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Chronic *Listeria* Infection in SCID Mice: Requirements for the Carrier State and the Dual Role of T Cells in Transferring Protection or Suppression[1]

Vatsala Bhardwaj, Osami Kanagawa, Paul E. Swanson, and Emil R. Unanue[2]

Listeriosis in mice with the SCID mutation results in a chronic infection. The chronic infection is characterized by abundant granulomas and neutrophil infiltrates. Both lesions were particularly noticeable in the liver. In the liver, about 95% are granulomas with 5% microabscesses involving intrahepatic infection. The majority of *Listeria* resided in membrane-bound vacuolar structures of the macrophages and not in the cytosol. Three manipulations resulted in alterations in the equilibrium between granulomas and liver microabscesses, with massive transfer of the infection to the hepatocyte and dissolution of the granulomas: depletion of neutrophils and neutralization of IFN-γ and TNF-α. We did not find a role for IL-12, IL-10, or nitric oxide. Adoptive transfer studies showed a decisive role for both $CD4^+$ and $CD8^+$ T cells for an effective immune response, i.e., clearance of bacteria, granuloma formation with lymphocytes, and disappearance of microabscess. Clearance of *Listeria* was induced by transfer of $CD8^+$ T cells from mice with targeted disruption of the IFN-γ structural gene (IfgTM1KO), even in the presence of neutralizing mAb to IFN-γ. In marked contrast, transfer of $CD4^+$ T cells from IfgTM1KO mice exacerbated the infection in the chronically infected SCID mice, resulting in increased mortality with dissolution of the granulomas and severe hepatic infection with neutrophil infiltration. Thus, these data indicate that both IFN-γ-dependent and -independent mechanisms are operative in the context of a chronic listerial infection. ***The Journal of Immunology,*** **1998, 160: 376–384.**

Previous studies from our laboratory examined the course of acute infection with *Listeria monocytogenes* in the SCID mouse (Ref. 1, reviewed in Ref. 2). While deficient in lymphocyte immunity, the SCID mouse provides important information on how natural or T-independent mechanisms of immunity operate. From the study in SCID mice the role of various cytokines was ascertained. SCID mice developed partial protection to *Listeria monocytogenes*: they mobilized neutrophils early in the infection and activated the macrophage, in part, by virtue of IFN-γ released by NK cells (3). However, SCID mice did not develop sterilizing immunity, harboring *Listeria monocytogenes* organisms for many weeks.

This study evaluates the chronic carrier state of *Listeria monocytogenes* infection in the SCID mouse. We wanted to compare the parameters of natural resistance in the chronic infected SCID mouse with those from SCID or normal mice with acute listeriosis. The purpose was to examine whether the basic parameters of resistance would change during chronic infection. Chronic infection with many intracellular pathogens may not result in the complete elimination of the microbe, leading to the speculation of whether the state of resistance and immunity changes and/or deteriorates with time. A second component that we evaluated here is the role of the T cells transferred to SCID mice that are chronically infected. Is a chronic *Listeria* state of infection effective in priming lymphocytes, and if so, how is the lymphocyte carrying out sterilizing immunity? Along these lines, it is important to stress that although macrophage activation by IFN-γ was a sine qua non for *Listeria* protection (1, 4), this activation did not bring about sterilizing immunity, which required T cells. The results of *Listeria monocytogenes* infection in the SCID mouse are highly eloquent in this regard: the development of extensive macrophage activation with participation of nitric oxide ($NO^{\cdot}$)[3], (5), yet a chronic state of infection developed in which the infective organism and the host entered into a state of apparent equilibrium.

Materials and Methods

Animals

C.B-17/ICR SCID and C.B-17/ICR (C.B-17) mice (6) were bred in pathogen-free conditions at Washington University (St. Louis, MO). Mice were used at 6 to 12 wk of age. BALB/c mice and BALB/c-IfgTM1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 6 to 8 wk of age. The latter mice did not express IFN-γ as a result of a genetic ablation of the structural gene for IFN-γ (7).

Abs and reagents

RB6-8C5 is a rat mAb (IgG2b) specific for murine neutrophils initially produced by R. Coffman (DNAX, Palo Alto, CA) (8–10). The anti-IL-10 mAb, 2A5, and Red-T, a hamster anti-IL-12 mAb, were previously described and used by Tripp, Beckerman, and Unanue as described in Reference 11 and Tripp, Kanagawa, and Unanue, as described in Reference 12. Rat mAb was purified by affinity chromatography using standard procedures. Hamster anti-mouse TNF mAb, TN3-19.12 (13), and hamster anti-mouse IFN-γ mAb, H22.1 (4) were kindly donated by Dr. R. D. Schreiber (Washington University, St. Louis, MO). These reagents were used as purified Ig and were endotoxin free, as determined by the *Limulus* assay (Whittaker Bioproducts, Walkersville, MD). RL172 (anti-CD4) and 3.155 (anti-CD8) were used as culture supernatants. FITC-conjugated rat anti-CD4 mAb (clone CT-CD4) and FITC-conjugated rat anti-CD8 mAb (clone CT-CD8a) were purchased from Caltag Labs., San Francisco, CA. Aminoguanidine hemisulfate salt (Sigma Chemical Co., St. Louis, MO) was

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[3] Abbreviations used in this paper: $NO^{\cdot}$, nitric oxide; KO, knock-out.

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injected twice daily for 6 days (5) at a concentration of 6 mg in 0.5 ml of pyrogen-free saline.

Generation of splenic cells

Immune cells were derived from mice after a primary or a secondary immunization. For primary infection, C.B-17 mice were immunized i.p. with 5×10^3 viable *Listeria* (strain EGD), in 0.2 ml of pyrogen-free saline (LD_{50}:10^4). The secondary infection was initiated 14 days after the primary infection with 5×10^4 live *Listeria*, injected i.p. in 0.2 ml of pyrogen-free saline. All the mice survived the secondary infection with this dose of *Listeria* compared with naive mice, all of which died. In experiments utilizing unfractionated splenic cells from naive mice, single cell suspensions were made and RBC were lysed with Tris ammonium chloride. Cells were sieved through a Nytex filter (Becton, Dickinson, Lincoln Park, NJ), and resuspended at 5×10^8 cells per ml in pyrogen-free saline. For the adoptive transfer of T cell subsets, after lysis of the RBC, negative selection of $CD4^+$ and $CD8^+$ T cells from primed or naive mice was conducted by incubating 5×10^7 cells/ml with 10% v/v of undiluted culture supernatants of anti-CD4 (RL172) and anti-CD8 (3.155) Ab and 1:10 dilution of rabbit serum as a source of complement (Low-tox rabbit complement; Pel-Freeze Biologics, Rogers, AR) for 45 min at 37°C. Two rounds of treatment were performed. This protocol consistently resulted in reduction of the targeted subsets to <2% of the total spleen cells as assessed by flow cytometric analysis using FITC-conjugated anti-CD4 and anti-CD8 mAbs. Subsequently, cells were sieved through a Nytex filter, and resuspended at 5×10^8 cells per ml in pyrogen-free saline (see Fig. 6 for one representative result that includes data on the treated cells). (Treatment with both anti-CD4 and anti-CD8 essentially eliminated all CD3-bearing T cells. Operationally, the CD4-depleted spleen cells are called CD8 T cells and vice versa.)

Adoptive immunization

The SCID mice were injected i.v. with 10^4 *Listeria*, usually 14 to 30 days before transfer of 10^8 immune splenic cells. In some experiments, spleen cells from naive mice were transferred. The number of *Listeria* in the organs of recipient SCID mice were quantitated at the time points indicated in the figure legends, i.e., 3, 6, and 15 days. Spleens and livers were homogenized in 10 mM PBS containing 0.05% Triton X-100, and then were plated as serial dilutions onto brain-heart infusion agar (Difco, Detroit, MI). Bacterial colonies were quantitated after 24 h of growth at 37°C. Immune spleen cells were obtained from CB.17 mice either 10 days following a primary infection or 3 days following a secondary challenge.

Histologic and electron microscopic procedures

At various times after *Listeria* infection, SCID mice were killed, and sections of liver and spleen were fixed for 24 h in 10% neutral-buffered formalin. Sections were taken from chronically infected SCID mice following i.p. administration of aminoguanidine hemisulfate or anti-cytokine Abs. Similarly, sections from chronically infected mice were taken following adoptive transfer of unfractionated or negatively selected $CD4^+$ or $CD8^+$ splenic cells. The tissues were routinely processed for staining with hematoxylin and eosin. Samples for electron microscopy were fixed in cacodylate-buffered glutaraldehyde, postfixed in OsO_4, stained en-bloc with uranyl acetate, and infiltrated with a methacrylate resin. Thin sections were mounted as 200-mesh copper or nickel grids, stained with lead citrate, and examined with a Philips CM10 electron microscope. (Philips Electronic Instruments Co. Eindhoven, The Netherlands).

Results

Listeria *infection in SCID mice is chronic*

To establish a model of chronic infection, SCID mice were infected i.v. with 10^4 *Listeria* and the number of viable organisms in the spleen and liver determined in groups of mice at various times after infection. Figure 1 shows that the mice were unable to eliminate the *Listeria* from the liver, with the bacterial numbers ranging from 0.1×10^4 to 7.7×10^5. Similar results were obtained in the spleen, with the *Listeria* counts ranging from 0.1×10^4 to 5.4×10^5. After 28 days, a proportion of mice (10–20%) died of *Listeria* infection.

Sequential histologic analysis of the infected livers from the carrier mice showed two types of lesions. By 24 h, distinct microabscesses were found (14, 15), which increased in number up to 5 days postinfection (Fig. 2*A*). By day 7, a granulomatous lesion was

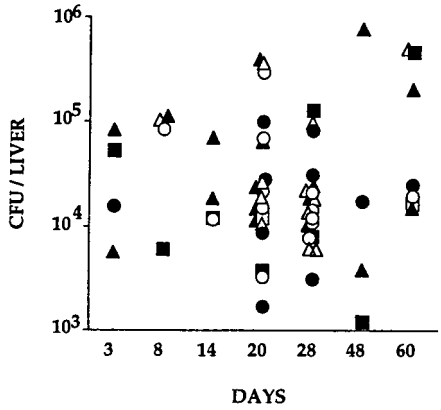

FIGURE 1. Chronicity of *Listeria* infection in the SCID mice. SCID mice were infected with 10^4 *Listeria* i.v., and *Listeria* numbers in liver were enumerated in groups of mice, 3 to 60 days following infection. Each symbol represents an individual mouse. A similar range of values was observed in the spleen. The geometric mean values for liver and spleen, respectively, were as follows: day 20: 2.5×10^4 and 2.0×10^4, $n = 20$; day 28: 1.6×10^4 and 1.9×10^4, $n = 20$; day 48: 2.7×10^4 and 3.27×10^4, $n = 4$; and day 60: 6.6×10^4 and 2.3×10^4, $n = 7$.

observed that essentially consisted of a compact collection of mononuclear phagocytes, devoid of lymphoid cells (Fig. 2*B*). From day 14 to day 60, of a total of 20 to 30 lesions per section, about 95% were granulomas and 5% were microabscesses. Thus, microabscesses were always found, but these constituted a minor component after the first 5 days, up to the 60 days of observation. In the spleen, the histologic picture progressed from a periarteriolar collection of neutrophils in early infection to granulomas of different sizes localized in the white pulp after day 14 of infection, and mixed with foci of neutrophils.

The *Listeria* were localized in the day 21 liver lesion by electron microscopy. The *Listeria* were found in the mononuclear cells of the granuloma, while hepatocytes were totally devoid of them. A total of 97% (42 of 43) of the *Listeria* were in membrane-bound vacuolar structures (phagolysosomes), with the majority appearing intact (Fig. 2*C*). Only one *Listeria* was found in the cytosol.

Elements essential for maintaining a Listeria*-carrier state in the chronically infected SCID Mice*

Previous work with SCID mice showed that neutrophils (8), IL-1 (16), IFN-γ (1), TNF-α (17), IL-12 (18), and $NO^{\cdot}$ (5) were necessary for resistance to acute *Listeria* infection. Similar requirements were found for acute listeriosis in normal mice (reviewed in Ref. 2). In this first report, we evaluated the roles of two major cytokines, IFN-γ and TNF, plus those of IL-12, IL-10, and $NO^{\cdot}$. Specific inhibitors were administered shortly before initiation of infection, or in the case of $NO^{\cdot}$, 2 days after i.p. inoculation with live organisms. We sought to examine the role of these mediators in a chronic infective state by the i.p. administration of anti-cytokine Abs to SCID mice infected with 10^4 *Listeria* 14 to 30 days earlier. Aminoguanidine hemisulfate was injected twice daily, as described in *Materials and Methods*, for the purpose of inhibiting the inducible $NO^{\cdot}$ synthetase. In both cases the mortality, bacterial counts, and histology of the livers were assessed at 40, 96, or 144 h after treatment. The course of the infection was easier to follow in the liver, where the two histologic lesions were easily outlined.

Administration of anti-granulocyte (RB6-8C5) mAb had a profound effect by increasing splenic and liver *Listeria* burden (Fig. 3*A*). This effect was seen as early as 40 h, e.g., the liver *Listeria*

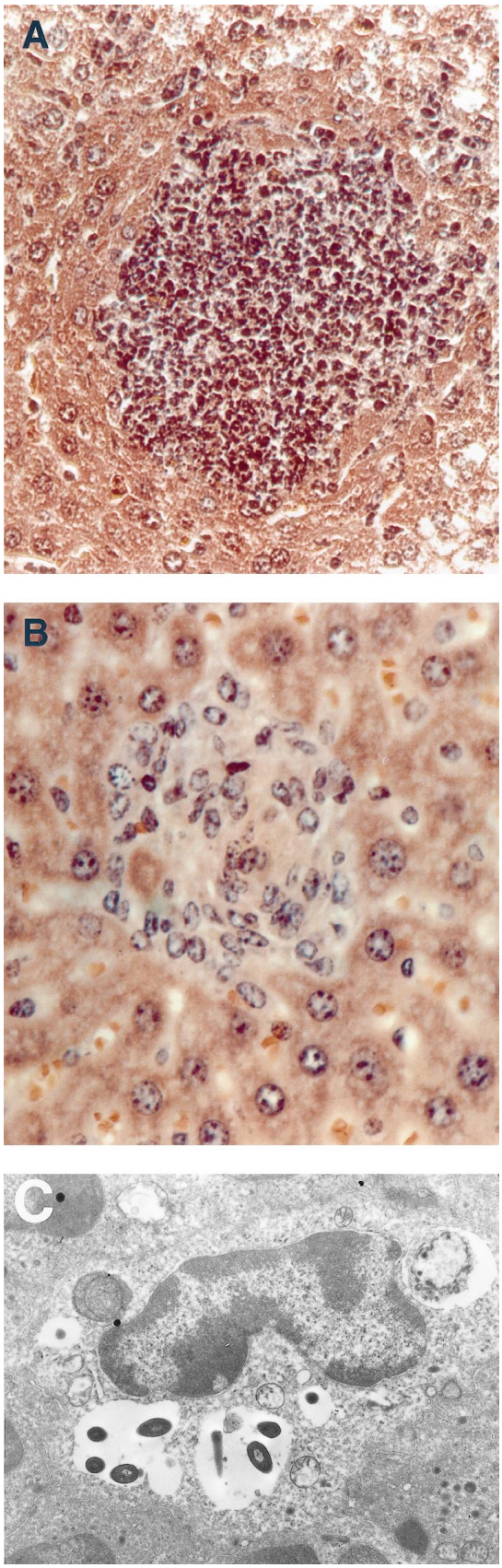

FIGURE 2. Histologic and ultrastructural analysis of chronic liver lesions and localization of *Listeria*. *A,* Liver section from a SCID mouse infected with 10^4 *Listeria* i.v. for 5 days shows neutrophilic microabscesses. Identical neutrophilic lesions were also present at later time points, i.e., days 14, 21, 28, 48, and 60. *B,* Liver section from a SCID mouse infected with 10^4 *Listeria* i.v. for 21 days shows a compact collection of mononuclear cells. The granulomas contained very few neutrophils or lymphocyte-like cells and were of different sizes, i.e., from a few macrophages to large clusters, as shown here. No multinucleated giant cells were found. *C,* Electron micrograph of liver from SCID mice infected with 10^4 *Listeria* i.v. for 21 days. Intact *Listeria* are identified in membrane-bound vacuoles (phagolysosomes) in mononuclear cells. *C,* Original magnification ×5200.

numbers in two mice at 40 h was 4.4×10^6 and 6.2×10^6. There was also an increase in mortality of these mice; in two separate experiments, three of five and two of five mice given anti-granulocyte mAb died by day 6. Similar enhancement of liver and splenic bacterial loads, but with a delayed time kinetics (after 2 days) was seen after administration of anti-TNF-α (TN3-19.12) and anti-IFN-γ (H22.1) mAbs (Fig. 3, *A* and *B*).

Anti-IL-10 mAb (2A5) given at 200 or 400 μg did not change the *Listeria* load (Fig. 3*C*). Inhibition of endogenous $NO^{\cdot}$ by injection of aminoguanidine hemisulfate also had no effect on the in vivo growth of *Listeria* (Fig. 3*D*). Similarly, inhibition of IL-12 by administration of anti-IL-12 mAb, Red-T (250 μg) had no effect on the bacterial load (Fig. 3*D*).

The histologic lesions in mice depleted of neutrophils or given the mAbs that neutralized IFN-γ or TNF-α were essentially similar in that the net result was infection of hepatocytes. Histology of livers from mice treated with RB6-8C5 showed large confluent zones of liquefactive necrosis, with *Listeria* present extracellularly and in necrotic hepatocytes in the center (Fig. 4*A*). The livers of mice treated with anti-TNF-α mAb also showed abundant *Listeria* present in the microabscess with a viable rim of hepatocytes around a central necrotic area (Fig. 4*B*). Neutrophils were present to variable degrees in the infective foci. Histologic sections from anti-IFN-γ mAb (H22.1)-treated mice also showed a dramatic increase in the number and size of neutrophilic lesions (Fig. 4*C*). Very importantly, granulomas were no longer identifiable in any of these mice. A Gram stain demonstrated abundant extracellular *Listeria*, and in the hepatocytes adjacent to the microabscess (Fig. 4*D*). The spleen lesions in anti-TNF- or anti-IFN-γ-treated mice were characterized by numerous foci of neutrophils dispersed through both the red and white pulp.

There was no effect observed on the histology of the livers from infected mice that had been given anti-IL-10 mAb, anti-IL-12 mAb, or aminoguanidine hemisulfate (data not shown). The histopathology was identical to that found in the untreated mice.

The mAbs were tested at doses that exceeded their neutralizing effect by two- to threefold. Indeed, the efficacy of the anti-IL-12 mAb was tested by injecting the Abs 1 h before acute infection of SCID mice with live *Listeria* (5×10^3), as previously described (18). Similarly, aminoguanidine hemisulfate was administered shortly after (day 0) i.p. inoculation of SCID mice with live *Listeria*, as was described by Beckerman et al. (5). Both treatments resulted in a significantly higher number of cultured *Listeria* in spleens and livers of infected mice (i.e., for aminoguanidine and Red-T; >2 log increase over control mice). The anti-IL-10 mAb had been previously evaluated (11).

Adoptive transfer of immune or naive splenic cells is protective

Having evaluated some of the parameters important in maintaining a carrier state in chronically infected SCID mice, we examined whether adoptive transfer of immune splenic cells could produce sterilizing immunity. Immune splenic cells were derived from immunocompetent C.B-17 mice 10 days following a primary i.p. infection with 5×10^3 *Listeria*. Figure 5*A* shows that by day 6

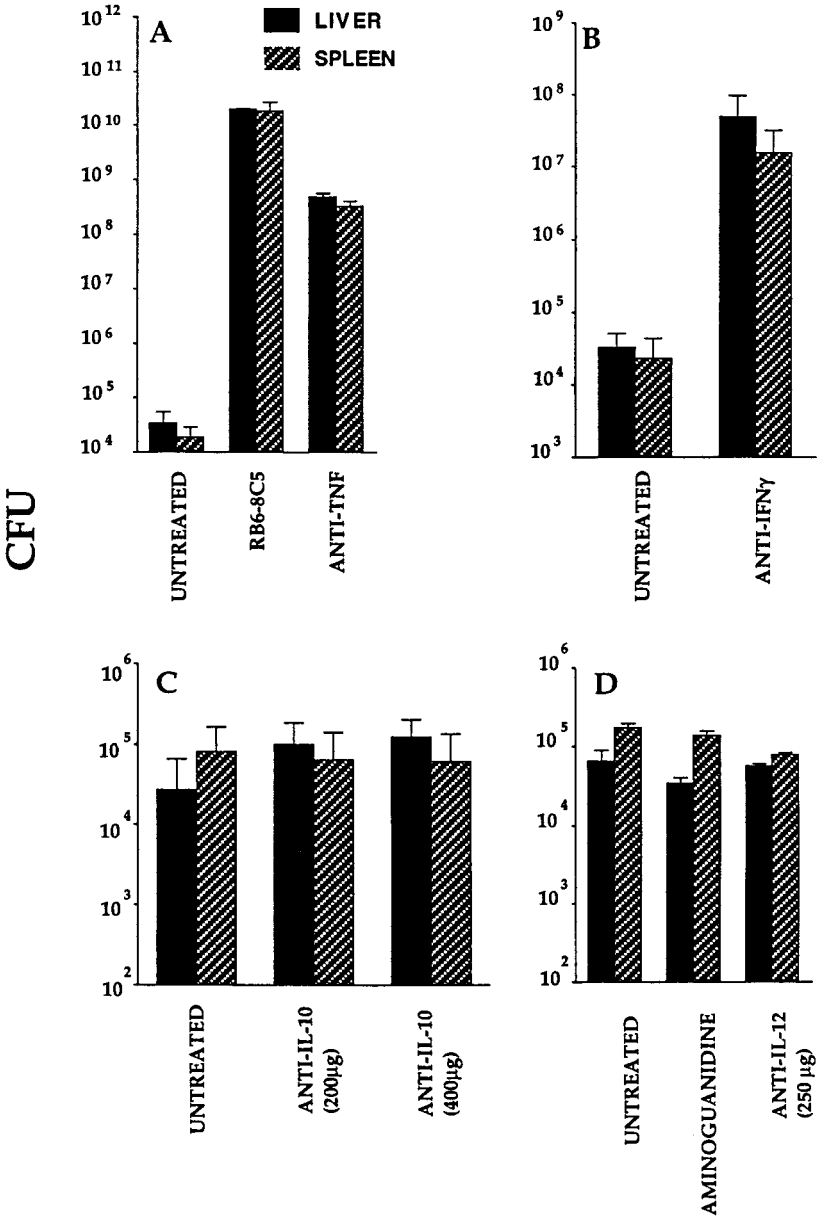

FIGURE 3. Neutrophils as well as endogenous TNF-α and IFN-γ are important in chronic *Listeria* infection. SCID mice were injected on day 14 postinfection with (*A*) 300 μg of RB6-8C5 and 200 μg of anti-TNF-α mAb; (*B*) 200 μg of anti-IFN-γ mAb; (*C*) 200 and 400 μgs of anti-IL-10 mAb; (*D*) 6 mg of aminoguanidine hemisulfate salt, twice daily, as indicated in *Materials and Methods*; and 250 μg of anti-IL-12 mAb. *Listeria* was quantitated in the spleens and livers on day 20 postinfection (i.e., 6 days after treatment). The data indicate mean ± SD and are representative of two separate experiments, each of which included three to five mice.

post-transfer of 10^8 splenic cells, the *Listeria* infection was substantially cleared (the minimal level of detection of *Listeria* in organs is $\leq 10^2$). Similar results were obtained by transfer of immune splenic cells derived from mice that received a secondary challenge 14 days later with 5×10^4 *Listeria*. (Transfer of 5×10^7 splenic cells also led to clearance of *Listeria* but there was greater variability among the mice.) The protection was specifically mediated by T cells since immune splenic cells depleted of both $CD4^+$ and $CD8^+$ cells with Ab and complement treatment were not effective (Fig. 5*B*) in clearing the chronic infection.

We also sought to examine whether naive T cells (i.e., T cells from untreated mice) could bring about protective immunity. Figure 5*C* shows that 10^8 naive cells were equally effective in protecting immunity, though with a delayed time kinetic (by day 15 instead of day 6).

Histologic analysis of post-transfer livers showed well-formed, medium-sized to large granulomas, with lymphocytes (Fig. 5*D*). There was complete disappearance of the microabscess. Thus, specific immunity to *Listeria* could be induced by adoptive transfer of either immune or naive T cells from immunocompetent mice.

Both $CD4^+$ and $CD8^+$ T cells can resolve a chronic Listerial *infection*

Our intent was to determine whether $CD4^+$ and $CD8^+$ T cell subsets were effective in clearing *Listeria* in chronically infected mice. Figure 6*A* shows the results of transfers of spleen cells from naive C.B-17 mice depleted of either $CD4^+$ or $CD8^+$ T cells by treating them with mAb and complement treatment in vitro. The negatively selected cells led to sterilizing immunity, by day 15, and to granulomas containing lymphocytes. Granulomas identical to those shown in Figure 5*D* were seen following transfer of either $CD4^+$ or $CD8^+$ T cells.

Figure 6*B* shows that treatment with anti-CD4 or anti-CD8 mAb removed the targeted cell population, as determined by cytofluorometric analysis of the splenic cells. We also followed the course of these negatively selected splenic cells in vivo after adoptive

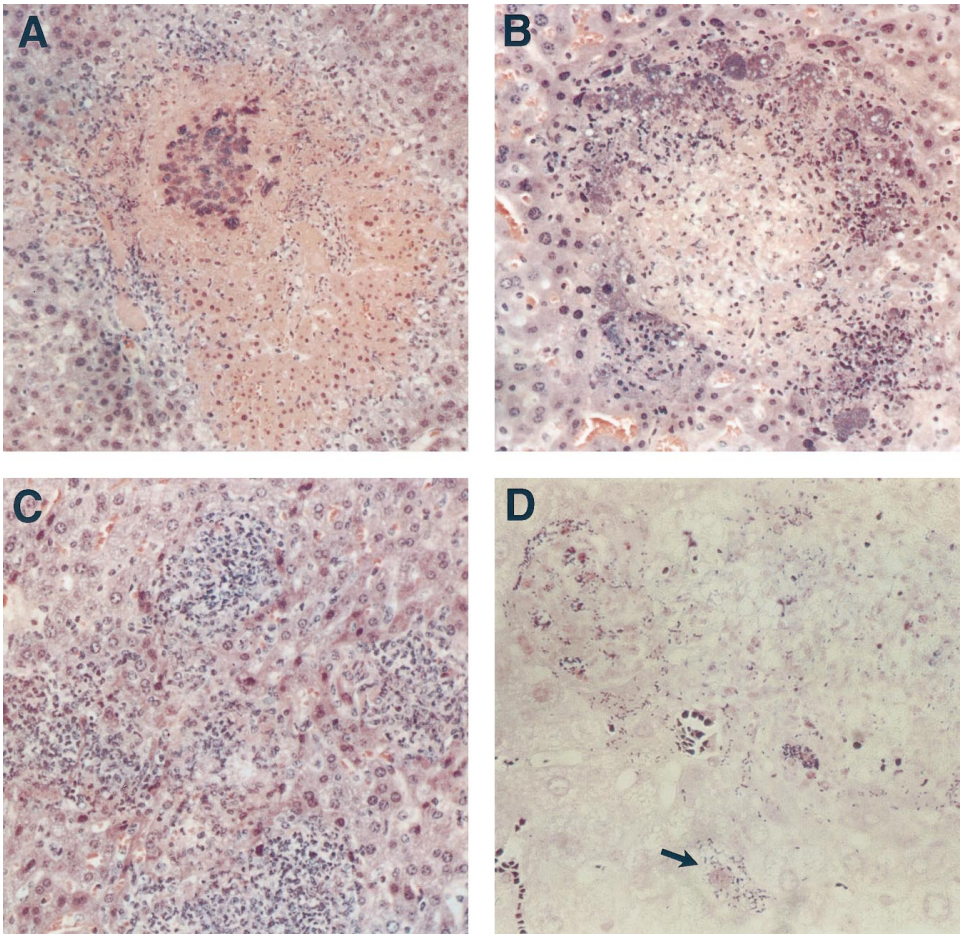

FIGURE 4. Both neutrophilic depletion and neutralization of endogenous TNF-α and IFN-γ in a chronic infection in SCID mice result in hepatic colonization by *Listeria*. Sections of liver were stained with hematoxylin and eosin. Mice were treated with: (*A*) 300 μg of anti-granulocyte mAb; (*B*) 200 μg of anti-TNF-α mAb; (*C*) 200 μg of anti-IFN-γ mAb. Large neutrophilic abscess and reversal of the histopathology from granulomas to liver microabscess were found in *B* and *C*. *D,* Gram stain of slide depicted in *C* shows *Listeria* extracellularly and in cytoplasm of the hepatocyte at the periphery of the abscess (arrow).

transfer into chronically infected mice. We found that the expected T cell depletion was essentially complete at 14 days post-transfer, i.e., in mice given negatively selected $CD4^+$ T cells, there was no residual staining for CD8, and vice versa. Thus, both $CD4^+$ and $CD8^+$ T cells were effective in mediating an antibacterial immune response. We have not conducted a titration of each T cell subset to determine their relative efficiency.

T cell protection is mediated by both IFN-γ-dependent and -independent mechanisms: identification of a T cell suppression of resistance

We have now performed a set of initial experiments attempting to characterize the mode of action of T cells. We report here the results of using T cells lacking expression of IFN-γ. Naive splenic cells from homozygous IFN-γ-null gene, i.e., knock-out (KO) mice (IfgTM1) were transferred into chronically infected SCID mice. The IFN-γ gene KO mice have been shown to be exquisitely sensitive to primary infection with *Listeria* (19). As shown in Figure 7 (*A* and *B*), transfer of 10^8 naive splenic cells from IFN-γ gene KO mice protected as efficiently as the spleen cells from wild-type BALB/c controls.

The $CD8^+$ T cells from IFN-γ gene KO mice were also effective. We also transferred $CD8^+$ T cells from IFN-γ gene KO mice into chronically infected SCID mice in the presence of neutralizing Ab to IFN-γ (H22.1). Figure 7*C* shows that $CD8^+$ T cells protected when the neutralizing mAb H22.1 was administered either at 5 or 10 days after transfer of T cells. Mice given mAb H22.1 before adoptive transfer of $CD8^+$ T cells from IFN-γ KO mice were not protected and died by day 15. Groups of control chronically infected mice not transferred with cells and injected by mAb H22.1 at day 0 were susceptible to infection as was also shown in Figure 3.

Interestingly, transfer of $CD4^+$ T cells from IFN-γ gene KO animals led to different results, with exacerbation of disease and increased mortality. In three experiments, 7 of 17 mice had died by 2 wk after transfer. This compared with 0 of 11 of control infected mice not given $CD4^+$ T cells, or 0 of 12 transferred with wild-type CD4 T cells. Histology of liver lesions from these mice revealed multiple large neutrophilic abscesses with massive infection of liver cells admixed with neutrophils and lymphocytes. Granulomas were not present. The histologic picture was identical to that found in mice given anti-TNF or anti-IFN-γ mAb (Fig. 4, *B* and *C*). Likewise, the spleen contained numerous foci of infection rich in neutrophils.

Discussion

The present results address certain aspects of chronic *Listeria* infection in the SCID mouse. Four conclusions can be made. 1) The chronic carrier state of *Listeria* infection involves an equilibrium

FIGURE 5. Adoptive transfer of *Listeria*-immune or naive splenic cells to chronically infected SCID mice results in protective immunity. *A,* 10^8 immune splenic cells from C.B-17 mice were transferred to chronically infected SCID mice, and *Listeria* numbers in liver and spleen were determined 3 and 6 days later. The data represent the mean ± SD of values from three to four individual mice, and are representative of three separate experiments. *B,* Protection is specifically mediated by T cells: 10^8 immune splenic cells from C.B-17 mice depleted of $CD4^+$ and $CD8^+$, by Ab and complement treatment, were transferred to chronically infected SCID mice, and *Listeria* in liver and spleen were enumerated 6 days later. The data represent the mean ± SD of values from five to six individual mice. *C,* 10^8 naive spleen cells from C.B-17 mice were transferred to chronically infected SCIDs. *Listeria* in liver and spleen were enumerated 6 and 15 days later. The data represent the mean ± SD of values from four individual mice and are representative of two separate experiments. *D,* Liver sections from a SCID mouse that received 10^8 *Listeria*-immune splenic cells show well-formed granulomas at day 6. Similar histology is seen at day 15 in mice given naive cells.

in which a continuous interaction takes place between the two major effector cells, i.e., the macrophage and the neutrophil, and with the participation of two cytokines, IFN-γ and TNF-α. 2) Lymphocytes from naive donors could be transferred and primed in the chronic infected mice, indicating that Ag presentation could take place. 3) $CD8^+$ T cells could bring about sterilizing immunity without the apparent participation of IFN-γ. 4) In contrast to $CD8^+$ T cells, $CD4^+$ T cells from IFN-γ gene KO animals have an active suppressive role, bringing about a profound disturbance of the fine equilibrium between the host and the microbe.

The livers of the chronically infected SCID mice exhibited two distinct types of pathologic lesions, i.e., microabscess and a granuloma, the latter composed of a compact collection of mononuclear cells without lymphocytes. Most of the lesions in chronic infection were of the latter type. We believe that there is an equilibrium between both of the lesions, with the microabscess representing a "spillover" of *Listeria* from the granuloma. This equilibrium is indeed maintained by neutrophils and macrophages, and by at least two cytokines (IFN-γ and TNF). It is likely that the granulomas of the SCID mouse may not be effective in limiting the spread of *Listeria*. Once *Listeria* escapes the granuloma, it enters the hepatocyte as it occurs in the early blood-born infection. In the hepatic parenchymal cells, neutrophils represent the first, if not only, line of defense, limiting the extent of growth and dissemination of *Listeria*. Ample precedent for this observation has been found in the many reports demonstrating the effects of neutrophil depletion in acute listeriosis (8–10). In the framework of chronic infection in the SCID mice, depletion of neutrophils resulted in increased mortality, extensive hepatic colonization, and pronounced exacerbation of infection, reaffirming that neutrophils play a major role in controlling the intrahepatocyte infection. Thus, the new information herein is showing, first, the continuous interplay between these two forms of inflammation that involve neutrophils and macrophages and, second, the leakiness, or partial ineffectiveness, of the chronic granuloma in the absence of effective T cell immunity.

A second feature to note in the context of the chronic infection was the requirement of IFN-γ and TNF-α. Neutralization of IFN-γ and TNF-α resulted in the loss of the granuloma <—> microabscess equilibrium, skewing the process toward infection of the hepatocytes. Although we did not evaluate the cellular source of either of the cytokines, we presume that NK cells are involved, as in the acute infection. IFN-γ is likely to be maintaining the activated macrophage state as evaluated previously in the acute infection. The exact role of

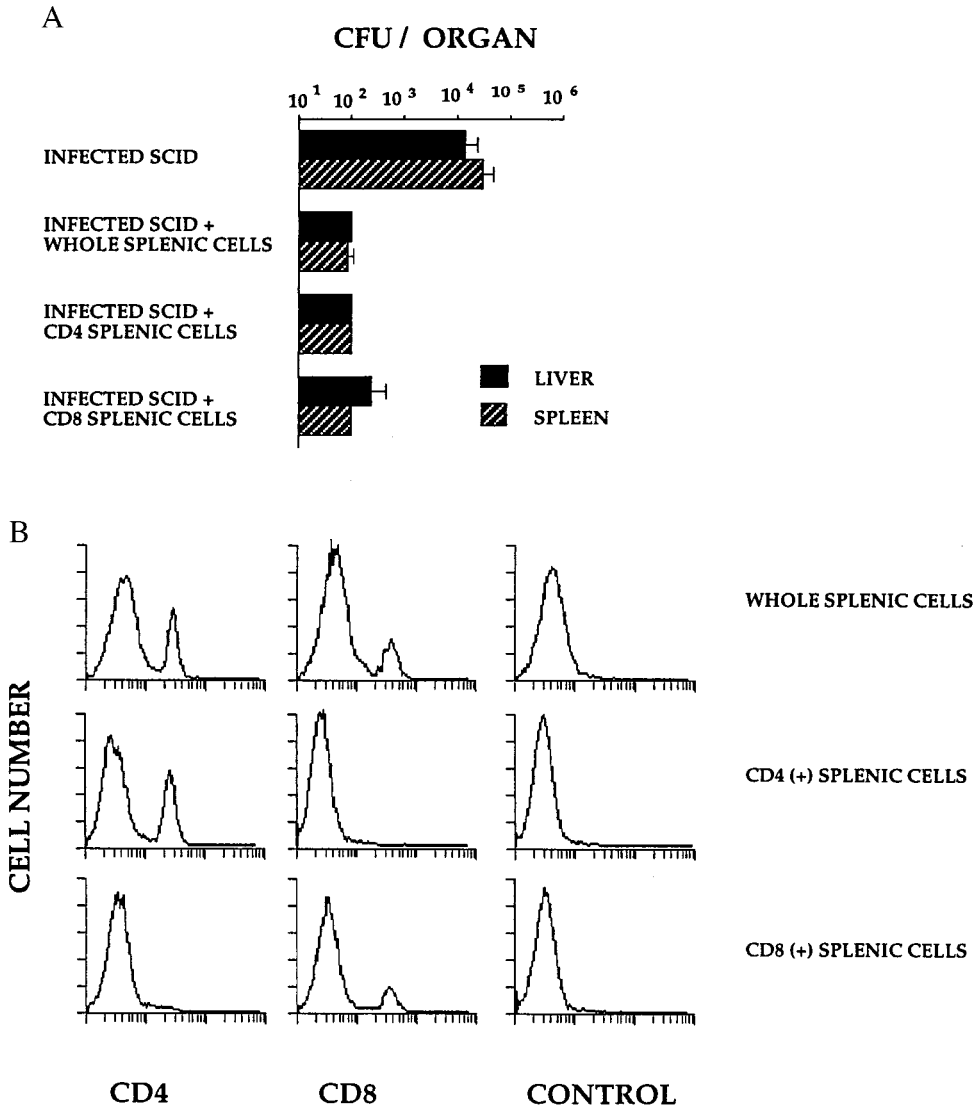

FIGURE 6. Both $CD4^+$ and $CD8^+$ T cells are equally important in resolving a chronic listerial infection. *A*, 10^8 naive splenic cells or 10^8 negatively selected $CD4^+$ or $CD8^+$ cells from C.B-17 mice were transferred into chronically infected SCID mice. CFU in spleen and liver were determined 15 days later. Results are expressed as mean ± SD for groups of four mice, and are representative of four separate experiments. *B*, Flow cytometric analysis of cell surface markers on whole or negatively selected $CD4^+$ or $CD8^+$ spleen cells from naive C.B-17 mice. Spleen cells were stained with FITC-conjugated anti-CD4 or FITC-conjugated anti-CD8 mAb.

TNF is not clear. TNF, together with IL-12, is required for NK cells to produce IFN-γ. Previously, we found that the effects of anti-TNF Abs in vivo could not be entirely corrected by IFN-γ, indicating that its target may be more than one cellular locus (18).

We found that the process of resistance in the chronic state was not using IL-12 in contrast to its central role in acute listeriosis both in normal and SCID mice (3, 12, 18). Similar results were found in chronic response to *Toxoplasma gondii* by Gazzinelli et al. (20). Our laboratory had also previously noted that the secondary response to *Listeria* in normal mice was considerably less affected by IL-12 (12). Also important to note is that the maintenance of chronic resistance was not affected by neutralization of $NO^{\cdot}$. This is in contrast to the role of $NO^{\cdot}$ in the early stage of *Listeria* infection (5, 21). Neutralization of IL-10 also had a minimal effect on bacterial replication or liver morphology of *Listeria*-infected mice. At this point we cannot distinguish whether these molecules were not produced or simply were produced but were not functionally relevant.

The granulomatous lesion in chronic *Listeria* infection showed several other interesting features. Our results with electron microscopic studies of the liver lesion at day 21 showed *Listeria* in membrane-bound vacuolar structures within mononuclear phagocytes and not in the cytoplasm. Moreover, the majority of the bacteria appeared intact. In an experiment run in parallel with the present ones we found, as expected, that in early infection many of the *Listeria* were found in the cytosol of macrophages, surrounded by the typical condensation of actin fibrils. Thus, the state of the infectious process is one feature that conditions the intracellular location of this microbe. The explanation may lie in the study by Portnoy et al. (22), indicating that IFN-γ activation of resident peritoneal macrophages in culture prevented escape of *Listeria* from the phagosome.

Another observation that is relevant in the context of the localization of *Listeria* is the protection conferred by $CD8^+$ T cells. We had previously argued that the localization of *Listeria* in the cytosol was the main factor that determined the activation and involvement of

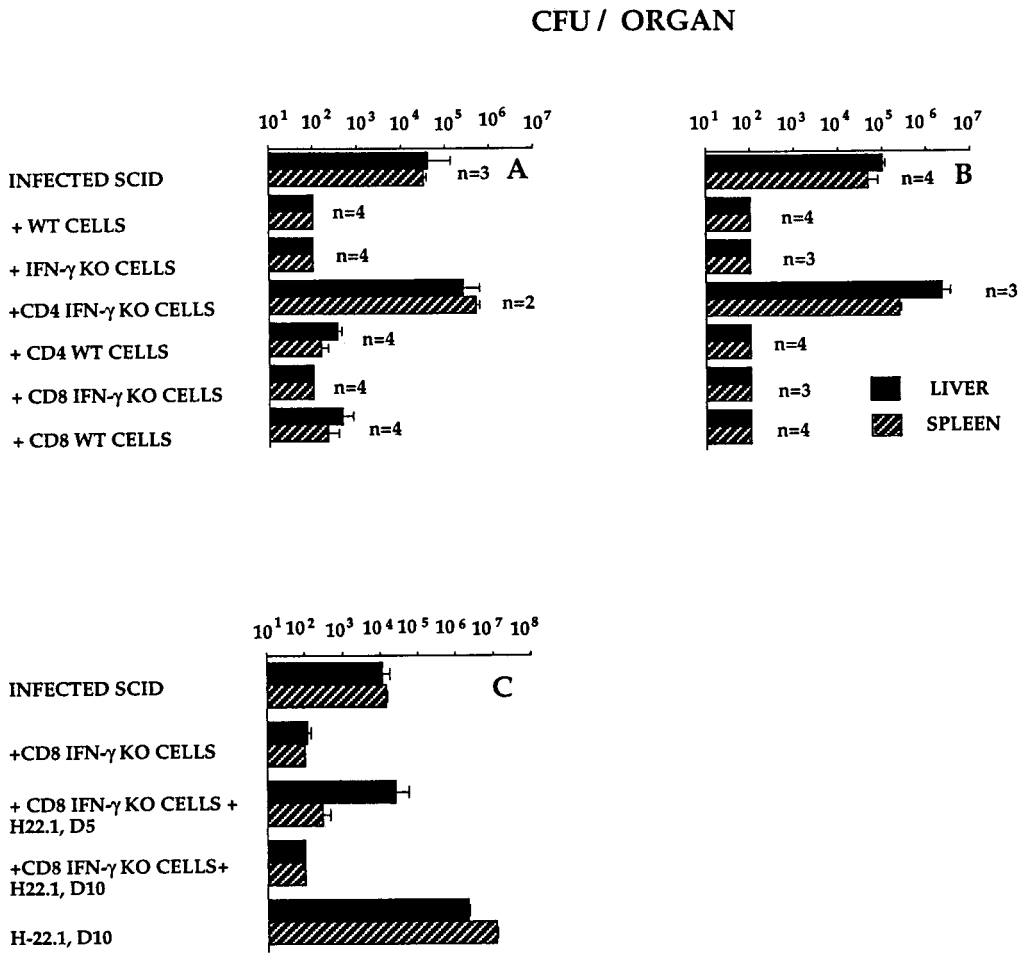

FIGURE 7. Adoptive transfer of immunity to *Listeria* requires both IFN-γ-dependent and -independent mechanisms. *A* and *B,* 10^8 naive splenic cells or 10^8 negatively selected $CD4^+$ or $CD8^+$ T cells from BALB/c (wild type) or BALB/c-IfgTM1 mice (IFN-γ KO) were transferred into chronically infected SCID mice. *Listeria* numbers in spleen and liver were determined 15 days later. Results of two representative experiments are expressed as mean ± SD for groups of three to four mice. One-third of chronically infected SCID mice given 10^8 $CD4^+$ T cells from BALB/c-IfgTM1 mice died in the experiment shown in *A*. *C,* 10^8 negatively selected $CD8^+$ T cells from BALB/c-IfgTM1 mice were transferred into chronically infected SCID mice in the presence of 250 μg of neutralizing mAb to IFN-γ (H22.1) (given immediately or at day 5 or day 10) after cell transfer. *Listeria* counts were estimated 15 days after transfer. (In controls, the chronically infected SCID mice given H22.1 were dead by day 15.) Results are expressed as mean ± SD for groups of three to four mice.

CD8 T cells (23). But as shown herein, CD8 T cells were activated even with limited localization of *Listeria* to the cytosol. Of course, there are quantitative issues here that need to be taken into consideration. It is possible that the extent of T cell priming may vary greatly depending on the amounts of *Listeria* in cytosol and/or in vacuoles. Regardless, the results do make a point that the relationship in vivo between localization of the microbe and the resultant CD4 or CD8 response may not be absolute. Intravesicular microbes have been shown to be effective in donating peptides for class I MHC binding (24), an issue that is supported by the present findings.

The transfer of naive T cells and the induction of protective immunity indicates that the APC in chronically infected mice were still effective in priming. Experiments presented in this study clearly showed that both $CD4^+$ and $CD8^+$ T cells could contribute equally to the elimination of *Listeria* and granuloma (with admixed lymphocytes) formation, although detailed quantitative comparisons were not performed. Protection mediated by $CD8^+$ T cells, as shown in earlier studies from Bevan's laboratory (19, 25), can take place by an IFN-γ-independent pathway, presumably via perforin-mediated cytotoxicity. We are currently investigating the role of $CD8^+$ T cells, which could resolve the infection either by cytotoxic mechanisms, or could modulate the inflammatory response. In the present study, although we can conclude that $CD8^+$ T cells operated without production of their own IFN-γ, nevertheless a participation of endogenous IFN-γ cannot be entirely ignored. The $CD8^+$ T cells did bring about protection if by the fifth day IFN-γ was neutralized by mAb, but this did not occur when neutralization took place earlier. In the case of the Harty and Bevan experiment (19), they found $CD8^+$ T cells transferring protection to IFN-γ KO mice, ruling out cooperativity with IFN-γ.

However, it is noteworthy that the secondary response in conventionally immunized mice was highly susceptible to neutralization of IFN-γ (12). Clearly, in the Harty and Bevan experiments (19), which used well-primed CD8 T cells, and in ours, herein, using a contained infection, the CD8 T cells could operate well. In contrast, the systemic challenge in a resting mouse with memory T cells may impose more demands on the cellular system and require macrophage activation via IFN-γ. Experiments in progress are addressing how $CD8^+$ T cells operate in producing sterilizing immunity.

This requirement of IFN-γ is no more eloquent in the results with $CD4^+$ T cells from IFN-γ KO mice. The results of adoptive

transfers with $CD4^+$ T cells from IFN-γ gene KO mice were dramatic: a major suppressive effect that altered the balance between granulomatous and neutrophilic lesions. This suppressive effect was superseded when the CD4 cells were mixed with CD8 T cells, although at this time we have not explored using different ratios of one or the other. At face value, the protective role of CD8 T cells-IFN-γ KO predominated. How the CD4 T cells lacking IFN-γ production are operating is now under evaluation. Perhaps they directly or indirectly produce negative regulatory cytokines (like IL-10) or perhaps they differentiate to Th2 subsets that produce cytokines like IL-4 that may inhibit the granuloma. Regardless, these in vivo experiments demonstrate with great eloquence the profound suppressive effect of lymphocytes in disturbing a fine balance of chronic infection. The present series of results poise our investigations into the physiology of a chronic infective state and the nature of the regulation exhibited by T cells.

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