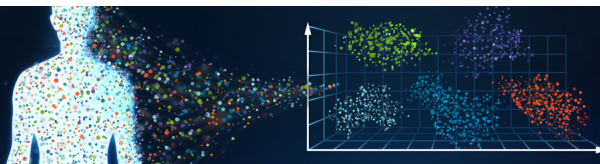


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# Recruitment of Hepatic NK Cells by IL-12 Is Dependent on IFN- $\gamma$ and VCAM-1 and Is Rapidly Down-Regulated by a Mechanism Involving T Cells and Expression of Fas<sup>1,2</sup>

William E. Fogler,<sup>3\*</sup> Kirk Volker,<sup>†</sup> Morihito Watanabe,\* Jon M. Wigginton,<sup>‡</sup> Philip Roessler,\* Michael J. Brunda,<sup>§</sup> John R. Ortaldo,\* and Robert H. Wilttrout<sup>4\*</sup>

NK cells have been shown to be important antitumor or antiviral effector cells in the liver. In the present study we have examined the factors that regulate the initial recruitment and subsequent fate of hepatic NK and T cells in mice treated with IL-12 or IL-2. Daily administration of IL-12 caused a rapid initial increase in NK cells followed by a subsequent decrease that coincided with an accumulation of T cells. The recruitment of hepatic NK cells by IL-12, but not the subsequent T cell infiltrate, was abrogated in IFN- $\gamma^{-/-}$  mice. In contrast, daily administration of IL-2 caused a sustained increase in liver-associated NK cells that was not diminished in IFN- $\gamma^{-/-}$  mice. The IL-12-induced recruitment in both hepatic NK and T cells was abrogated by in vivo treatment with anti-VCAM-1 mAbs, while treatment with anti-ICAM-1 Abs decreased only the recruitment of T cells in the IL-12-treated mice. The rapid loss of newly recruited hepatic NK cells in IL-12-treated mice did not occur in SCID mice or in B.MRL-*Fas*<sup>lpr</sup> (*Fas*<sup>-</sup>) and B6.Smn.C3H-*Fas*<sup>gld</sup> (*FasL*<sup>-</sup>) mutant mice, suggesting that T cells can actively eliminate hepatic NK cells through a Fas-dependent mechanism. These findings also imply that during the endogenous innate immune response to infectious agents or tumors or in the host response induced by cytokine therapies, the biologic effects of NK cells may be limited by T cell-mediated effects. *The Journal of Immunology*, 1998, 161: 6014–6021.

Natural killer cells comprise a population of large granular, non-T lymphocytes that act as multifunctional effector cells against infectious and neoplastic diseases (1). Specifically, NK cells produce and respond to a variety of proinflammatory cytokines, act as a primary component of the initial host response to many microbial agents, and mediate the spontaneous lysis of certain tumor, allogeneic, and bone marrow-derived target cells (1–4). Considerable insight has been recently provided into the molecular mechanisms of cytokine responsiveness (5–7) and cytolytic activity (8, 9) of NK cells. However, considerably less is known about the biology of these cells in vivo.

The recruitment of leukocytes from the blood into sites of infection or tumor development represents a critical early step in the

development of an effective immune response. Several recent reports have contributed to a better understanding of the mechanisms involved in the in vivo recruitment of NK cells from the vasculature into lymphoid and/or nonlymphoid organ sites within the host (2, 10–12). A number of biochemical and biologic agents have been shown to stimulate, in a cytokine-dependent manner, the recruitment of NK cells from the peripheral circulation in both lymphoid and nonlymphoid tissues (2, 11, 13). In particular, the treatment of mice with the potent cytokine-inducing agent poly-ICLC leads to a TNF-dependent increase in NK cell number and lytic activity within the liver (10), which is also dependent on the expression of VCAM-1/VLA-4<sup>5</sup> (11). Similarly, infection of mice with murine CMV (MCMV) or induction of IFN- $\alpha/\beta$  results in a unique redistribution of NK cells to the splenic marginal zone, and there is recruitment of NK cells to the liver following infection of the liver with MCMV and lymphocytic choriomeningitis virus (2, 14, 15). Depletion of NK cells has been shown to increase the susceptibility of mice to MCMV (16) and herpes simplex virus (17). More recently, new information has emerged that some viruses (18) can induce an IFN- $\alpha/\beta$ -dependent inhibition of IL-12 production that could contribute to the pathogenesis of such viral infections.

These findings coupled with the documented effects of IL-12 on NK- and T cell-mediated functions in vitro (reviewed in Ref. 19) and the demonstrated ability of these cells to mediate antimicrobial or anti-tumor functions in vivo suggest that a better understanding of the effects of IL-12 on NK and T cell recruitment and function in a model organ such as the liver may provide new insight into the in vivo effector and immunoregulatory roles of these cells. In addition, recent evidence showing that IL-12 and

\*Laboratory of Experimental Immunology, Division of Basic Sciences, and <sup>†</sup>Science Applications International Corp. Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21702; <sup>‡</sup>Pediatric Oncology Branch, National Institutes of Health, Bethesda, MD 20892; and <sup>§</sup>Department of Oncology, Hoffmann-La Roche, Inc., Nutley, NJ 07110

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<sup>3</sup> Current address: EntreMed, Inc., 9610 Medical Center Dr., Suite 200, Rockville, MD 20850.

<sup>4</sup> Address correspondence and reprint requests to Dr. Robert H. Wilttrout, Laboratory of Experimental Immunology, National Cancer Institute-Frederick Cancer Research and Development Center, Building 560, Room 31–93, Frederick, MD 21702-1201. E-mail address: wilttrout@mail.ncifcrf.gov

<sup>5</sup> Abbreviations used in this paper: VLA-4, very late antigen-4; MCMV, murine cytomegalovirus; FasL, Fas ligand; rmIL-12, recombinant murine IL-12; FCA, two-color cytometric analysis; tVCAM-1, truncated VCAM-1; rhIL-2, recombinant human IL-2.

IL-2 use parallel but distinct intracellular signaling pathways (20), reciprocally up-regulate the expression of each other receptors (19, 21), additively or even synergistically enhance the induction of cytokines and effector activity (22–25), and possess synergistic antitumor activity (26–30) suggests that a better understanding of the *in vivo* biology of these cytokines could help optimize their therapeutic utility. The present studies were undertaken to investigate and compare the mechanisms by which exogenous administration of IL-2 or IL-12 causes the recruitment of NK vs T cells to the liver and to determine the potential cross-regulatory interactions that might occur between these two leukocyte subsets. The results demonstrate that the recruitment of NK vs T cells to the liver in response to IL-12 vs IL-2 differs quantitatively and qualitatively, and that the ultimate disappearance of newly recruited NK cells is dependent on T cells and a Fas/FasL-mediated mechanism. These findings suggest that the effectiveness of biologic therapies may be limited in some organ sites as a consequence of the induction of NK-T cell interactions that ultimately lead to the elimination of specific lymphocyte populations.

## Materials and Methods

### Mice

Specific pathogen-free C57BL/6 and C57BL/6 SCID mice (6–8 wk of age) were obtained from the Animal Production Area, National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The following spontaneous or induced mutant mouse strains were purchased from The Jackson Laboratory (Bar Harbor, ME): B6.MRL-*Fas*<sup>gpr</sup>, B6.Smn.C3H-*FasL*<sup>gld</sup>, and C57BL/6J-*Icam1*<sup>tm1Bay</sup>. C57BL/6 IFN- $\gamma$ <sup>-/-</sup> (GKO) mice were obtained from our own mouse colony that was derived from mice donated by Dr. Dyana Dalton, Genentech (South San Francisco, CA). Mice were kept under specific pathogen-free conditions and were provided sterilized mouse chow (Ziegler Brothers, Gardner, PA) and water *ad libitum*. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23, 1985).

### Reagents

Recombinant human IL-2 (Tecin;  $3.2 \times 10^7$  U/mg) and recombinant murine IL-12 (rmIL-12;  $7 \times 10^6$  U/mg) were provided by Hoffmann-La Roche (Nutley, NJ). Stock aliquots of rmIL-12 in sterile Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free Dulbecco's PBS were stored at -70°C until use. The rmIFN- $\gamma$  (sp. act. =  $4.7 \times 10^6$  U/mg) was provided by Genentech. For *in vivo* administration the stock solutions were diluted as necessary with PBS containing 0.1% sterile-filtered B6 mouse serum and used within 48 h. The following hybridomas were obtained from American Type Culture Collection (Manassas, VA): YN1/1.7.4 (CRL-1878, rat anti-mouse CD54) and M/K2.7 (CRL-1909, rat anti-mouse CD10<sup>b</sup>). The mAbs were isolated from clarified ascites by affinity chromatography using GammaBind-Plus according to the manufacturer's instructions (Genex, Gaithersburg, MD), dialyzed into sterile Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free PBS, and stored at -20°C. Control IgG was isolated from rat serum as described above.

### Isolation and analyses of hepatic mononuclear cells

Leukocytes were obtained from the livers of control and cytokine-treated mice by a modified version (31) of a previously described procedure (32). Briefly, livers were first perfused with 25 ml of Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free PBS and excised. Cell suspensions were generated after a 2-min disruption in a stomacher (Tekmar, Cincinnati, OH). The resulting cell suspensions were centrifuged at  $500 \times g$  for 5 min, and the pellet was resuspended and filtered through 100- $\mu$ m nylon mesh. The cellular filtrate was centrifuged at  $500 \times g$  for 5 min, and the pellet was resuspended to 35 ml in Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free PBS. The cell suspension was then underlaid with 13 ml of Lympholyte-MJ (Cedar Lane Laboratories, Ontario, Canada) and centrifuged at  $1400 \times g$  for 30 min at 20°C. After centrifugation, 10 ml of the interface was aspirated, mixed with 40 ml of Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free PBS, and centrifuged at  $500 \times g$  for 5 min. The resulting cell pellet was resuspended in 0.2% BSA in Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free PBS for two-color cytometric analysis (FCA) or in RPMI supplemented with 5% FBS for NK activity as described below.

Cells were labeled with optimally titrated Abs, and cellular fluorescence was determined on  $1.5 \times 10^4$  cells using a FACScan analyzer (Becton Dickinson, San Jose, CA). NK cells or T cells were detected using R-

phycoerythrin-labeled NK1.1 (clone PK136) or FITC-labeled anti-CD3 (clone 145-2C11) obtained from PharMingen (San Diego, CA). In certain experiments the DX-5 Ab (provided by Lewis Lanier, DNAX, South San Francisco, CA) was substituted for NK1.1. The data were analyzed by the use of FACScan research and LYSYS software programs written for the Hewlett Packard Consort 30 microcomputer integral to the FACScan analyzer (Becton Dickinson). The percentage of cells bearing a particular phenotypic marker was determined on a pool of five livers per group, and the number of cells bearing that marker was then calculated by multiplying the percentage by the mean of the total number of cells isolated from the pooled livers. Lytic unit activity was assessed with a 4-h <sup>51</sup>Cr release assay against YAC-1 target cells as described previously (11).

### Isolation of total cellular RNA and Northern blot analysis

Total RNA was isolated from the liver using Trizol reagent (Life Technologies, Gaithersburg, MD). Five micrograms of total RNA was separated in a 1% agarose gel in 1 $\times$  MOPS buffer. After the electrophoresis, the gel was treated with 0.05 N NaOH for 20 min and then neutralized in 5 $\times$  MOPS buffer. The RNA was transferred overnight to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL) with 10 $\times$  SSC. The hybridization and detection were performed either with <sup>32</sup>P-labeled probe, according to the Hybond-N manufacturer's protocol or with digoxigenin-labeled antisense riboprobe, as follows. The murine ICAM-1 cDNA clone Ly-47 in pBluescript SK<sup>-</sup> and murine VCAM-1 cDNA clone Ly-59 were purchased from American Type Culture Collection. Clone Ly-59 was used as a template for the PCR with the sense (5'-AAGGTGAGGACG GAGGGGT-3') and the antisense (5'-CAGAGATCGTTGTATTCTGG-3') primers to amplify the 481-bp murine VCAM-1 cDNA. The plasmid, pmVCAM-1, was constructed by subcloning the PCR product into the vector pCR2.1 with a TA cloning kit (Invitrogen, San Diego, CA) such that the T7 promoter produces the antisense strand. This plasmid was capable of detecting and discriminating both full-length murine VCAM-1 as well as an alternative splice variant, truncated VCAM-1 (tVCAM-1). The riboprobes for murine ICAM-1 (Ly-47) and VCAM-1 (pmVCAM-1) were generated by T3 and T7 RNA polymerase with digoxigenin, respectively. The digoxigenin-labeled RNA probe was diluted to 100 ng/ml with DIG Easy Hyb buffer (Boehringer Mannheim, Indianapolis, IN) and was incubated with the membrane at 65°C overnight after prehybridization. The membrane was then washed twice in 0.1 $\times$  SSC/0.1% SDS at 65°C for 15 min. The detection of bound digoxigenin-labeled probes was performed according to the DIG System User's Guide for Filter Hybridization (Boehringer Mannheim) with disodium 3-(4-methoxyphospho{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1<sup>3,7</sup>]decan}-4-yl)phenyl phosphate (CSPD) as a substrate.

### Statistical analyses

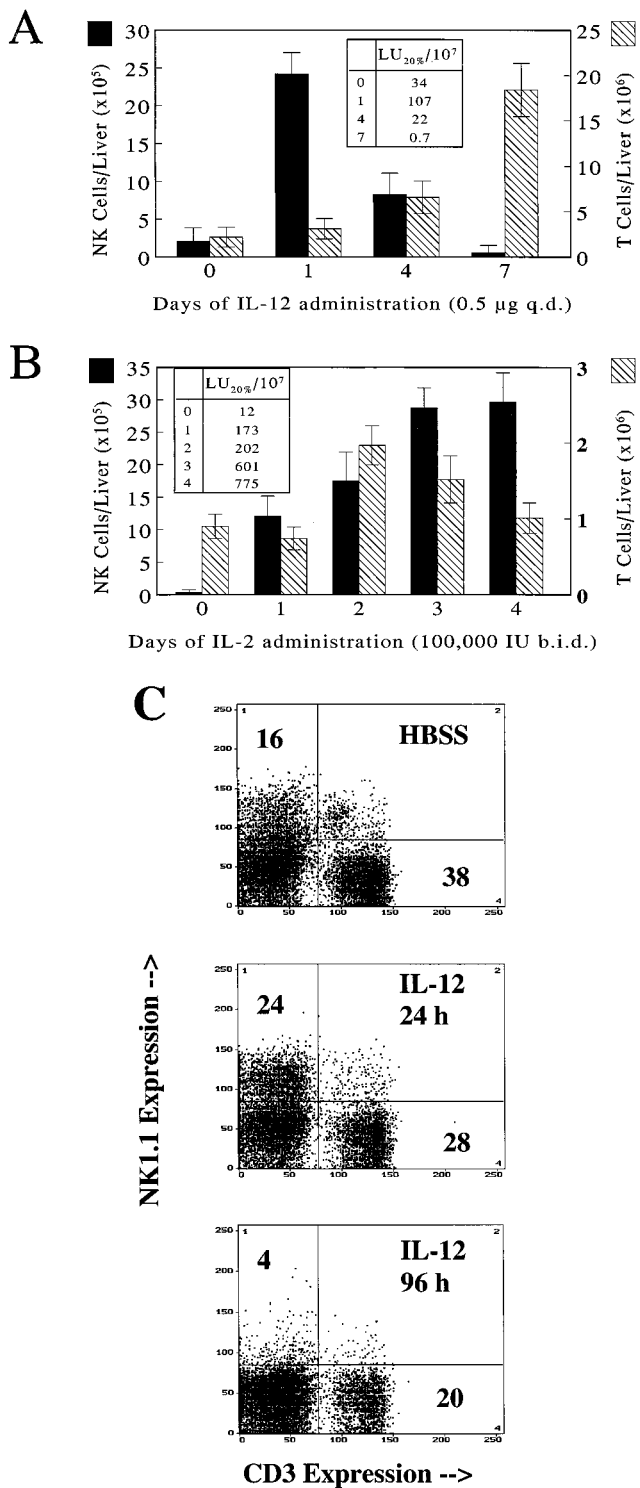
Experimental results were analyzed for their statistical significance by Student's *t* test. Results were considered statistically significant for  $p < 0.05$ .

## Results

### Contrast between IL-12- and IL-2-induced alterations in hepatic NK and T cell populations

Initial studies were performed to determine the unstimulated, homeostatic levels of resident hepatic NK and T cells in C57BL/6 mice. The results show that the percentage of NK1.1<sup>+</sup>, CD3<sup>-</sup>, and NK1.1<sup>-</sup>, CD3<sup>+</sup> cells were 16 and 38%, respectively (data not shown). In four separate determinations, the mean  $\pm$  SD numbers of resident hepatic NK and T cells were  $2.9 \pm 1.3 \times 10^5$  and  $1.9 \pm 0.3 \times 10^6$ , respectively.

IL-12-induced alterations in hepatic NK and T cells were investigated in C57BL/6 mice injected *i.p.* with 0.5  $\mu$ g of rmIL-12 daily for 7 consecutive days, and the livers were processed for the *ex vivo* determination of NK1.1<sup>+</sup>, CD3<sup>-</sup>, or NK1.1<sup>-</sup>, CD3<sup>+</sup> cells on days 1, 4, and 7 of treatment. Twenty-four hours after IL-12 administration, the number of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells recovered from the liver was increased approximately 10-fold over homeostatic levels to  $24.4 \times 10^5$  ( $p < 0.01$ ; Fig. 1A). The observed increase in hepatic NK cells was due largely to an increase in both lymphoid cell number as well as the frequency of NK1.1-expressing cells from 16% on day 0 to 24% on day 1 (Fig. 1C). Coinciding with the observed rmIL-12-induced increase in liver-associated NK cells was a corresponding increase in NK lytic activity by 24 h



**FIGURE 1.** Alterations in the number and frequency of NK and T cells induced in the liver by administration of rmIL-12. C57BL/6 mice were injected i.p. daily with either 0.5  $\mu\text{g}$  of rmIL-12 or 100,000 IU of human rIL-2 for 1–7 days. Leukocytes were isolated from the livers, and the numbers of NK1.1<sup>+</sup>, CD3<sup>-</sup> and NK1.1<sup>-</sup>, CD3<sup>+</sup> cells were determined by FCA for IL-12 (A) or IL-2 (B). C depicts changes in the frequency of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells on days 0, 1, and 4 in mice treated with IL-12. NK activity was determined as outlined in *Materials and Methods*.

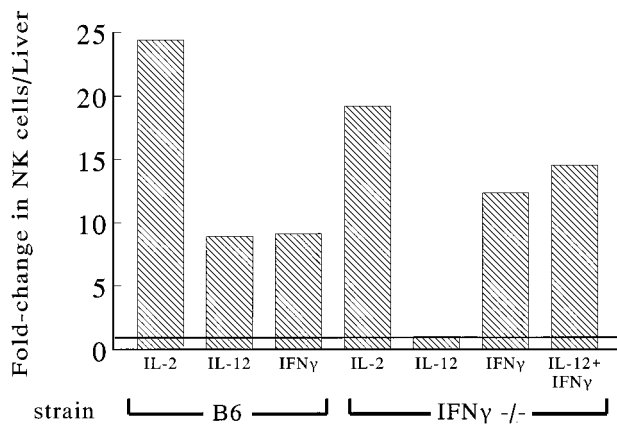
(Fig. 1A). The initial increase in hepatic NK cell number and lytic activity following rmIL-12 administration was not sustained by continued IL-12 administration, since the number of liver-associated NK cells declined to 8.3 and 0.8  $\times 10^5$  by days 4 and 7,

respectively (Fig. 1A). This decrease in NK1.1<sup>+</sup>, CD3<sup>-</sup> NK cells occurred largely because of a dramatic decrease in frequency from 24% on day 1 to 4% on day 4 (Fig. 1C). Concomitant with the decrease in hepatic NK cell number and lytic activity was an increase in liver-associated CD3<sup>+</sup>, NK1.1<sup>-</sup> cells to 7.2 and 18.8  $\times 10^6$  on days 4 and 7, respectively, of rmIL-12 administration ( $p < 0.01$ ; Fig. 1A).

To determine the IL-2-induced alterations in hepatic NK and T cells, C57BL/6 mice were injected i.p. with 1  $\times 10^5$  IU rhIL-2 twice daily for 4 consecutive days, and the livers were processed on days 1–4 of treatment. Similar to rmIL-12, 24 h after rhIL-2 administration, the number of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells recovered from the liver was increased approximately 10-fold over homeostatic levels ( $p < 0.01$ ; Fig. 1B). However, in marked contrast to the results of rmIL-12 injection, continued IL-2 administration resulted in a further increase in hepatic NK cell number, and by day 3 and 4 of treatment was approximately 26-fold greater than that in unstimulated mice ( $p < 0.01$ ; Fig. 1B). The increase in liver-associated NK cells induced by rhIL-2 was reflected in the marked augmentation of hepatic NK lytic activity (Fig. 1B). In addition, by 48 h of administration rhIL-2 induced a transient 2-fold increase in the number of NK1.1<sup>-</sup>, CD3<sup>+</sup> cells recovered from the liver ( $p < 0.01$ ), which returned to homeostatic levels by 96 h (Fig. 1B). Taken together these data demonstrate that compared with rhIL-2, rmIL-12 induces a rapid, but unsustainable, increase in liver-associated NK cells and lytic activity. Moreover, the data suggest that concurrent with the observed decrease in hepatic NK cell number induced by 4 days of rmIL-12 administration, there is a marked increase in the number of liver-associated T cells.

*IL-12 induction of hepatic NK cell, but not T cell, infiltrates is dependent on IFN- $\gamma$*

Regulatory events that could contribute to the observed pattern of IL-12-induced liver-associated lymphocyte alterations were next investigated. Previous studies have shown that many, but not all, the in vivo effects of IL-12 can be attributed to the induction of IFN- $\gamma$  production (33, 34). To study the role of IFN- $\gamma$  in mediating the observed IL-12-induced changes in hepatic leukocyte subsets, C57BL/6 and B6 mice deficient in IFN- $\gamma$  (IFN- $\gamma^{-/-}$ ) were injected i.p. with 0.5  $\mu\text{g}$  of rmIL-12 daily for 4 consecutive days, and the livers were processed for the determination of NK and T cell numbers on days 1 and 4, respectively. In agreement with the data summarized in Fig. 1A, 24 h after rmIL-12 administration the number of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells recovered from the livers of wild-type C57BL/6 mice was augmented approximately ninefold (to 4.9  $\times 10^6$  cells/liver) over the unstimulated level (5.5  $\times 10^5$  cells/liver;  $p < 0.01$ ; Fig. 2). In contrast, under identical treatment conditions, the injection of rmIL-12 into B6 IFN- $\gamma^{-/-}$  mice did not result in a significant increase in liver-associated NK cells (3.8  $\times 10^5$ , 4.2  $\times 10^5$ , and 4.8  $\times 10^5$  cells/liver on days 0, 1, and 4, respectively). However, by day 4 of continued rmIL-12 administration, a comparable increase in the number of liver-associated T cells was observed in both the C57BL/6 and B6 IFN- $\gamma^{-/-}$  strains of mice (Fig. 3). These data demonstrate that the IL-12-induced increase in hepatic NK cells, but not T cells, was dependent on endogenous IFN- $\gamma$ . This conclusion was supported by additional experimental groups showing that comparable levels of IL-12-induced liver-associated NK cell numbers were observed 24 h following the i.p. injection of 2.5  $\times 10^5$  U of rmIFN- $\gamma$  to both C57BL/6 and B6 IFN- $\gamma^{-/-}$  mice, and this effect was not further enhanced in the IFN- $\gamma^{-/-}$  strain by rmIL-12 (Fig. 2). The role of endogenous IFN- $\gamma$  in the rhIL-2-induced hepatic NK cell infiltration was determined using C57BL/6 and B6 IFN- $\gamma^{-/-}$  mice that were treated i.p. with 1  $\times 10^5$  U of rhIL-2 twice daily. Twenty-four hours later

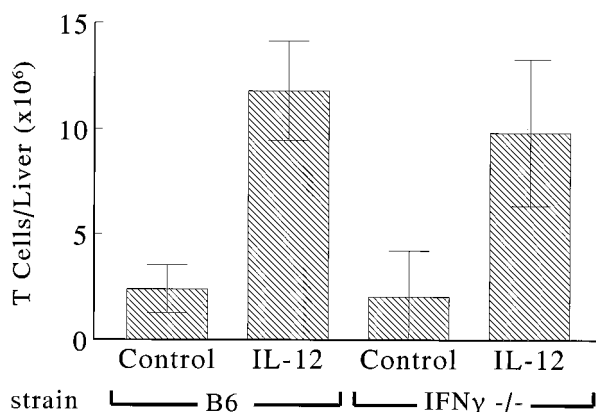


**FIGURE 2.** Role of IFN- $\gamma$  in IL-12-induced changes in hepatic NK cells. Wild-type C57BL/6 mice or C57BL/6 mice exhibiting targeted disruption of the IFN- $\gamma$  gene (IFN- $\gamma$ <sup>-/-</sup>) were injected i.p. with 0.5  $\mu$ g of rmIL-12, 2.5  $\times 10^5$  U of rmIFN- $\gamma$ , or 100,000 IU of rhIL-2. Leukocytes were isolated 24 h later from the livers, and the number of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells was determined.

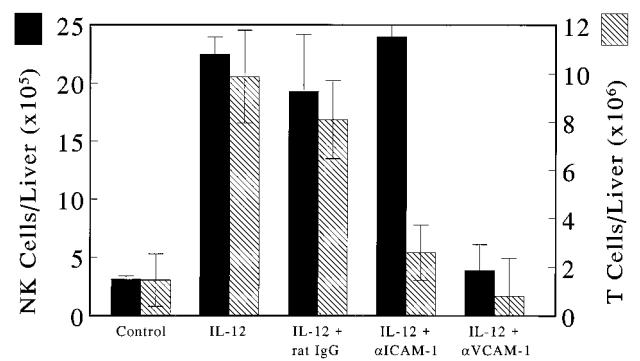
the livers were processed for the determination of NK cells. Under these experimental conditions a similar fold increase in the number of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells was obtained from the livers of C57BL/6 and B6 IFN- $\gamma$ <sup>-/-</sup> treated with rhIL-2 (Fig. 2). Taken together, these data demonstrate that endogenous IFN- $\gamma$  was both critical and specific for hepatic NK cell infiltration induced by rmIL-12.

*IL-12-induced hepatic NK cell infiltration is strictly dependent on VLA-4/VCAM-1 interaction, while T cell infiltration proceeds through a mechanism dependent on the expression of both VCAM-1 and ICAM-1*

Previous studies from our laboratory have shown that the biologic response modifier poly-ICLC induces the recruitment of NK cells to lungs and liver via a TNF- $\alpha$ -dependent mechanism (10), and that this is at least partly mediated through VLA-4/VCAM-1 interaction (11). The involvement of VLA-4 and VCAM-1 interaction was also observed for the IL-2-induced NK cell infiltration into lungs and liver (11). To investigate the potential regulatory role of VCAM-1 in mediating the observed IL-12-induced alterations in hepatic leukocyte subsets, C57BL/6 mice were injected i.p. with 0.5  $\mu$ g of rmIL-12 daily for 4 consecutive days and with 500  $\mu$ g of either  $\alpha$ -VCAM-1 mAb or control rat IgG immediately



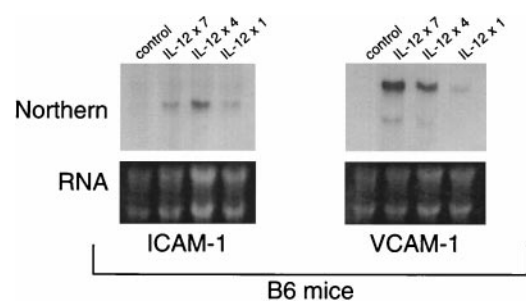
**FIGURE 3.** Role of IFN- $\gamma$  in IL-12-induced changes in hepatic T cells. Wild-type or IFN- $\gamma$ <sup>-/-</sup> C57BL/6 mice were injected i.p. for 4 days with 0.5  $\mu$ g of rmIL-12. Leukocytes were then isolated from the livers, and the number of NK1.1<sup>+</sup>, CD3<sup>+</sup> cells was determined.



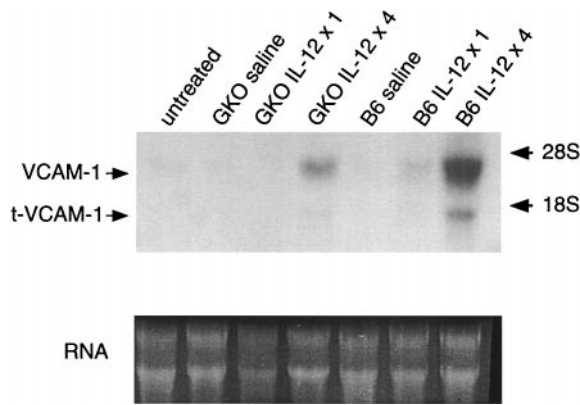
**FIGURE 4.** Role of VCAM-1 and ICAM-1 expression in IL-12-induced recruitment of NK and T cells to the liver. C57BL/6 mice were injected i.p. with 0.5  $\mu$ g of rmIL-12 daily for 1 or 4 days. Some mice also received i.p. injections of 500  $\mu$ g of  $\alpha$ -VCAM-1,  $\alpha$ -ICAM-1 mAb, or control rat IgG on days 1 or on days 1 and 3. Leukocytes were then isolated on day 1 for assessment of the effects on the number of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells and on day 4 for assessment of the effects on the NK1.1<sup>+</sup>, CD3<sup>+</sup> cell subset.

before the start of rmIL-12 treatment and again on day 3. The livers were then processed for the quantitation of NK and T cells on days 1 and 4, respectively. Twenty-four hours after the initial injection of rmIL-12 alone or in combination with control rat IgG, the number of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells recovered from the liver was increased approximately eight- to ninefold over the control homeostatic level to 23.3 and 19.2  $\times 10^5$ , respectively ( $p < 0.01$ ; Fig. 4). Coinjection of  $\alpha$ -VCAM-1 mAb inhibited the rmIL-12-induced NK cell infiltration of liver such that the number of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells was not increased over the level observed in control mice (Fig. 4). Similar results were obtained on day 4 for the inhibition of rmIL-12-induced hepatic T cell infiltration by treatment of mice with  $\alpha$ -VCAM-1 mAb (Fig. 4).

Because LFA-1/ICAM-1 interaction is also important in mediating lymphocytic distribution patterns, subsequent experiments investigated the influence of  $\alpha$ -ICAM-1 mAb on IL-12-induced hepatic NK and T cell infiltration. Using an experimental design identical with that outlined above, coinjection of  $\alpha$ -ICAM-1 mAb resulted in a 75% inhibition of the rmIL-12-induced increase in liver-associated T cells ( $p < 0.01$ ), but did not alter the NK cell infiltration pattern (Fig. 4). To investigate whether the apparent differential regulation of NK and T cell infiltration patterns by ICAM-1, compared with VCAM-1, was due to a difference in the kinetics of their induction by rmIL-12, total hepatic RNA was purified from unstimulated C57BL/6 mice and from mice treated i.p. with 0.5  $\mu$ g of rmIL-12 for 1, 4, and 7 days for assessment of ICAM-1 and VCAM-1 gene expression by Northern blot analyses.



**FIGURE 5.** Kinetics of hepatic gene expression of ICAM-1 and VCAM-1 in mice treated with IL-12. C57BL/6 mice were injected i.p. daily for 1, 4, or 7 days with 0.5  $\mu$ g of rmIL-12. Total liver RNA was extracted at various times, and ICAM-1 and VCAM-1 gene expression was detected by Northern blot analysis.



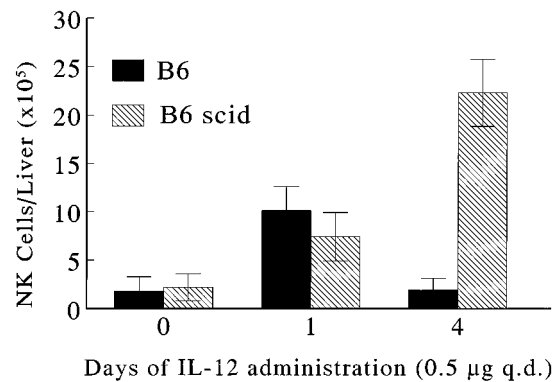
**FIGURE 6.** Relationship between IFN- $\gamma$  induction and VCAM-1 and tVCAM-1 gene expression during rmIL-12 induction of hepatic cell infiltration. C57BL/6 wild-type and IFN- $\gamma^{-/-}$  mice were injected i.p. with 0.5  $\mu$ g of rmIL-12. Twenty-four hours later the livers were harvested, total RNA was extracted, and Northern blot analysis of VCAM-1 gene expression was performed.

The data presented in Fig. 5 show that the expression of both VCAM-1 and ICAM-1 in the liver is not detected in unstimulated mice. However, within 24 h following rmIL-12 administration the genes for both ICAM-1 and VCAM-1 were expressed, and their expression further increased with continued treatment. Taken together these data demonstrate that IL-12-induced recruitment of hepatic NK cells is strictly dependent on the VLA-4/VCAM-1 interaction, while T cell infiltration proceeds via both VCAM-1- and ICAM-1-dependent pathways.

The relationship between endogenous IFN- $\gamma$  and VCAM-1 expression for the induction of hepatic NK cell infiltration by rmIL-12 was next investigated. Total hepatic RNA was purified from unstimulated C57BL/6 and B6 IFN- $\gamma^{-/-}$  mice 24 h after the i.p. administration of 0.5  $\mu$ g of rmIL-12 for assessment of VCAM-1 gene expression by Northern blot analyses. The data shown in Fig. 6 confirm that there was no detectable endogenous expression of VCAM-1 in the livers of unstimulated C57BL/6 or B6 IFN- $\gamma^{-/-}$  mice. Following administration of rmIL-12, expression of full-length VCAM-1 was detected by 24 h in C57BL/6 mice but remained undetectable in B6 IFN- $\gamma^{-/-}$  mice (Fig. 6). At this time point, the induction of hepatic NK cell infiltration was observed in C57BL/6, but not B6, IFN- $\gamma^{-/-}$  mice (Fig. 2). By 4 days of IL-12 administration there was a pronounced induction of both VCAM-1 and tVCAM-1 mRNA expression in C57BL/6 wild-type mice, with a lower level of expression in IFN- $\gamma^{-/-}$  mice. Overall, these data demonstrate that the regulatory pathway for the rmIL-12-induced recruitment of hepatic NK cells involves both the production of endogenous IFN- $\gamma$  and the subsequent expression of the VCAM-1 adhesion molecule.

#### *The disappearance of IL-12-induced hepatic NK cells does not occur in SCID mice*

Because the increase in IL-12-induced hepatic NK cell number is rapidly lost, we speculated that some active down-regulating event had been induced. Since there was an increase in the appearance of hepatic T cells that coincided with the decline in hepatic NK cells, we further speculated that T cells might be contributing to the loss of the NK cells. This hypothesis was tested using T cell-immunodeficient C57BL/6 SCID mice (Fig. 7). These SCID mice were injected i.p. with 0.5  $\mu$ g of rmIL-12 daily for 4 consecutive days, and the number of liver-associated NK cells was determined on days 1 and 4. Twenty-four hours after the initial injection of IL-12,



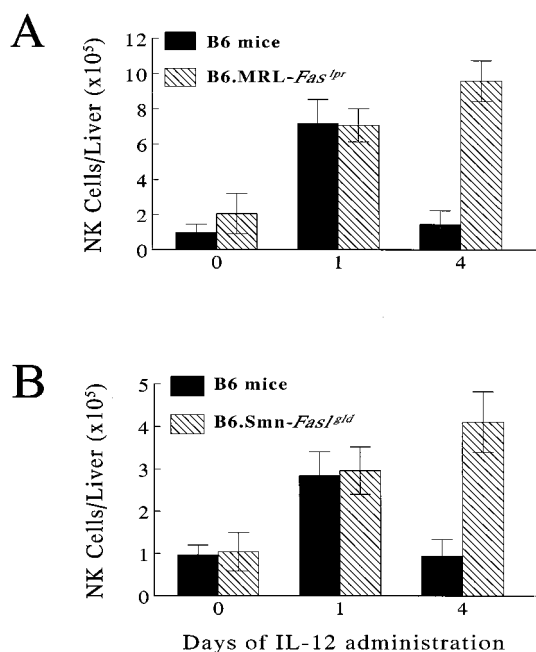
**FIGURE 7.** The disappearance of IL-12-induced hepatic NK cells is abrogated in SCID mice. C57BL/6 SCID mice were injected i.p. with 0.5  $\mu$ g of rmIL-12 daily for 1 or 4 days, and the number of hepatic NK1.1<sup>+</sup>, CD3<sup>-</sup> cells was determined by FCA.

an approximately threefold increase in the number of hepatic NK cells was observed in B6 SCID mice ( $p < 0.01$ ). However, unlike the results obtained in wild-type C57BL/6 mice, in which a rapid loss of these cells then occurs, there was instead a further increase in the number of liver-associated NK cells in B6 SCID mice. Since SCID mice are devoid of functional T or B cells, these results demonstrate that either T or B cells are involved in the loss of hepatic NK cells. Since the repeated administration of IL-12 results in an increase in T cells but no change in hepatic B cell numbers in immunocompetent mice, the results implicate a role for T cells in NK cell disappearance.

#### *Fas/Fas ligand interactions are involved in mediating the eventual disappearance of IL-12-induced hepatic NK cells*

Since the loss of IL-12-induced hepatic NK cells occurred coincident with a recruitment of hepatic T cells and did not occur in SCID mice, we then speculated that because activated T cells often express FasL, these infiltrating T cells might be eliminating the IL-12-recruited NK cells from the liver by a Fas/FasL-dependent mechanism. Such a mechanism could be somewhat analogous to the known involvement of Fas/FasL interactions in restricting immigration of activated lymphocytes into the testes, the anterior chamber of the eye, and the placenta (35–37), except that in this case the effect could be mediated by the recruited T cells.

To investigate whether Fas/Fas ligand interaction also was involved in mediating the decrease in hepatic NK cells following continuous IL-12 administration, C57BL/6, B6.MRL-Fas<sup>lpr</sup> (B6 Fas<sup>-</sup>), or B6.Smn.C3H-Fas<sup>gld</sup> (B6 FasL<sup>-</sup>) mice were injected i.p. with 0.5  $\mu$ g of rmIL-12 daily for 4 consecutive days, and the number of liver-associated NK cells was determined on days 1 and 4 of treatment. Twenty-four hours after IL-12 administration into C57BL/6, B6 Fas<sup>-</sup>, or B6 FasL<sup>-</sup> mice, the number of hepatic NK cells was increased approximately 7-, 3.5-, and 2.9-fold, respectively ( $p < 0.01$ ; Fig. 8). In agreement with Fig. 1A, the induction of hepatic NK cell infiltration by rmIL-12 in C57BL/6 mice was transient, and continued treatment resulted in a decrease in newly recruited hepatic NK cells such that by day 4 the number of liver-associated NK cells approximated that observed in unstimulated mice. In contrast, continuous treatment of B6 Fas<sup>-</sup> or B6 FasL<sup>-</sup> mice with rmIL-12 for 4 days resulted in the sustained induction of liver-associated NK cell infiltration and an approximately 5- or 4.7-fold increase, respectively, in NK cell number compared with that in unstimulated mice ( $p < 0.01$ ). These effects occurred despite similar fold increases in hepatic T cells and only minimal changes in the CD4<sup>+</sup>/CD8<sup>+</sup> ratio on days 1 and 4 in both Fas<sup>-</sup> and



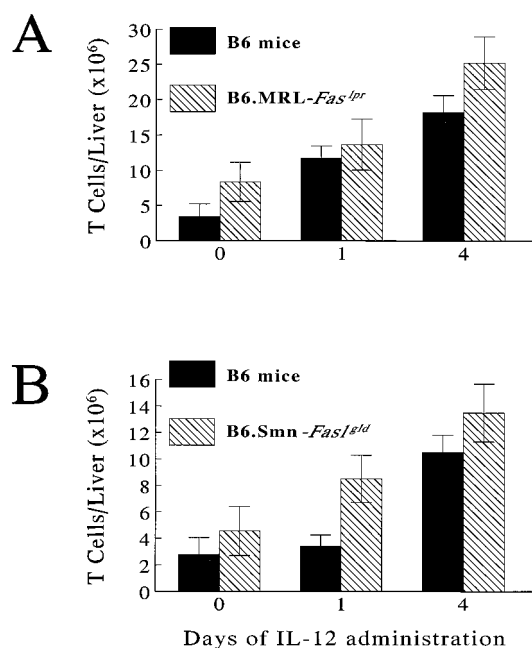
**FIGURE 8.** Persistence of IL-12-induced hepatic NK cells in B6.MRL-*Fas*<sup>lpr</sup> (*Fas*<sup>-</sup>; A) and B6.Smn.C3H-*Fas*<sup>gld</sup> (*FasL*<sup>-</sup>; B) mice. Mice were injected i.p. daily with 0.5  $\mu$ g of rmIL-12 for 1 or 4 days. Leukocytes were isolated from the liver on days 1 and 4, and the number of NK1.1<sup>+</sup>, CD3<sup>-</sup> cells was determined by FCA.

*FasL*<sup>-</sup> mutant mice (Fig. 9). These results demonstrate that the T cell-coincident loss of IL-12-induced hepatic NK cells is also dependent on a *Fas*/*FasL*-mediated event.

## Discussion

In the present study we have demonstrated that the systemic administration of rmIL-12 into mice results in temporal alterations in intrahepatic NK cells characterized by an early infiltration that is dependent on IFN- $\gamma$  and VCAM, followed later by infiltration of T cells and concurrent loss of NK cells via a *Fas*/*FasL*-dependent mechanism. Moreover, we have shown that the observed influx of T cells occurs via a IFN- $\gamma$ -independent, VCAM-1- and ICAM-1-dependent mechanism. Previous studies that have investigated the hepatic lymphocyte compartment in IL-12-treated mice have noted an increased number of CD8<sup>+</sup> T cells and NK cells, but not of CD4<sup>+</sup> T cells (38). This is the first report showing that the regulatory pathways involved in the IL-12-mediated induction of liver-infiltrating NK cells and T cells are distinct, and further suggest that once in the hepatic parenchyma, these lymphocyte populations may undergo a dynamic interaction that determines their tissue life span.

The pattern of IL-12-induced lymphocyte infiltration of the hepatic parenchyma was in marked contrast to that obtained following injection of mice with rhIL-2, in which an IFN- $\gamma$ -independent increase in the intrahepatic lymphocyte population was primarily restricted to the NK cell compartment and was of long duration. The immunomodulatory effects mediated by IL-12 or IL-2 on T and NK cells are in part overlapping and involve induced production of cytokines (39–41), enhanced transcription and production of several granule-associated proteins (42, 43), increased expression of cell surface adhesion molecules (44, 45), enhancement of cytotoxicity, and increased proliferation of activated cells (1–5, 19). Although these observations suggest that IL-12 and IL-2 may share common activation pathways, differences in their effects



**FIGURE 9.** Hepatic T cell infiltration in B6.MRL-*Fas*<sup>lpr</sup> (*Fas*<sup>-</sup>; A) and B6.Smn.C3H-*Fas*<sup>gld</sup> (*FasL*<sup>-</sup>; B) mice in response to IL-12. Mice were injected i.p. daily with 0.5  $\mu$ g of rmIL-12 for 1 or 4 days. Leukocytes were isolated from the liver on days 1 and 4, and the number of NK1.1<sup>-</sup>, CD3<sup>+</sup> cells was determined by FCA.

have also been reported. Only IL-2 is mitogenic for resting NK cells, inducing their proliferation in vitro and in vivo, whereas IL-12 induces proliferation only in preactivated NK and T cells, and does so less effectively than IL-2 (46, 47). Recent studies have suggested that the functional differences observed in IL-2- and IL-12-stimulated NK and T cells may depend at least in part on differential gene regulation (48). The markedly contrasting impact of IL-12 or IL-2 treatment on liver-associated NK and T cells as seen in our studies suggests that the nonoverlapping effects of these two cytokines predominate during the induction, infiltration, and tissue residence of these lymphocyte populations.

Many of the in vivo immunomodulatory effects of IL-12 have been ascribed to its ability to induce IFN- $\gamma$  production. Therefore, the various activities ascribed to IL-12 represent the sum of activities attributable to IFN- $\gamma$ -dependent and IFN- $\gamma$ -independent effects of this cytokine. For example, IL-12 is pivotal for the differentiation of naive CD4<sup>+</sup> Th0 cells to cells with a Th1 phenotype through regulation of IFN- $\gamma$ , and although both IL-2 and IL-12 induce IFN- $\gamma$  production, IL-2 is relatively inefficient for inducing Th1 cell differentiation (49). The use of mice deficient in IL-12 (50), IFN- $\gamma$  (51, 52) or IFN- $\gamma$ R (33) have proven instrumental in studying this complex relationship. In our studies the induction of the hepatic T cell infiltrate by rmIL-12 was similar in IFN- $\gamma$ <sup>-/-</sup> mice and wild-type mice. These data imply that although a regulatory circuit may exist between IL-12 and IFN- $\gamma$  for T cell differentiation, induction of IFN- $\gamma$  following IL-12 administration is not involved in the subsequent recruitment and expansion of the intrahepatic T cell compartment.

In contrast to that caused by IL-2, the IL-12-mediated increase in the intrahepatic NK cell population was abrogated in IFN- $\gamma$ <sup>-/-</sup> mice, indicating a requirement for endogenous IFN- $\gamma$  production in NK cell mobilization and infiltration. In this regard, it is important to note that the administration of rmIFN- $\gamma$  was sufficient to induce increased hepatic NK cellularity to levels comparable to those observed with IL-12 alone and was not further augmented by

concomitant IL-12 treatment. The importance of IFN- $\gamma$  in mediating the antiviral effects of NK cells in the liver has been previously reported by Tay and Welsh (53), who demonstrated that production of IFN- $\gamma$  by hepatic NK cells was the predominant mechanism for regulating MCMV synthesis in the liver. However, the mechanism through which IL-12 induction of IFN- $\gamma$  leads to the initial NK cell infiltration of hepatic parenchyma is currently unknown, but may involve the up-regulation of specific cell surface adhesion molecules. NK cells can be induced to extravasate from peripheral blood and infiltrate into both lymphoid and non-lymphoid organs in response to a variety of pathologic conditions or specific biologic response modifiers (1–4). An initial event in this process involves NK cell adhesion to endothelial barriers. Previous studies from our laboratory have shown that the VCAM-1/VLA-4-dependent interaction is critical for the induction of NK cell infiltration by poly-ICLC- and IL-2 into the lungs, liver, and tumor lesions after mobilization from the bone marrow, and that the principal site of interaction during this critical event is the interface between peripheral blood and endothelium (11). In the current study we have shown that 1) the expression of the genes encoding VCAM-1 and ICAM-1 in the liver is augmented by IL-12 treatment; and 2) the initial increase in IL-12-induced hepatic NK cells is attenuated by blocking Abs directed against VCAM-1, but not ICAM-1. Further, the induction of hepatic VCAM-1 RNA is minimal in IFN- $\gamma^{-/-}$  mice 24 h after IL-12 treatment, and this correlates with a concurrent lack of NK cell infiltration in these mice as well. Taken together these results demonstrate a link among IL-12, IFN- $\gamma$ , and VCAM-1 in the regulation of NK cell infiltration into the hepatic parenchyma.

Perhaps even more interesting than the mechanism(s) governing the initial recruitment of hepatic NK cells by IL-12 is the very rapid subsequent loss of this population. Our experimental results performed in various normal and mutant mouse strains demonstrate that the loss of these NK cells occurs in conjunction with marked local T cell recruitment, is absent in T-deficient mice, and depends on Fas/FasL-mediated events. There is precedent for the Fas/FasL-mediated loss of leukocytes in several model systems. A major area of interest in this regard derives from studies that reported the killing of Fas<sup>+</sup> T cells by FasL<sup>+</sup> tumor cells (54–56). These results provide evidence that tumor cells can in some situations protect themselves from effector T cells attempting to infiltrate and destroy neoplasms. Further, immune privilege in some normal tissues is maintained via expression of FasL that serves to initiate Fas-mediated apoptosis of potentially dangerous infiltrating leukocytes (35–37, 57, 58). There also is some evidence that one leukocyte population may under some conditions destroy another, as highly Ag-specific CTL (anti-H-2<sup>k</sup>) have been reported to lyse syngeneic bystander BALB/c (H-2<sup>d</sup>) Con A blasts in a Fas/FasL-dependent, perforin-independent manner (59). Other studies have reported that CD4<sup>+</sup> T cells stimulated with anti-CD3 mAb or PMA and ionomycin induced apoptosis in cocultured inactivated CD8<sup>+</sup> T cells, and this effect was inhibited by addition of soluble Fas mAb (60).

To our knowledge, these results provide the first evidence for a unique Fas/FasL-dependent regulatory event in which where there is a Fas/FasL-mediated elimination of newly recruited NK cells in vivo and show that this effect occurs in the absence of any overt Ag stimulation of the T cell compartment. These results support the hypothesis that Ag-independent, cytokine-mediated events are sufficient in vivo for the loss of a leukocyte subset. We speculate that this effect is T cell dependent because it occurs coincident with the appearance of T cells in the liver and is absent in T cell-deficient SCID mice. Such a down-regulatory process may be vital for preventing tissue damage or other undesirable effects during the evo-

lution of an innate immune response and may provide a self-limiting safeguard mechanism for unrestrained effects of activated leukocytes. The technology to formally investigate the role of Fas and FasL through detection on the appropriate leukocyte subsets is now under development, and future studies associating the expression of these molecules with apoptotic functions should soon be possible. Alternatively, such effects might also result in a premature down-regulation of a beneficial immune response. Studies supporting these suggestions have been reported by Welsh and colleagues (61), who have shown that T cells activated by a viral infection become susceptible to apoptosis after repeated stimulation through the TCR.

Another intriguing possibility is that in some cases the Fas/FasL-dependent elimination of one leukocyte subset by another could be deleterious to a more beneficial role of some effector cells, and therefore limit overall therapeutic efficacy. For example, some studies investigating the antitumor activity of IL-12 have concluded that NK cells are not the primary cell type involved in this process (62). However, one interpretation of our results could be that in some organ sites in T cell-competent mice, treatment with IL-12 can result in a relatively rapid depletion of NK cells, thereby severely limiting their potential contribution to any beneficial therapeutic effect. The absence of such cells might contribute to the observed difficulties in the treatment of diseases such as various chronic infections or metastatic disease in organ sites such as the liver. Therefore, it remains possible that the antitumor activity of IL-12 could be further augmented in some anatomical sites by the continued presence of an NK cell infiltrate. A broader implication of these findings is that the transition between an initial innate immune response and a subsequent adaptive response may not always proceed in a manner that takes full advantage of all available effector cells. In particular, while the rapid down-regulation of an early innate response by a subsequent adaptive response may serve a protective role in limiting organ damage during the immune response to acute infections, it may hamper the full, sustained engagement of mechanisms required to induce complete regression of cancers or some chronic infectious diseases.

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