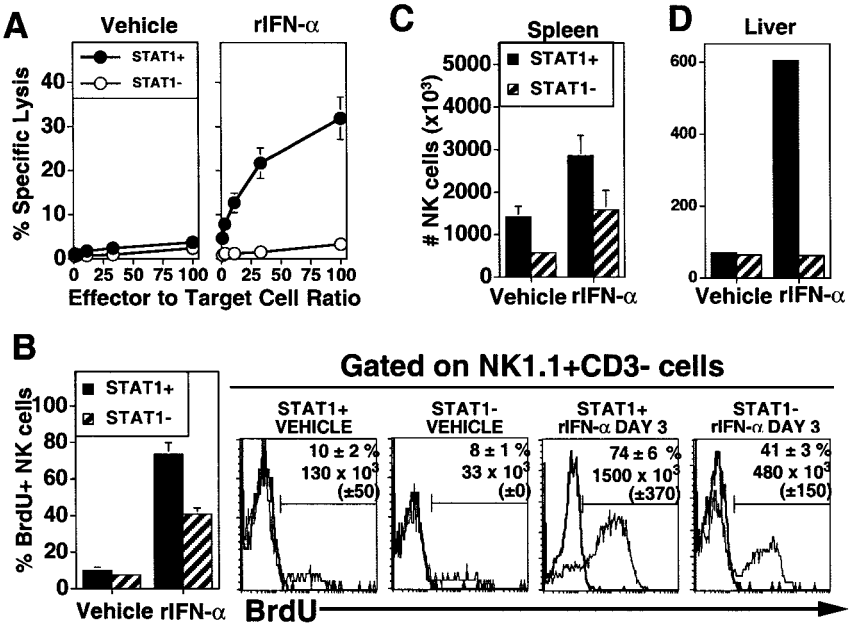

FIGURE 3. STAT1 requirements for rIFN-α-mediated induction of NK cell cytotoxicity and proliferation in vivo. C57BL/6 and C57BL/6 STAT1$^-$ mice were treated with i.p. injections of 1×10^5 U of rIFN-α or vehicle on days 0, 1, and 2 and given BrdU-containing drinking water at time 0. Responses were evaluated on day 3. *A*, Analysis of NK cell cytotoxicity. *B*, Accumulation of proliferating NK cells. Histograms shown are representative of BrdU labeling within gated NK1.1$^+$CD3$^-$ splenic leukocytes, with shaded histograms representing BrdU labeling and line histograms representing the corresponding isotype-matched mAb control. Numbers given in histograms are proportions and yields of proliferating NK cells. *C* and *D*, Enumeration of total numbers of NK cells in spleens (*C*) and livers (*D*) of untreated or rIFN-α-treated wild-type or STAT1-deficient mice. In all panels, shown are averages ± SEM of three mice per group.

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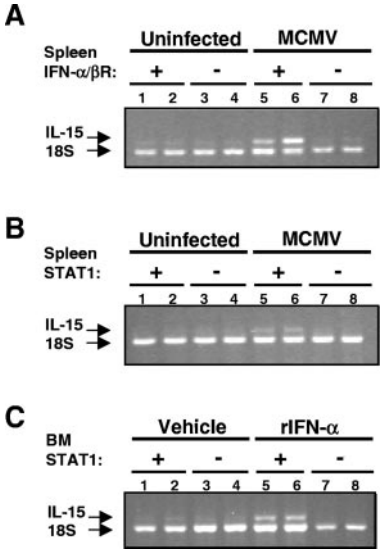

FIGURE 4. IFN-αβR and STAT1 requirements for induction of IL-15 mRNA in vivo. 129SvEv, 129SvEV IFN-αβR$^-$, C57BL/6, and C57BL/6 STAT1$^-$ mice were uninfected or infected i.p. with 1×10^4 PFU MCMV for 1.5 days (*A* and *B*). A separate group of C57BL/6 and C57BL/6 STAT1$^-$ mice also were given three daily doses of rIFN-α or vehicle on days 0, 1, and 2 (harvest on day 3) (*C*). Following treatment, spleens and bone marrow cells were obtained and total RNA was prepared for IL-15 mRNA evaluation by semiquantitative RT-PCR. *A*, Evaluation of IFN-αβR$^-$ requirements for splenic induction of IL-15 mRNA following MCMV infection. IL-15 and 18S ribosomal rRNA are shown as indicated. Samples in *lanes 1* and *2* are from uninfected 129SvEv; *lanes 3* and *4* uninfected IFN-αβR$^-$; *lanes 5* and *6* MCMV-infected 129SvEv; and *lanes 7* and *8* MCMV-infected IFN-αβR$^-$ mice. *B*, Evaluation of STAT1 requirements for splenic induction of IL-15 mRNA following MCMV infection. IL-15 and 18S ribosomal rRNA are shown as indicated. Samples in *lanes 1* and *2* are from uninfected C57BL/6; *lanes 3* and *4* uninfected STAT1$^-$; *lanes 5* and *6* MCMV-infected C57BL/6; and *lanes 7* and *8* MCMV-infected STAT1$^-$ mice. *C*, STAT1 requirements for rIFN-α induction of IL-15 mRNA in the bone marrow. Samples in *lanes 1* and *2* are from vehicle-treated C57BL/6; *lanes 3* and *4* vehicle-treated STAT1$^-$; *lanes 5* and *6* rIFN-α-treated C57BL/6; and *lanes 7* and *8* rIFN-α-treated STAT1$^-$ mice.

STAT1, mediates enhanced expression of the NK cell survival cytokine IL-15.

IL-15 requirements for NK cell responses in vivo

IL-15 effects and requirements for NK cell responses were evaluated in vivo. Under the conditions examined, NK cell cytotoxicity and serum IFN-γ production were not elicited to detectable levels following rIL-15 administration (data not shown), but the factor readily induced splenic NK cells to incorporate BrdU (Table III). The responses were observed across several different strains (Table III and data not shown). Concordantly, total NK cell numbers increased in both spleens (Table III) and livers (data not shown) of rIL15-treated mice. The effects on NK cells did not require IFN-αβ, as IL-15 stimulated NK cells in IFN-αβR$^-$ mice to incorporate BrdU and to accumulate to levels similar to those from IFN-αβR$^+$ animals (Table III). IL-15 treatments of STAT1-deficient mice also induced NK cell proliferation (data not shown). Thus, IL-15, independent of IFN-αβ and STAT1, had potent effects in inducing NK cell proliferation and accumulation.

To examine the role of endogenous IL-15 in promoting NK cell responses following MCMV infections or rIFN-α administration, endogenous IL-15 functions were neutralized by treatment with αIL-2/IL-15Rβ mAb F(ab)′ to block the receptor or by treatments with recombinant sIL-15Rα to block the factor. Blocking endogenous IL-15 functions with either αIL-2/IL-15Rβ F(ab)′ or sIL-15Rα did not inhibit the IFN-αβ-dependent induction of NK cell cytotoxicity or the IL-12-driven NK cell IFN-γ expression (data not shown). In contrast, IL-2/IL-15Rβ blockade had dramatic effects on the accumulation of proliferating NK cells, both in proportions (Fig. 5*A*) and numbers (Fig. 5*B*). Because IL-2/IL-15Rβ F(ab)′ treatment could block the functions of other cytokines, sIL-15Rα was a more specific inhibitor of endogenous IL-15. Similar to IL-2/IL-15Rβ, sIL-15Rα treatment dramatically reduced both proportions (Fig. 5*C*) and numbers (Fig. 5*D*) of NK cells incorporating BrdU. Administration of rIFN-α yielded responses similar to those observed after MCMV infection (Fig. 5, *E* and *F*), except that NK cell IFN-γ production was not observed (data not shown). Anti-IL-2/IL-15Rβ treatment during rIFN-α treatment also blocked accumulation of proliferating NK cells (Fig. 5, *E* and F). Interestingly, IL-15 blockade, especially after treatment with anti-IL-2/IL-15Rβ, also decreased NK cell proportions in uninfected mice (data not shown). Taken together, the data demonstrated that IL-15 and its downstream effects are induced by type 1 IFNs, and that although it is not critical for induction of NK cell cytotoxicity or IFN-γ expression, the cytokine is required for the maintenance and/or accumulation of proliferating NK cells.

Table III. *IFN-αβ-independent IL-15-mediated activation of NK cell responses in vivo*[a]

Strain	Treatment	% BrdU$^+$	No. of NK Cells ($\times 10^4$) Spleen
C57BL/6	Vehicle	13 ± 2	167 ± 10
C57BL/6	rIL-15	44 ± 3	261 ± 34
129 SvEv	Vehicle	16 ± 3	80 ± 11
129 SvEv	rIL-15	45 ± 4	208 ± 32
129 IFN-αβR$^-$	Vehicle	14 ± 1	44 ± 1
129 IFN-αβR$^-$	rIL-15	42 ± 5	87 ± 17

[a] rIL-15 was administered to C57BL/6, 129SvEv, or 129 IFN-αβR$^-$ mice as described in *Materials and Methods*. Responses were evaluated 1 day after the third rIL-15 injection. Shown are means ± SEM of three mice per group. rIL-15 induced significant changes ($p < 0.05$) in the NK cell parameters shown above, as compared to untreated animals.

Discussion

This report dissects the signaling pathways for in vivo NK cell responses to type 1 IFNs and IL-12, and defines a critical role for IFN-αβ-induced endogenous IL-15 expression in regulating NK cells during viral infections. Under these conditions, the type I IFNs promote NK cell cytotoxicity and proliferation, whereas IL-12 elicits IFN-γ production. IFN-γ production, cytotoxic activity, and proliferation were shown in this study to occur in overlapping populations of NK cells. STAT1, but not STAT4, was required for the IFN-αβ-dependent induction of cytotoxicity. In contrast, the IL-12-dependent NK cell IFN-γ response was dependent on STAT4, but not STAT1. STAT1, but not STAT4, was required for accumulation of the cells. Impaired NK cell expansion in STAT1$^-$ mice was due to failure to induce IL-15 in response to IFN-αβ. Taken together, the studies delineate the STAT pathways required for induction of specific NK cell functions in vivo. They suggest that the distinct immunoregulatory effects mediated by IFN-αβ and IL-12 are due at least in part to nonoverlapping signaling pathway requirements for induction. They demonstrate the nonredundant but critical functions for the innate cytokines IFN-αβ, IL-12, and IL-15 in regulating NK cell responses during viral

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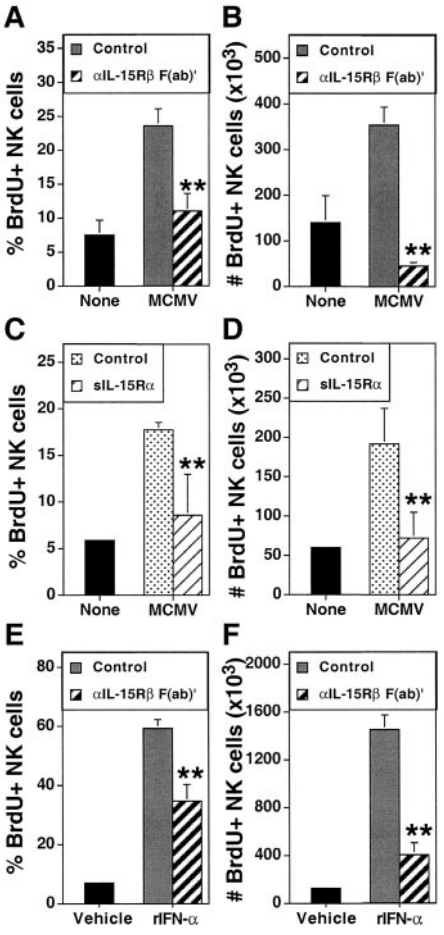

FIGURE 5. IL-15 requirements for induction of NK cell proliferation following MCMV infection or rIFN-α administration. C57BL/6 or endotoxin-resistant C3H/HeJ mice were uninfected, infected i.p. with 1×10^4 PFU MCMV, or given three daily doses of rIFN-α on days 0, 1, and 2 and had BrdU-containing drinking water at time 0. In addition, infected or treated mice also were given anti-IL-15Rβ mAb F(ab)′ or sIL-15Rα, along with appropriate controls (see *Materials and Methods*). Responses were evaluated 1.5 days after MCMV infection and 24 h after the third rIFN-α injection. *A*, Evaluation of anti-IL-15Rβ F(ab)′ effects on NK cell proportions and (*B*) numbers incorporating BrdU after MCMV infection of C57BL/6 mice. *C*, Evaluation of sIL-15Rα treatment effects on NK cell proportions and (*D*) numbers incorporating BrdU following MCMV infection of C3H/HeJ mice. *E*, Analysis of anti-IL-15Rβ F(ab)′ effects on NK cell proportions. *F*, Numbers incorporating BrdU following rIFN-α administration of C57BL/6 mice. NK cells are identified as NK1.1$^+$CD3$^-$ for *A*, *B*, *E*, and *F* and as DX5$^+$CD3$^-$ for *C* and *D*. Shown are averages ± SEM of three mice per group. **, $p < 0.02$ compared with control treatments.

infections. Lastly, they establish an IFN-$\alpha\beta$-STAT1-IL-15 axis critical for maintenance of NK.

By differentiating between signals for induction of cells to proliferate, to acquire effector functions, and to maintain these populations in vivo, the complexity of NK cell regulation during an infectious process is revealed (Fig. 6). In the endogenous milieu of multiple cytokine stimulations of the same cell subsets, individual cytokines nevertheless retain unique functions. During MCMV infections, even though IFN-$\alpha\beta$ and IL-12 are accessing overlapping NK cell populations, IL-12, through STAT4, exclusively regulates IFN-γ. Likewise, the IFN-$\alpha\beta$ cytokines, through STAT1-dependent signaling, uniquely regulate NK cell cytotoxicity and proliferation. NK cell activation by IFN-$\alpha\beta$ does not appear to confer intrinsic survival capabilities. Survival signals, conferred by IL-15

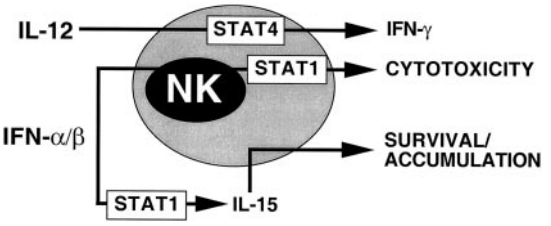

FIGURE 6. A model for coordinate regulation of NK cell responses during viral infection by IFN-$\alpha\beta$, IL-12, and IL-15. Following viral infection, biologically active IL-12, when elicited, signals through STAT4 for activation of NK cell IFN-γ production. The IFN-$\alpha\beta$ cytokines, through STAT1, induce NK cell cytotoxicity and through STAT1-dependent mechanisms, stimulate IL-15 expression. IL-15 promotes NK cell survival and/or accumulation following cell activation.

elicited in response to IFN-$\alpha\beta$, are required to sustain the NK cell response following activation. The observation that STAT1 is critical for induction of cytotoxicity is consistent with another recent report demonstrating that the signaling molecule is required for basal and dsRNA-induced NK cell killing (40). STAT1 is also required for a variety of IFN-γ-dependent effects (41). At 1.5 days after MCMV infection, IFN-γ is not a major regulator of NK cell cytotoxicity, because this cytokine is just being produced by NK cells. In the absence of IFN-$\alpha\beta$ functions, a modest contribution by IFN-γ to NK cell cytotoxicity can be observed at later times after infection. However, in the presence of IFN-$\alpha\beta$ functions, IFN-γ contribution to cytotoxicity is not readily observed (K. B. Nguyen and C. A. Biron, unpublished observations). Thus, during immune responses to viral infections, multiple cytokines are induced, and each of these has unique roles in regulating distinct aspects of the NK cell responses with IFN-$\alpha\beta$-inducting cytotoxicity, IL-12-inducing IFN-γ production, and IFN-$\alpha\beta$-induced IL-15 supporting expansion.

The STAT1- and STAT4-dependent effects on NK cell responses documented in this study occurred independently of their antiviral functions. At 1.5 days after MCMV infection, STAT4 deficiency did not affect MCMV replication in the spleen, and lack of STAT1 only modestly increased splenic viral burden (*Materials and Methods*). Moreover, parallel studies conducted administering recombinant cytokines to STAT-deficient mice yielded similar results to viral infections. However, both STAT1- and STAT4-deficient mice succumb to MCMV between 5 and 7 days after infection (data not shown), indicating that these molecules also are necessary for antiviral defense. However, these effects are likely also to be downstream of their numerous immunoregulatory functions.

Although certain studies have suggested the role for IL-15 in maintaining memory T cell responses and for maintaining long-term protection to viruses (16, 42, 43), these are the first studies to our knowledge to define and document the expression and function of IL-15 during acute viral infections and to critically define this factor's role in regulation of NK cell functions. The importance for endogenous IL-15 in NK cell development (16, 17, 22, 23), pathogenesis during collagen-induced arthritis (34), and NK cell IFN-γ production following endotoxin challenge (20) have been demonstrated. One of the challenges for identifying specific IL-15 functions in vivo has been the lack of reagents selectively neutralizing this cytokine without eliminating NK cells. As mice genetically deficient in either the IL-15R α-chain or IL-15 do not develop NK cells (22, 23), they would not have been informative for the questions being addressed in this study, i.e., effects of induced IL-15 on NK cell responses to viral infections. Thus, for our studies, in vivo

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IL-15 functions were evaluated by two complementary, short-term approaches (Fig. 5). First, the shared IL-2/IL-15R β-chain was blocked by administering F(ab)′′ before infection or rIFN-α administration. Second, the soluble rIL-15Rα was given to neutralize endogenous factor. The sIL-15Rα used was generated by recombinant technology and expression in Gram-negative bacteria (33, 34). It was purified and had no detectable endotoxin, i.e., LPS, using standard assays. However, because previous work from our laboratory has demonstrated that sensitivity to endotoxin can be increased 10- to 100-fold during viral infections (37, 38), and because treatment of both mutated and functional sIL-15Rα had stimulatory effects on NK cells in C57BL/6 mice, experiments with this reagent were performed in the endotoxin-resistant C3H/HeJ mice. Under these conditions, this very specific blocker of IL-15 functions inhibited the accumulation of NK cells.

We also demonstrated that administration of rIL-15 induced NK cell accumulation (Table III). In contrast, IL-15 was not crucial for either NK cell cytotoxicity or IFN-γ expression under the conditions of viral infections. This is in contrast to other works demonstrating that IL-15 can stimulate NK cell cytotoxicity in vitro and synergize with IL-12 for induction of NK cell IFN-γ production (16–23). Given differences in in vitro vs in vivo settings, the experimental conditions, and the amounts of IL-15 used in our studies and others, the results presented in this study do not necessarily contradict published work on the role of this cytokine in promoting NK cell cytotoxicity. Rather, we show that under conditions of short-term exposure in vivo, IL-15 effects on proliferation and cytotoxicity readily can be separated. Taken together, the results conclusively demonstrate the critical role for endogenous IL-15, induced during MCMV infection, for NK cell survival, and suggest that IL-15 functions are not static and may have different functions depending on the in vivo stimulus.

The observation that NK cells from MCMV-infected STAT1-deficient mice express higher levels of IFN-γ (Table II), is consistent with our previous report demonstrating that STAT1 acts to dampen and/or inhibit the NK cell IFN-γ response (30). STAT1-mediated inhibition of IFN-γ is downstream of IFN-$\alpha\beta$. Type 1 IFNs under normal conditions do not elicit IFN-γ production by NK cells. However, when STAT1 is absent or somehow negatively regulated, type 1 IFNs now can induce IFN-γ expression. Thus, in the absence of STAT1, the IFN-$\alpha\beta$ cytokines may have roles in the enhanced IFN-γ production during MCMV infection. In other studies, IL-12 administration to STAT1$^-$ mice also resulted in increased NK cell and serum IFN-γ as compared with STAT1$^+$ animals (K. B. Nguyen and C. A. Biron, unpublished observations). These results indicate that STAT1 has a role in dampening the IFN-γ responses to IL-12 in the absence of virus-induced IFN-$\alpha\beta$ production and in modulating IL-12 levels during viral infections. Such effects may be critical to the balance of beneficial vs detrimental effects of IL-12. Thus, STAT1 may be a general inhibitor of IFN-γ production, and the increased IFN-γ production by NK cells in STAT1-deficient MCMV-infected mice may be the result of heightened sensitivity of NK cells to IFN-γ production in response to cytokines, including IFN-$\alpha\beta$ and IL-12.

In summary, these studies characterize key cytokines and STAT molecules required for induction of NK cell effector functions during immune responses in vivo. They reveal IFN-$\alpha\beta$-dependent, STAT1-dependent mechanisms for induction of biological effects, separate signaling pathways for IL-12- as compared with IFN-$\alpha\beta$-mediated effects, and identify an IFN-$\alpha\beta$ to STAT1 to IL-15 cascade critical for NK cell responses in vivo. Given that particular NK cell responses may be more important in defense against certain infectious agents, and that NK cells have the potential to mediate detrimental effects if not appropriately regulated, the unique effects mediated by, and signaling pathways accessed by, IFN-$\alpha\beta$, IL-12, and IL-15, provide elegant mechanisms to precisely control these cells. Moreover, although in vitro and certain in vivo studies suggest a strong degree of overlap for immunoregulation between these factors, our studies show the nonredundancy of these cytokines for regulation of protective responses under physiologic conditions. These results emphasize the need for characterization of cytokine effects and interactions under conditions of mixed milieus.

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