Compartmental Differences in NK Cell Responsiveness to IL-12 During Lymphocytic Choriomeningitis Virus Infection

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Some, but not all, viral infections induce endogenous IL-12 to drive NK cell IFN-γ production and downstream antiviral defenses during innate immune responses. Even though lymphocytic choriomeningitis virus (LCMV) can be sensitive to IFN-γ-mediated antiviral effects, infections with this agent do not elicit IL-12 or early IFN-γ in immunocompetent hosts. Studies presented here demonstrate that LCMV infections of mice not only fail to induce IL-12, but also modify responsiveness to exogenous IL-12 for IFN-γ production. IFN-γ responses induced by IL-12 administration were greatly diminished in splenic populations, but significantly increased in serum and hepatic leukocytes, during the early course of LCMV infections. The IFN-γ production was NK cell dependent, and the compartmental dichotomy between spleen and liver was also demonstrated in response to in vitro IL-12 stimulation. Although infections did increase proportions and numbers of liver NK cells, changes in responsiveness for IFN-γ expression could not be explained by cell redistribution. Corroborating changes in proportions of NK cells induced to express intracellular IFN-γ protein within the compartments were observed. The reduction in ability of splenic populations to produce IL-12-induced IFN-γ after infection by LCMV was associated with decreased efficacy of administered IL-12 for promoting IFN-γ-dependent antiviral effects in the spleen. Concomitantly, the maintenance of hepatic population IFN-γ production was associated with preserved efficacy of administered IL-12 to elicit IFN-γ-dependent antiviral effects in the liver. Taken together, these results demonstrate modifications of compartmental responses to IL-12 by viral infections and the consequences of these changes for efficacy of cytokine therapy. The Journal of Immunology, 2000, 164: 994–1001.

Interleukin-12 is a highly potent inducer of IFN-γ synthesis, and endogenous expression of IL-12 is critical for NK cell IFN-γ production during innate responses to a variety of agents (1–6). However, early IL-12 and IFN-γ are induced in some, but not all, viral infections. In particular, the cytokine proteins are not readily detectable during infections of immunocompetent mice with lymphocytic choriomeningitis virus (LCMV) (4, 5, 7, 8), even though this virus can be sensitive to IFN-γ-dependent antiviral effects (9). The lack of early IFN-γ expression is associated with a lack of demonstrable role for NK cells in defense against this virus (4, 5, 7, 8). It is not clear whether the absence of NK cell IFN-γ is simply a result of IL-12 absence and/or a consequence of other virus-induced changes. If the lack of a response is exclusively the result of poor IC-12 induction, administration of the cytokine may provide a therapeutic modality for inducing NK cell IFN-γ and promoting resistance at early times of infection.

The studies presented here were undertaken to evaluate efficacy of administered IL-12 for promoting NK cell IFN-γ responses and downstream antiviral effects during LCMV infections. The results demonstrate compartmental differences between splenic and hepatic leukocytes for IFN-γ induction by IL-12, with an infection-induced loss of splenic, but not hepatic, leukocyte IL-12 responsiveness for IFN-γ production. The decrease in splenic cell responsiveness was associated with diminished efficacy of IL-12 intervention to induce antiviral states, whereas maintenance of hepatic cell responsiveness was associated with preserved antiviral efficacy. This striking dichotomy in compartmental IFN-γ production and antiviral efficacy in response to exogenous IL-12 suggests differential pathways regulating NK cells in specific microenvironments and has implications for cytokine therapy for eliciting effects at particular sites.

Materials and Methods

Mice

Male C57BL/6 (Taconic Laboratory Animals and Services, Germantown, NY) and T and B cell-deficient C57BL/6-J-SCID and C57BL/6J immunocompetent mice (The Jackson Laboratory, Bar Harbor, ME) were used for these studies. Also used were E26 mutant mice, containing high copy numbers of human CD3ε transgenes and deficient in NK and T cells (10), and C57BL/6-J-IFN-γ−/− mice (11) (a generous gift from Herbert W. Virgin, IV, Washington University, St. Louis, MO) maintained through brother × sister matings in breeding facilities at Brown University (Providence, RI). All mice were used between 5 and 9 wk of age. Experiments were conducted in accordance with institutional guidelines for animal care and use.

In vivo treatment protocols

Infections were established by i.p. injection with 2 × 10⁶ PFU of LCMV Armstrong strain, clone E350. This isolate of LCMV replicates to high titers in spleen, but is relatively nonhepatotropic. Some studies used 2 × 10⁶ PFU of the Armstrong or the hepatotropic LCMV isolate, WE, for establishing infections. Murine recombinant IL-12 (12) (sp. act. 4.9 × 10⁹ U/mg), a gift from Genetics Institute (Andover, MA), was diluted in vehicle (PBS supplemented with 5% heat-inactivated C57BL/6 mouse serum from Taconic Laboratory Animals and Services) and administered i.p. for in vivo treatments. Effects of IL-12 administration on viral titers were investigated by initiating IL-12 injections at different times relative to day of infection. Some experiments used daily IL-12 treatments beginning either 1 day before (day −1) or 1 day after (day +1) LCMV infection. In these studies, mice received daily IL-12 treatments up to, and including,
day 2 after infection. In other experiments, only a single dose of IL-12 was given. In all treatment studies, the spleens were harvested on day 3. In vivo effects of IL-12 for induction of IFN-γ were examined using a single injection of IL-12 given to infected or day 0, 1, or 2 LCMV-infected mice. One day after IL-12 administration, mice were anesthetized with methoxyflurane (Pittman-Moore, Mundelein, IL), and whole blood was collected into heparinized tubes for preparation of serum and/or PBMCs. Spleens were harvested from killed mice into ice-cold RPMI 1640 (RPMI 1640 media supplemented with NaHCO3, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin from Life Technologies, Grand Island, NY), followed by perfusion of the hepatic portal vein with 5 ml PBS and harvesting of livers for isolation of leukocytes.

Isolation of leukocytes

Whole blood, collected into 2 ml RPMI 1640 with heparin, was overlaid on 3 ml Histopaque-1083 (Sigma, St. Louis, MO) and centrifuged according to manufacturer’s directions (800 x g for 15 min at room temperature). PBMCs were harvested at the interface. To obtain splenic leukocytes, spleens were minced, passed through nylon mesh (Tetko, Kansas City, MO), and treated with ammonium chloride. Hepatic leukocytes were prepared using published methods (13) with modifications. Briefly, livers were forced through steel mesh and digested with 0.05% collagenase IV and 0.004% DNase I (Sigma) in RPMI 1640 at 37°C for 30 min. Digested slurry was passed through nylon mesh and treated with ammonium chloride. Cell suspensions were then layered on two-step discontinuous Percoll gradients (Pharmacia Fine Chemicals, Piscataway, NJ) in D-PBS (Life Technologies) for density separation. Hepatic leukocytes were harvested at the 35/80% Percoll interface after centrifugation at room temperature for 30 min at 900 x g. Cell yields and viabilities were determined by trypan blue exclusion (Life Technologies).

Generation of conditioned media (CM) and cytokine measurements

To investigate induction of IFN-γ production after in vivo IL-12 administration, leukocytes were plated in flat-bottom microtiter plates at 104 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT). Plates were incubated for 24 h at 37°C in a 5% CO2 incubator before collection of supernatants and storage at −80°C. To determine in vitro sensitivity of leukocytes to IL-12, plated leukocytes were supplemented with 100 μl/well of indicated concentrations of IL-12 prepared in 10% FBS-RPMI 1640 to a final volume of 200 μl/well. Cytokine levels in CM and serum were determined by sandwich ELISA, as previously described (7, 14, 15).

Flow cytometric analysis

Cells were blocked with 20% FBS-PBS supplemented with 100 μg/ml mouse IgG (Sigma), stained using R-PE-conjugated anti-NK1.1 (PK136) and CyChrome-conjugated anti-TCR-β (H57-597) or isotype control Abs (PharMingen, San Diego, CA), and fixed with 4% paraformaldehyde in PBS. More than 25,000 events were collected within a leukocyte acquisition gate using a FACScalibur (Becton Dickinson, San Jose, CA). Argon laser output was operating at 15 mW at 488 nm. Data were acquired and analyzed using CellQuest software (Becton Dickinson). Intracellular staining of IFN-γ protein was conducted as described (16). Briefly, cells were first incubated for 5 h at 37°C in 10% FBS-RPMI 1640 containing 10 μg/ml brefeldin A (Sigma), harvested, and blocked with 20% FBS-PBS containing mouse IgG. Biotinylated anti-NK1.1 (PK136) and CyChrome-conjugated anti-TCR-β (H57-597), followed by streptavidin-conjugated APC (PharMingen), were used for surface staining. Cells were fixed with 4% formaldehyde in PBS, then treated with permeabiliation buffer (1% saponin in 0.5% BSA-PBS with 0.006% NaN3; Sigma). Samples were blocked in permeabiliation buffer containing 300 μg/ml rat IgG (Sigma) followed by staining with R-PE-conjugated anti-mouse-IFN-γ (XMG1.2) or isotype control Ab (PharMingen). Specificity of intracellular IFN-γ staining was demonstrated by blocking with unlabeled soluble IFN-γ or unconjugated XMG1.2. Samples were acquired using a FACScalibur with the first laser operating as described above and a second laser operating at a wavelength of 635 nm. More than 100,000 events were collected within a leukocyte acquisition gate.

Plaque assays

LCMV titers were quantitated as previously described (17). Briefly, monolayers of Vero cells were grown until confluent. Frozen organs were homogenized, and debris was removed by centrifugation. Serially diluted supernatants were incubated in duplicate on cell monolayers for 1.5 h at 37°C. Cell monolayers were overlaid with 10% FBS-2× Media 199 (Life Technologies) mixed with equal part 1% SeaKem ME agarose (FMC Bio-products, Rockland, ME). Plates were incubated for 4 days at 37°C and overlaid with 10% FBS-2× Media 199/1% ME agarose containing a 6.7% neutral red solution (Life Technologies) to visualize plaques. LCMV standards and negative controls were included in each assay.

Statistical analyses

Results are given as means ± SEM. Statistical analyses using 2-tailed homoscedastic Student’s t tests were run on Microsoft Excel 98 (Microsoft Corporation, Redmond, WA). ANOVA tests were run on StatView 4.5 (Abacus Concepts, Berkeley, CA) where indicated.

Results

Effects of LCMV infection on IL-12-elicited IFN-γ responses in spleen and serum

To characterize the effects of viral infection on the efficacy of IL-12 administration for IFN-γ induction, uninfected and LCMV-infected C57BL/6 mice were vehicle treated or treated with a single 1-μg dose of the cytokine. One day after injection, splenic leukocytes and serum samples were harvested. IFN-γ production by leukocytes was assessed in culture media conditioned for 24 h without further ex vivo stimulation (CM). Cells isolated from all mice treated with vehicle injections failed to produce detectable IFN-γ in culture (Table I). Cells isolated from uninfected mice treated with IL-12 for 1 day produced 47.9 pg/106 cells of IFN-γ (Table I; C57BL/6, uninfected). Those isolated from mice treated with IL-12 on day 2 and harvested on day 3 of infection produced only 6.3 pg/106 cells of IFN-γ (Table I; C57BL/6, LCMV infection day 3), representing an infection-induced inhibition of >80%. This inhibition was reproducibly observed in over seven independent experiments, comprising an aggregate of 18 samples that demonstrated an average IFN-γ production of 80.9 ± 24.3 pg/106 cells by splenic leukocytes from IL-12-treated infected but only 24.5 ± 5.9 pg/106 cells by those from IL-12-treated infected mice (significantly different; p ≤ 0.02). The IFN-γ was primarily produced by NK cells because it was present in T and B cell-deficient SCID but absent in NK and T cell-deficient E26 mice (Table I). In contrast to the infection-induced inhibition of splenic leukocyte production, IL-12-induced IFN-γ levels were significantly augmented in serum during the infection. In this compartment, IL-12 treatment of uninfected mice induced 218.5 ± 45.2, and of LCMV-infected mice induced 265.6 ± 330.3 pg/ml serum IFN-γ (p < 0.001; Table II). The serum responses were also shown to be NK cell dependent because they were observed in SCID but not E26 mice (Tables II). Thus, LCMV infection results in dramatically inhibited IL-12-elicited IFN-γ production by splenic leukocytes but augmented serum IFN-γ, and these effects appear to be at the level of NK cell responsiveness to the cytokine. As the enhancement in serum IFN-γ levels occurs concomitantly with diminished splenic production, the results suggest that other compartments are contributing to IL-12-induced circulating levels of IFN-γ.

Kinetics of virus-induced changes in compartmental responses to IL-12

To expand characterization of responses in spleen and serum, as well as to extend studies to cells in other compartments known to harbor significant proportions of NK cells, i.e., blood (circulating PBMCs) and liver, time course experiments were conducted with populations isolated from these different sites. Uninfected mice and mice infected with LCMV for 0, 1, or 2 days were treated with a single dose of vehicle control or 1 μg IL-12. One day following treatment, i.e., 0 (uninfected), 1, 2, or 3 days after infection, sera were collected, and cells were isolated to prepare CM. IL-12 induced significant levels of serum IFN-γ, and these levels were
progressively increased during the course of LCMV infection. Mice treated for 1 day with IL-12, on the day of infection (0) or day 1 or day 2 after infection, respectively, exhibited 2-, 6-, and 10-fold greater serum IFN-γ levels as compared with treated uninfected mice (−1) (Fig. 1A). In contrast, the ability of splenic leukocytes to produce IFN-γ was severely attenuated as infection progressed such that administration of IL-12 1 day or later after infection resulted in up to 80% decreases in IFN-γ production (Fig. 1B). Interestingly, PBMCs from IL-12-treated mice failed to produce detectable levels of IFN-γ at any time point examined, suggesting that circulating leukocytes were not significant contributors to serum IFN-γ (Fig. 1C). In contrast, hepatic leukocytes were a rich source of IFN-γ under these conditions, with cells from mice receiving IL-12 on day 2 and isolated on day 3 of infection producing >5-fold more IFN-γ, reaching >2000 pg/10⁶ cells (Fig. 1D). Estimated total production based on cell yields from specific organs indicated that IFN-γ production by cells from IL-12-treated uninfected or day 2-infected mice was, respectively, 3,322 (±234) and 355 (±53) pg/total cells isolated from spleen, but 2,477 (±443) and 10,278 (±970) pg/total cells isolated from livers. Thus during infection, cells in the liver sustain their ability to respond to IL-12 and synthesize IFN-γ, and the compartment is a significant source of the factor.

Characterization of cell subset distribution following LCMV infection and IL-12 administration

As production of IFN-γ was primarily dependent upon NK cells (Tables I and II), experiments were conducted to determine whether changes in NK cell subset distributions accounted for the compartmental differences. Flow cytometric analyses were conducted to quantitate classical NK cells (NK1.1⁺TCR-β⁻). For these studies, cell populations were prepared from uninfected and LCMV-infected C57BL/6 mice that had been untreated, vehicle treated, and/or IL-12 treated for 1 day. Although infections did induce significant increases in proportions and numbers of liver NK cells, differences in subset distribution could not account for the observed decreases in splenic, or increases in liver, cell IFN-γ production. As compared with the average of 1.7% (±0.2) in IL-12-treated, uninfected mice, NK cell proportion in the spleens of mice administered a single 1 µg dose of IL-12 on day 2 and harvested on day 3 of infection averaged 2.1% (±0.3) (Table III). The modest change in NK cell proportions was neither significant nor

Table I. Infection-induced changes in splenic leukocyte IFN-γ responses to IL-12 administration

| Mouse Strain   | LCMV Infection | Treatment*       | Splenic Leukocyte CM (pg/10⁶ cells) |
|----------------|----------------|-----------------|-------------------------------------|
| C57BL/6 (NK⁺, T⁺) | Uninfected     | Vehicle         | <1.8                                 |
|                | Day 3          | IL-12           | 47.9 ± 31.6                          |
|                |                | IL-12           | 6.3 ± 1.0                            |
| SCID (NK⁺, T⁻, B⁻) | Uninfected     | Vehicle         | <1.8                                 |
|                | Day 3          | IL-12           | 378.4 ± 34.5                         |
|                |                | IL-12           | 217.8 ± 33.9*                        |
| E26 (NK⁻, T⁻)   | Uninfected     | Vehicle         | <0.5                                 |
|                | Day 3          | IL-12           | 7.1 ± 4.2                            |
|                |                | IL-12           | <11.2 ± 13.1                         |

* Vehicle control or 1-µg IL-12 injections were administered i.p. to uninfected or LCMV-infected mice 24 h prior to cell isolation as described in Materials and Methods.

Table II. Infection-induced changes in serum IFN-γ responses to IL-12 administration

| Mouse Strain   | LCMV Infection | Treatment*       | Serum (pg/ml) |
|----------------|----------------|-----------------|---------------|
| C57BL/6 (NK⁺, T⁺) | Uninfected     | Vehicle         | <17.6         |
|                | Day 3          | IL-12           | 218.5 ± 45.2  |
|                |                | IL-12           | 49.4 ± 3.4    |
|                |                | II2-12          | 2655.6 ± 330.3* |
| SCID (NK⁺, T⁻, B⁻) | Uninfected     | Vehicle         | <17.6         |
|                | Day 3          | IL-12           | 343.6 ± 38.2  |
|                |                | IL-12           | 25.8 ± 1.4    |
|                |                | II2-12          | 3788.5 ± 463.5* |
| E26 (NK⁻, T⁻)   | Uninfected     | Vehicle         | <9.8          |
|                | Day 3          | IL-12           | <41.0 ± 27.2  |
|                |                | IL-12           | <14.8 ± 6.1   |

* Vehicle control or 1-µg IL-12 injections were administered i.p. to uninfected or LCMV-infected mice 24 h prior to serum collection as described in Materials and Methods.

* Values are means ± SEM from three mice per group.

* p < 0.001 as compared to uninfected controls treated with IL-12 in the same mouse strain.
congruent with the >80% infection-induced inhibition of splenic leukocyte IFN-γ production (Fig. 1). Similarly, differences in hepatic NK cell percentages (Table III) could not fully account for the >5-fold enhancement in hepatic leukocyte IFN-γ production after IL-12 administration during infection (Fig. 1). Taken together, these studies demonstrate that compartmental changes in NK cell proportions following LCMV infection fail to satisfactorily account for infection-induced changes in IFN-γ induction within each particular compartment or across compartments.

Differences in compartmental responses to IL-12 in culture

To directly evaluate intrinsic cell populations as opposed to potential secondary effects in vivo, IL-12 responsiveness was also evaluated in culture. For these experiments, leukocytes were isolated from C57BL/6 mice uninfected or LCMV infected for various periods, and IFN-γ production was examined by 24-h culture with the cytokine. Splenic leukocytes from uninfected animals produced high levels of IFN-γ upon in vitro exposure to IL-12, reaching 39,399.0 ± 10,895.5 pg/ml (at a final cell concentration of 5 × 10⁶ cells/ml). However, those prepared during the course of the infection were progressively attenuated such that on day 2 their IFN-γ production was reduced by >95% to only 924.5 ± 427 pg/ml (Fig. 2). In contrast, hepatic leukocytes from uninfected animals produced 1,518 pg/ml of IFN-γ and from day 2-infected mice produced >8-fold higher levels, reaching 13,492 pg/ml. Thus, in vitro reflected in vivo responses of the populations to IL-12.

Table III. NK cell proportions in spleen and liver: effects of LCMV infection and/or IL-12 administration

| Treatment | Spleen | | Liver | |
|-----------|--------| | | |
| | Uninfected | Day 3 | Uninfected | Day 3 |
| | No. Expts./ No. Mice | % NK Cells | No. Expts./ No. Mice | % NK Cells | No. Expts./ No. Pools | % NK Cells | No. Expts./ No. Pools | % NK Cells |
| None | 3/10 | 2.3 (±0.1) | 1/3 | 3.0 (±0.1) | 3/4 | 11.0 (±3.7) | 1/1 | 19.3 |
| Vehicle | 3/10 | 2.3 (±0.2) | 3/10 | 3.4 (±0.2) | 3/5 | 6.5 (±1.2) | 3/5 | 27.0 (±1.1) |
| IL-12 | 3/10 | 1.7 (±0.2) | 3/10 | 2.1 (±0.3) | 3/5 | 7.7 (±1.1) | 3/5 | 22.2 (±2.8) |

* C57BL/6 mice were either uninfected or infected with LCMV. Leukocytes were isolated from untreated mice or mice treated i.p. with vehicle control or 1 µg IL-12 for 24 h prior to cell isolation. Classical NK cells (NK1.1+ TCR-β+ phenotype) were quantitated by flow cytometry.

** Percentages were derived from results compiled from the indicated number of independent experiments and number of samples in the respective treatment groups. Splenic samples were obtained from individual mice, whereas due to cell yield limitations hepatic samples were pooled from several individual mice to yield the listed number of pools analyzed.

*** Values are means ± SEM for indicated total numbers of experiments and individual mice analyzed.

Values are means ± SEM for indicated total numbers of experiments and pooled samples analyzed.
cell proportions. Hence, compartmental differences in IL-12 responsiveness induced during viral infection are maintained by isolated cell populations stimulated with the cytokine in vitro.

**Identification of IFN-γ-expressing cell subsets**

To directly quantitate proportions of NK cells induced to express IFN-γ protein, normal or SCID C57BL/6 mice were treated with vehicle or IL-12 without infection or on day 2 of infection. One day later, splenic and hepatic cells were isolated, stained for intracellular IFN-γ protein expression, without further stimulation in culture and analyzed by flow cytometry. Experiments examining total IFN-γ-positive populations demonstrated that specific staining for IFN-γ under these conditions occurred almost exclusively in the classical NK cell subset, i.e., NK1.1+TCR-β populations, in both spleens and livers (data not shown). These results confirmed and extended the studies presented above indicating that NK cells were the primary source of IFN-γ (Tables I and II). Evaluation of the percentages of positive splenic cells demonstrated that IL-12 treatment induced 16% (± 2.0) of the NK cells in uninfected mice to express IFN-γ. In contrast, only 10% (± 1.0) of the splenic NK cells in infected mice were IFN-γ positive after treatment, representing a 38% decrease in the proportion of responders (Fig. 3A). SCID mice, naturally enriched in NK cells, also demonstrated similar reductions after infection (Fig. 3A). Thus, both the percentages and numbers of splenic NK cells induced to express IFN-γ by administered IL-12 were decreased as a result of LCMV infection. Moreover, the level of expression within individual positive cells was decreased as determined by mean channel of fluorescence intensity (data not shown).

In contrast, NK cell populations from the liver either maintained or increased in IL-12 induction of intracellular IFN-γ expression during infection. Similar proportions of NK cells isolated from immunocompetent C57BL/6 mice were induced by IL-12 administration to express IFN-γ, comprising 24% and 23% of the NK cell subset in uninfected and infected mice, respectively (Fig. 3B). The trends were also observed in liver populations from IL-12-treated SCID mice, where percentages of IFN-γ-expressing NK cells increased from 25% to 32% after infection (Fig. 3B). Thus, in agreement with patterns of IFN-γ responses observed in CM, these flow cytometric results demonstrate that NK cells are a primary source for IFN-γ after IL-12 treatment, and that relative proportions of IFN-γ-expressing NK cells are decreased in spleen, but maintained or enhanced in liver, after LCMV infection.

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**FIGURE 2.** Differential splenic and hepatic IFN-γ production capacities in response to in vitro IL-12 stimulation after LCMV infection. Splenic (A) and hepatic leukocytes (B) were isolated from uninfected mice or mice infected for the indicated lengths of time. Cells were cultured for 24 h in 96-well microtiter plates at 10⁶ cells in 100 µl per well, supplemented with 100 µl of either media or the indicated concentration of IL-12. Culture supernatants were tested for IFN-γ production by sandwich ELISA. Results are means ± SEM from three individual mouse per group, except for hepatic leukocytes, which were tested as a pool of three livers per group. Differences between uninfected and day 2-infected spleens and livers are representative of results from three separate experiments. Significant inhibition of IL-12-induced splenic IFN-γ production was observed after infection as compared with uninfected controls (*, p < 0.02) (A).

**FIGURE 3.** Intracellular IFN-γ protein expression in NK cells after IL-12 administration. Splenic (A) and hepatic (B) leukocytes were isolated from uninfected or day 3 LCMV-infected C57BL/6 or SCID mice treated with vehicle control or 1 µg IL-12 24 h before harvesting. Cells were cultured for 5 h with brefeldin A without restimulation and stained for surface markers NK1.1 and TCR-β, followed by permeabilization and staining for intracellular IFN-γ. Filled areas on histograms (linear y-axes of fluorescence) in gated NK cell populations. Solid lines indicate background staining with isotype-matched control Ab. Splenic leukocyte profiles shown are representatives of individual mouse samples, whereas those of hepatic leukocytes are samples pooled from three mice. Percentages and cell yield numbers are given. Those from splenic populations are means from three individual mouse samples. Experiments were repeated at least twice with similar results in both strains of mice.
Requirements for antiviral IL-12 intervention in spleen

LCMV is reported to be sensitive to IFN-γ antiviral effects, but the infection itself does not induce IL-12 and/or early IFN-γ protein in immunocompetent hosts. The results presented above suggest that administered IL-12 may be used therapeutically to induce early IFN-γ expression and downstream antiviral effects, but that these effects may be limited to particular compartments due to infection-induced changes in NK cell responsiveness to IL-12. Studies were initiated to determine whether IL-12 administration could be used therapeutically during LCMV infection. The primary site of replication for LCMV is reported to be sensitive to IFN-γ antiviral effects, but that these effects may be limited to particular compartments due to infection-induced changes in NK cell responsiveness to IL-12. The experiments thus far have definitively shown a dichotomy in infection itself does not induce IL-12 and/or early IFN-γ protein in immunocompetent hosts. The results presented above suggest that administered IL-12 may be used therapeutically to induce early IFN-γ expression and downstream antiviral effects, but that these effects may be limited to particular compartments due to infection-induced changes in NK cell responsiveness to IL-12. Studies were initiated to determine whether IL-12 administration could be used therapeutically during LCMV infection. The primary site of replication for LCMV is reported to be sensitive to IFN-γ antiviral effects, but that these effects may be limited to particular compartments due to infection-induced changes in NK cell responsiveness to IL-12. The experiments thus far have definitively shown a dichotomy in requirements for antiviral IL-12 intervention in spleen.

FIGURE 4. Requirements in spleen for antiviral IL-12 intervention during LCMV infections. C57BL/6 mice were treated daily with various doses of either vehicle control or IL-12 starting 1 day after (A) or 1 day before (B) infection, with a single dose of either vehicle control or 1 μg IL-12 on different days relative to infection (C), or with daily treatments of vehicle control or 100 ng IL-12 per day initiated 1 day before infection in IFN-γ-deficient (IFN-γ−/−) or immunocompetent control (IFN-γ+/+) mice (D). All spleens were harvested on day 3 for determination of viral titers by LCMV plaque assays. Results are presented as means ± SEM of at least three mice per group. Statistical analyses demonstrated significant differences between IL-12- and vehicle control-treated groups as follows: Student’s t test, *p < 0.05 and †p ≤ 0.02; ANOVA, **p ≤ 0.0001.

Requirements for antiviral IL-12 intervention in liver

The experiments thus far have definitively shown a dichotomy in infection itself does not induce IL-12 and/or early IFN-γ protein in immunocompetent hosts. The results presented above suggest that administered IL-12 may be used therapeutically to induce early IFN-γ expression and downstream antiviral effects, but that these effects may be limited to particular compartments due to infection-induced changes in NK cell responsiveness to IL-12. Studies were initiated to determine whether IL-12 administration could be used therapeutically during LCMV infection. The primary site of replication for LCMV Armstrong strain used here is the spleen. In immunocompetent C57BL/6 mice, daily IL-12 treatments initiated 1 day after establishment of infection (day +1) resulted in no significant decreases in splenic viral titers, i.e., ~6.7–6.9 logs of virus were detected in both vehicle control and IL-12-treated groups (Fig. 4A). In contrast, daily IL-12 treatments initiated 1 day before infection (day −1) resulted in significant decreases of up to 1 log virus over vehicle control treatments (Fig. 4B). These results could not be explained by total aggregate dosage of cytokine received as statistically significant inhibitory effects on viral replication were observed with low-dose (1 ng/day) IL-12 initiated on day −1, whereas marginal effects at 1000-fold higher doses were detected when initiated on day +1. Furthermore, studies using only a single 1-μg dose of IL-12 on different days relative to infection demonstrated significant reductions in day 3 splenic viral titers when administered once on day −1, but not when administered any time thereafter (Fig. 4C). As these results directly mirrored the reductions in IL-12-induced splenic IFN-γ production after LCMV infection (Fig. 1), additional studies to demonstrate the IFN-γ-dependency of antiviral effects were performed. The antiviral effects of daily IL-12 treatment initiated on day −1 were indeed found to be dependent upon the induction of IFN-γ as treatments failed to reduce viral burden in C57BL/6 mice genetically deficient in IFN-γ (Fig. 4D). Taken together, these results suggest that the reduced ability of splenic cells to produce IL-12-induced IFN-γ after infection is correlated with biological consequences that narrow the window of opportunity for cytokine therapy to exert antiviral effects locally in the spleen.

FIGURE 5. Compartmental differences in requirements for antiviral IL-12 intervention. C57BL/6 mice were treated daily with either vehicle control or 100 ng IL-12 per day starting 1 day before or 1 day after LCMV infection with either 2 × 10^2 PFU of Armstrong (A and C) or WE (B and D). Spleens (A and B) and livers (C and D) were harvested on day 3 for determination of viral titers. Results are presented as means ± SEM of four mice per group. Compared with vehicle control treatments, significant reductions in LCMV titers were observed in spleen if IL-12 treatments were initiated before infection (*p ≤ 0.001), and in liver significant reductions were observed with treatments initiated before or after infection (**, p ≤ 0.001).
The synthesis of IFN-\(\gamma\)-induced NK cell IFN-\(\gamma\) is associated with affected abilities to elicit IFN-\(\gamma\) in splenic cell populations. In striking contrast, IFN-\(\gamma\)-deficient (IFN-\(\gamma^{-/-}\)) or immunocompetent control (IFN-\(\gamma^{+/+}\)) mice were infected with 2 \(\times\) 10^3 PFU of WE and treated daily with vehicle control or 100 ng IL-12 per day starting 1 day after infection. Splens and livers were harvested on day 3 for determination of viral titers. Results are presented as means ± SEM of five mice per group. IL-12 treatment induced significant reductions in viral titers over vehicle controls in the livers of IFN-\(\gamma^{-/-}\) mice (*, \(p \leq 0.001\)).

FIGURE 6. IFN-\(\gamma\) requirement for antiviral IL-12 intervention in liver. IFN-\(\gamma\)-deficient (IFN-\(\gamma^{-/-}\)) or immunocompetent control (IFN-\(\gamma^{+/+}\)) mice were infected with 2 \(\times\) 10^3 PFU of WE and treated daily with vehicle control or 100 ng IL-12 per day starting 1 day after infection. Splens and livers were harvested on day 3 for determination of viral titers. Results are presented as means ± SEM of five mice per group. IL-12 treatment induced significant reductions in viral titers over vehicle controls in the livers of IFN-\(\gamma^{-/-}\) mice (*, \(p \leq 0.001\)).

Although it is not yet known why LCMV infections do not induce endogenous IL-12 and NK cell IFN-\(\gamma\) responses in immunocompetent hosts, it is becoming clear that the lack of such responses is not exclusively a result of an absence of the ability to elicit IL-12. This laboratory has previously demonstrated infection-induced mechanisms in place to actively inhibit endogenous IL-12 expression (8). The studies presented here demonstrate that bypassing this inhibition by adding exogenous cytokine reveals another tier of regulation at the level of ability for IL-12 to induce IFN-\(\gamma\) in splenic NK cells. This repression of IL-12-induced IFN-\(\gamma\) production is reminiscent of human NK cell studies demonstrating particular conditions driving in vitro differentiation of NK1 cells to preferentially produce IFN-\(\gamma\), as compared with conditions driving NK2 cells to produce IL-5/IL-13 (18–20). However, the dichotomy in NK cell responsiveness between organ compartments is more similar to demonstrated hyporesponsiveness after sequential augmentation of splenic, but not hepatic, NK cells following treatments with biological response modifiers, such as maleic anhydride divinyl ether or heat-killed Propionibacterium acnes (13). Taken together with these other studies, our results suggest that localized responses are a result of unidentified microenvironmental influences in place to either negatively or positively regulate endogenous cytokine responses.

This report adds to characterization of how ongoing immune responses to infections may modify responses to administered cytokines, i.e., cytokine therapy. Other studies from our group, examining administered IL-12 during peak adaptive immune responses to LCMV, have shown dramatic synergistic effects between these and endogenous T cell responses in enhancing proinflammatory cytokine expression and cytokine-mediated toxicities (17, 21). This synergism limits tolerated doses of IL-12. The work presented here extends knowledge to the context of ongoing endogenous innate immune responses and has important implications for therapeutic use of IL-12 under these conditions. Our results indicate that, depending upon the nature of infection-induced modulation of innate immune responses within different compartments, achieving optimal antiviral defenses at particular sites may be limited to prophylactic regimens. This is consistent with observations from others that IL-12 treatments initiated before vesicular stomatitis virus infections significantly decrease viral titers in the central nervous system (22), but can also be effective postinfection (23); and that prophylactic is more effective than therapeutic IL-12 for enhancing host survival during lethal herpes simplex virus infections (24). Thus, different viral systems indicate that it is important to treat with IL-12 before infection to elicit maximal antiviral defenses at some sites. Our results indicate that LCMV infections can enhance IL-12 IFN-\(\gamma\) induction at sites other than the spleen, i.e., serum and liver. Therefore, IL-12 administration initiated during an established infection may be beneficial in particular organs if the cellular targets of a virus are localized in compartments with either maintained or enhanced IL-12 responsiveness, such as the liver. In this regard, IL-12 therapy may prove to be protective against infections of humans with hepatitis viruses sensitive to IFN-\(\gamma\) (25–28).

Surprisingly, the protective effects of IL-12 treatments in the spleen did not correlate with serum IFN-\(\gamma\) levels but with local IFN-\(\gamma\)-production. This dissociation of circulating IFN-\(\gamma\) responses from IFN-\(\gamma\)-mediated protective effects in spleens suggests that the cytokine must be locally produced to mediate antiviral effects at target sites. Other work from our group has been characterizing the requirements for NK cell-mediated defense during endogenous responses to murine cytomegalovirus (MCMV) infections in liver.
Those studies have similar implications. During MCMV infections, 1) NK cells mediate antiviral effects in liver through an IFN-γ-dependent pathway, 2) NK cells traffic to focal sites of viral Ag expression in this tissue, and 3) optimal antiviral defenses in liver require chemokine-dependent accumulation of NK cells (14, 29, 30). Taken together with the MCMV studies, the experiments presented here indicate that local NK cell responses must be regulated to deliver IFN-γ within particular tissues and that there is a requirement for local delivery even in the face of high serum IFN-γ.

In summary, the findings in this report demonstrate differential modification of compartmental responses to IL-12-induced IFN-γ production over the course of viral infection and highlight requirements for effective IL-12-mediated antiviral therapy resulting from these modifications. Furthermore, the data reveal intriguing differences between spleen and liver for regulation of NK cell IFN-γ responses during viral infection and suggest the existence of local conditions regulating cytokine responses.

Acknowledgments

We thank Drs. Leslie Cousens, Melanie Ruzek, and Thais Salazar-Mather, along with Stacey Carlton, Khuong (Ken) Nguyen, Michael Primiano, and Dr. Stanley Wolf for their help. We also thank Genetics Institute for the generous gift of recombinant murine IL-12, Dr. Philip Scott for his gift of sera, Dr. Cox Terhorst for providing original breeding pairs of E26, and Dr. Herbert W. Virgin, IV for breeding pairs of IFN-γ-deficient mice.

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