INFECTION AND IMMUNITY, May 1996, p. 1744–1749 Vol. 64, No. 5
0019-9567/96/$04.00+0

Control of Natural Killer Cell-Mediated Innate Resistance against the Intracellular Pathogen *Listeria monocytogenes* by γ/δ T Lymphocytes

CHRISTOPH H. LADEL,[1] CARMEN BLUM,[1] AND STEFAN H. E. KAUFMANN[1,2]*

Department of Immunology, University of Ulm, D-89070 Ulm,[1] and Max Planck Institute for Infection Biology, D-10117 Berlin,[2] Germany

Received 27 November 1995/Returned for modification 16 January 1996/Accepted 20 February 1996

***Listeria monocytogenes* is an intracellular bacterium which causes an acute infectious disease in mice. Initial host resistance depends on innate immunity mediated primarily by natural killer (NK) cells followed by specific α/β T cells, which are central to acquired specific immunity. γ/δ T lymphocytes seem to provide a link between the innate and the specific immune response. All these lymphocyte populations produce gamma interferon (IFN-γ), which, because of its macrophage-activating potential, is central to antibacterial protection. IFN-γ from NK cells not only contributes to early host resistance but also promotes development of protective T-cell responses of helper T type 1 (Th1) type. Here, we show that innate resistance and early IFN-γ production in listeriosis are markedly impaired in T-cell receptor (TCR)-δ$^{-/-}$ but not TCR-β$^{-/-}$ gene disruption mutant mice. By two-color cytofluorimetry, we demonstrate that NK cells rather than γ/δ T lymphocytes are the major cellular source of IFN-γ in immunocompetent mice and that IFN-γ production by NK cells is impaired in the TCR-δ$^{-/-}$ mutants. Probably, reduced tumor necrosis factor production in listeria-infected TCR-δ$^{-/-}$ mutants contributed to impaired NK cell activation. Our data reveal a novel function of γ/δ T cells as regulators of innate resistance against sublethal infection with an intracellular pathogen.**

Immunity against the intracellular pathogen *Listeria monocytogenes* encompasses two major waves of host response (14). Innate resistance is essential for early restriction of listerial growth (2, 3, 22). On day 4 to 5 of infection, specific α/β T cells, which ultimately achieve sterile elimination of the pathogen, have developed (2, 14, 22). The innate immune response is controlled primarily by natural killer (NK) cells. In experimental listeriosis, both NK cells and α/β T cells are potent producers of gamma interferon (IFN-γ), which activates antimicrobial macrophage functions (2, 14). Until recently, the innate and specific immune response were viewed as virtually independent host reactions. More recent data, however, suggest a closer link between these two responses. First, it has been shown that IFN-γ-producing γ/δ T cells are present at the site of listerial replication prior to α/β T cells and hence provide a link between innate and acquired immunity (9, 29). Second, convincing evidence that cytokines produced by the innate immune system determine the type of specific immune response has been presented (10, 20). In listeriosis, early production of interleukin-12 (IL-12) by macrophages and of IFN-γ by NK cells promotes development of protective acquired immunity dominated by helper T type 1 (Th1) cells (10). In addition, tumor necrosis factor (TNF) has been shown to enhance IFN-γ secretion by spleen cells, most probably by acting on NK cells (3).

The use of gene disruption mutant mice with defined immunodeficiencies has provided helpful tools for a deeper understanding of the role of distinct T-cell subsets and cytokines in fighting microbial pathogens (16, 17, 20, 22). Not only have such studies facilitated identification of the immune mechanisms which are vital for control of infectious diseases but also they have provided novel insights into the compensatory capacities of the immune system suffering from defined deficiencies. Thus, it has been shown that γ/δ T cells can transiently control *L. monocytogenes* infection in T-cell receptor (TCR)-β$^{-/-}$ mouse mutants. Conversely, acquired resistance to listeriosis is not affected in TCR-δ$^{-/-}$ mice, which clear infection just as well as their controls do (22). RAG-1$^{-/-}$ mutants, which lack all mature T and B lymphocytes because of a disruption in the recombinase-activating gene 1 but possess normal NK levels, die of *L. monocytogenes* infection (22). These findings emphasize the indispensable role of α/β T cells and the compensatory but insufficient role of γ/δ T cells in acquired resistance to *L. monocytogenes*. In this study, we have used T-cell-deficient mutant mice for analyzing the potential impact of T cells on innate resistance to sublethal listeriosis. Our data reveal exacerbation of disease and deficient IFN-γ production in RAG-1$^{-/-}$ and TCR-δ$^{-/-}$ but not TCR-β$^{-/-}$ mutant mice early after *L. monocytogenes* infection. Two-color staining for cell surface markers and intracellular IFN-γ emphasize that early IFN-γ production in *L. monocytogenes*-infected immunocompetent mice is a function primarily of NK1.1^{+} cells rather than of γ/δ T cells. These data suggest that control of IFN-γ-producing NK cells is mediated by γ/δ T cells in intracellular bacterial infections. Thus, not only is specific immunity regulated by the innate host response but also there is reciprocal regulation, namely, control of NK cells by γ/δ T lymphocytes.

MATERIALS AND METHODS

Mice and in vivo modulation of IFN-γ. T-cell-deficient mutant mice were created by the gene-targeting technique as described previously (11, 23, 24). In our experiments, TCR-γ/δ T-cell-deficient TCR-δ$^{-/-}$ mice, TCR-α/β T-cell-deficient TCR-β$^{-/-}$ mice, and RAG-1$^{-/-}$ mutant mice, lacking all mature T and B lymphocytes, were used. Mutant mice from the fourth to sixth backcrosses on the C57BL/6 background were bred and maintained in the animal area of the University of Ulm under strict specific-pathogen-free conditions and used in the experiments at 8 to 10 weeks, age and sex matched. Mice were treated intraperitoneally with anti-IFN-γ monoclonal antibodies (MAb) (clone R4-6A2; 0.5

* Corresponding author. Mailing address: Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, D-89070 Ulm, Germany. Phone: 0731/502-3361 or 3360. Fax: 0731/502-3367. Electronic mail address: Stefan.Kaufmann@Medizin.Uni-Ulm.DE.

mg intraperitoneally) (30) or with rat immunoglobulin (Immunotech, Marseille, France) on the indicated days.

Bacteria and infections. *L. monocytogenes* EGD was grown in tryptic soy broth (Gibco, Paisley, United Kingdom) until mid-log phase and stored at −70°C. For a given experiment, bacteria were thawed and diluted in phosphate-buffered saline (PBS). The intravenous infection volume was 0.2 ml per mouse via the lateral tail vein. For determination of CFU, spleens were homogenized with a Stomacher laboratory blender (Seward Medical, London, United Kingdom). CFU were determined by plating serial 10-fold dilutions of organ homogenates on tryptic soy agar (Gibco) and counting colonies after 24 h.

In vitro studies and stimuli used for in vitro stimulations. Spleen cells of *L. monocytogenes*-infected TCR-$\beta^{-/-}$, TCR-$\delta^{-/-}$, and RAG-$1^{-/-}$ mice and their respective +/− control mice were prepared by teasing spleens through stainless steel meshes. After lysis of erythrocytes with ammonium chloride buffer, the cells were resuspended in Iscove's modified medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin-streptomycin (Gibco) per ml, 5×10^{-5} M mercaptoethanol (Gibco), and 1 μg of indomethacin (Sigma, St. Louis, Mo.) per ml and seeded into 96-well plates (Nunc, Roskilde, Denmark) at 2×10^5 cells per well with heat-killed listeriae (HKL) or with concanavalin A (Sigma) as indicated in the figures and tables. After the cells were cultured for 2 days, supernatants were removed and stored until analyzed by enzyme-linked immunosorbent assay (ELISA). HKL were obtained by incubating a mid-log-phase culture of *L. monocytogenes* for 1 h at 70°C.

ELISA and determination of cytokines. IFN-γ ELISA was performed as described previously with two MAb (clones R4-6A2 and AN18-17.24; kind gifts of J. Langhorne, Max Planck Institute for Immunobiology, Freiburg, Germany) specific for IFN-γ in a two-site ELISA (17). Murine recombinant IFN-γ (kind gift of G. Adolf, Ernst-Boehringer Institute for Pharmaceutical Research, Vienna, Austria) was used to generate standard curves. The detection limit of the assay was 0.05 U of IFN-γ per ml. For detection of IL-12 by ELISA, the rat anti-IL-12 (p40) MAb C15.6.7 and C17.8 (kind gift of G. Trinchieri, Wistar Institute, Philadelphia, Pa.) were used as described previously (8). Murine recombinant IL-12 (kind gift of S. Wolf, Genetics Institute, Cambridge, Mass.) was used to calculate IL-12 contents. TNF was measured in a cytotoxicity assay with the TNF-sensitive cell line L929 (21). Murine TNF-α (Genzyme, Cambridge, Mass.) was used to obtain a standard curve. All measurements were done with the Spectra-Max equipment (Molecular Devices, Sunnyvale, Calif.) with the Softmax Pro software (Molecular Devices).

Cytofluorimetric analysis of IFN-γ producers. Mutant mice were infected intravenously with live *L. monocytogenes*. Spleen cells were obtained by preparing single-cell suspensions. These suspensions were cultured for 3 h with 1 μM monensin (Sigma) in Click's RPMI (Seromed) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin-streptomycin per ml, 5×10^{-5} M mercaptoethanol, and 1 μg of indomethacin per ml to accumulate intracellular IFN-γ (1, 12). Thereafter, cells were labelled with a biotinylated MAb against NK1.1 (clone PK136; American Type Culture Collection, Rockville, Md.) conjugated with biotin and subsequently detected with streptavidin-phycoerythrin (Gibco). All staining reagents were diluted in PBS–0.1% sodium azide (Sigma). Cells were fixed in 2% paraformaldehyde in PBS for 30 min. Intracellular IFN-γ was stained with fluorescein isothiocyanate-conjugated anti-IFN-γ MAb (clone R4-6A2) in PBS–0.5% bovine serum albumin (Sigma)–0.5% saponin (Sigma)–0.1% sodium azide. After being washed with PBS, 4×10^6 to 5×10^6 cells were analyzed in a FACScan (Becton-Dickinson, Mountain View, Calif.) with Lysis II software.

RESULTS

***L. monocytogenes* infection in T-cell-deficient mutant mice.** The major aim of this study was to analyze regulatory immune mechanisms involved in the early host response to *L. monocytogenes*. We therefore chose inocula of *L. monocytogenes* which are sublethal for all mouse strains used. Mice were infected intravenously with live *L. monocytogenes*, and bacterial growth in spleens and livers of infected mice was determined on days 1 and day 4 postinfection (p.i.). The data in Table 1 show CFU values in mice infected with 5×10^3 *L. monocytogenes* organisms. Similar results were obtained with sublethal doses ranging from 2×10^3 to 7×10^3 organisms. At very early time points after infection (4 h), no difference between the heterozygous control mice and the TCR-$\delta^{-/-}$ mice in bacterial load in livers (TCR-$\delta^{+/-}$, $\log_{10}$ CFU, 2.72; TCR-$\delta^{-/-}$, $\log_{10}$ CFU, 2.76) and spleens (TCR-$\delta^{+/-}$, $\log_{10}$ CFU, 2.13; TCR-$\delta^{-/-}$, $\log_{10}$ CFU, 2.23) was detectable after infection with 5×10^3 live *L. monocytogenes* organisms; i.e., TCR-$\delta^{+/-}$ and TCR-$\delta^{-/-}$ mice were equally resistant to listeriosis immediately after infection. However, a significant increase in bacterial load per organ on day 1 p.i. was detectable in TCR-$\delta^{-/-}$ and RAG-$1^{-/-}$ mutant mice compared with CFU in TCR-$\beta^{-/-}$ and heterozygous control animals (Table 1). On day 4 p.i., no significant differences in bacterial counts were detected among all the mouse strains. CFUs at later time points (up to day 21 p.i.) in the T-cell-deficient mutant mice used have already been published (22).

TABLE 1. Growth of *L. monocytogenes* in T-cell-deficient mice[a]

Group	Mice	$\log_{10}$ CFU per spleen[b]			
		Day 1 p.i.	Significant difference[c] (Δ log)	Day 4 p.i.	Significant difference[c]
1	+/−	5.3 ± 0.3		6.3 ± 0.2	
2	TCR-$\delta^{-/-}$	6.8 ± 0.7	vs 1 (1.5), vs 3 (2.0)	6.4 ± 0.5	NS[d]
3	TCR-$\beta^{-/-}$	4.8 ± 0.3	vs 2 (2.0), vs 4 (1.3)	5.7 ± 0.4	NS
4	RAG-$1^{-/-}$	6.1 ± 0.3	vs 1 (0.8), vs 3 (1.3)	6.3 ± 0.5	NS

[a] Mice were infected with 5×10^3 live *L. monocytogenes* organisms intravenously, and bacterial counts per spleen were determined by plating serial 10-fold dilutions on tryptic soy agar plates. After 24 h of incubation at 37°C, CFU were counted.

[b] Mean ± SD for four mice per time point. Data are from a representative experiment repeated twice.

[c] $P < 0.05$ (Student's t test).

[d] NS, not significant.

Cytokine production after in vitro stimulation of spleen cells from infected mice. We determined IFN-γ production by spleen cells from *L. monocytogenes*-infected T-cell-deficient mutants after in vitro stimulation with listerial antigen on days 1 and 4 p.i. (Fig. 1A and B). On day 1, only spleen cells from TCR-$\beta^{-/-}$ mutants and their heterozygous controls produced detectable amounts of IFN-γ. In contrast, TCR-$\delta^{-/-}$ spleen cells failed to produce IFN-γ on day 1. Even on day 4, IFN-γ production by TCR-$\beta^{-/-}$ and TCR-$\beta^{+/-}$ spleen cells exceeded IFN-γ secretion by TCR-$\delta^{-/-}$ spleen cells. RAG-$1^{-/-}$ spleen cells produced nondetectable to marginal IFN-γ on days 1 and 4, pointing to a defect in IFN-γ secretion by NK cells in the absence of T cells. Concanavalin A failed to induce IFN-γ secretion in RAG-$1^{-/-}$ and TCR-$\beta^{-/-}$ spleen cells. Hence, concomitant with impaired resistance, early secretion of IFN-γ was affected in *L. monocytogenes*-infected TCR-$\delta^{-/-}$ mice. The cytokine IL-12 is considered central to the development of IFN-γ-producing NK cells and α/β T cells (10). We therefore assessed whether differential IL-12 production would account for impaired early IFN-γ secretion and reduced innate resistance (Fig. 1C and D). The IL-12 production was impaired only in RAG-$1^{-/-}$ mutants, whereas TCR-$\beta^{-/-}$, TCR-$\delta^{-/-}$, and heterozygous mice produced comparable levels of IL-12 on days 1 and 4 p.i. Hence, we consider it unlikely that IL-12 exerts a major influence on impaired innate immunity to *L. monocytogenes* in TCR-$\delta^{-/-}$ mice. Because acquired resistance is not affected in TCR-$\delta^{-/-}$ mutants (22), our findings emphasize that IL-12 rather than IFN-γ is essential for the development of appropriate αβ T-cell responses to *L. monocytogenes.*

TNF triggers NK cells for IFN-γ production and cell-mediated lysis (3). Since TNF secretion in TCR-$\delta^{-/-}$ mutants is disturbed in different settings (6, 27), TNF secretion was determined (Table 2). On day 1 p.i., spleen cells from TCR-$\delta^{-/-}$ and RAG-$1^{-/-}$ mutants produced only minimal amounts of TNF after in vitro stimulation with HKL whereas control and TCR-$\beta^{-/-}$ splenocytes secreted higher, comparable concentrations of TNF. On day 4 p.i., TNF production was still impaired in RAG-$1^{-/-}$ mice whereas TCR-$\delta^{-/-}$ mutants had produced almost the levels of TNF observed in control mice. Therefore, similar to other infection models (6, 27), TNF production by

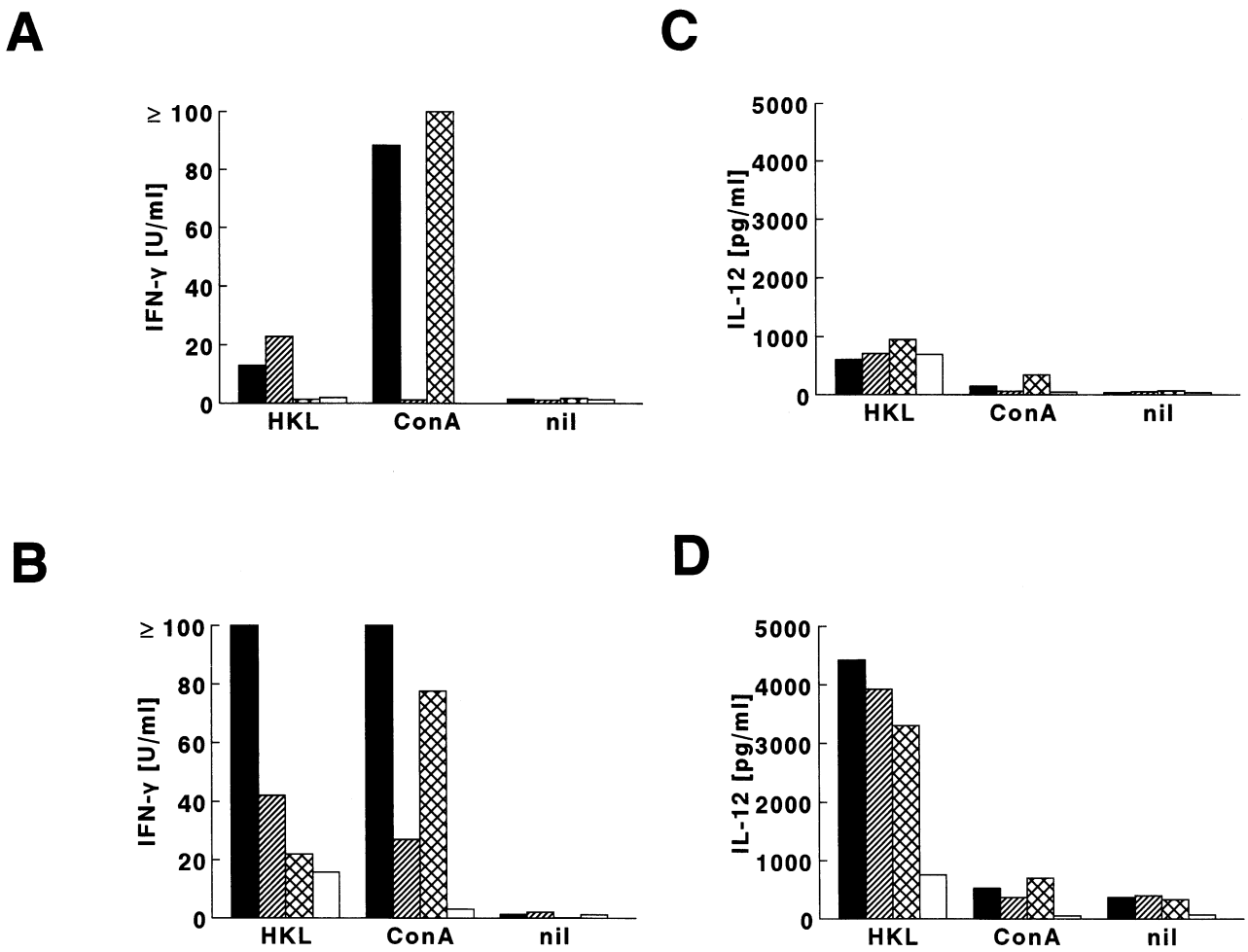

FIG. 1. IFN-γ and IL-12 production by spleen cells from *L. monocytogenes*-infected T-cell-deficient mice. (A and B) IFN-γ production by spleen cells after in vitro stimulation with HKL as the antigen or with concanavalin A (ConA) as the positive mitogen control. Data for day 1 (A) and day 4 (B) p.i. are shown. (C and D) IL-12 production after in vitro stimulation. Data for IL-12 production on day 1 (C) and day 4 (D) p.i. are shown. Data in all panels are from one representative experiment repeated at least twice. Solid bars, +/− control; striped bars, TCR-$\beta^{-/-}$; cross-hatched bars, TCR-$\delta^{-/-}$; open bars, RAG-$1^{-/-}$. Mean from triplicate determinations; standard deviation (SD), <10%.

spleen cells from TCR-$\delta^{-/-}$ mutants was transiently impaired in early listeriosis. Reduced TNF secretion could diminish activation of NK cells.

In vivo modulation of IFN-γ. Thus far, two major effector mechanisms have been identified in listeriosis (2, 5, 9, 14, 15, 26, 29). In the early phase of infection (before day 4 p.i.), NK cells and γ/δ T cells are the main mediators of defense against this pathogen (2, 5, 29). In the long term, however, this innate immune response fails to control listeriosis and needs to be supplemented by specific T-cell immunity (14, 15, 18, 22). After day 4 p.i., antigen-specific α/β T cells, which mediate eradication of listeriae and are responsible for a protective memory response, have developed (14, 15, 22, 26).

TABLE 2. TNF production by spleen cells from *L. monocytogenes*-infected T-cell-deficient mice[a]

Day p.i.	Stimulus	TNF production (pg/ml) by:			
		TCR-$\delta^{+/-}$	TCR-$\delta^{-/-}$	TCR-$\beta^{-/-}$	RAG-$1^{-/-}$
1	HKL[b]	121.4 ± 34.4	28.5 ± 1.0	136.8 ± 12.2	14.8 ± 8.6
	Nil	<1.5	<1.5	<1.5	<1.5
4	HKL[b]	173.4 ± 20.1	143.9 ± 12.2	231.5 ± 1.7	35.6 ± 17.5
	Nil	<1.5	<1.5	<1.5	<1.5

[a] Mice were infected with 5×10^3 live *L. monocytogenes* organisms, and their spleens were removed. At the time points indicated, 2×10^5 spleen cells were stimulated in vitro with HKL for 2 days. Thereafter, the supernatants were taken and analyzed in the L929 bioassay for TNF activity. Recombinant TNF was used to generate a standard curve, and values were calculated from this standard curve. Results are given as mean ± SD from triplicates from one experiment repeated twice.

[b] 10^6 HKL per well.

To elucidate whether a direct correlation between reduced IFN-γ production and impaired innate resistance existed, IFN-γ was neutralized in *L. monocytogenes*-infected gene disruption mutants by treatment with anti-IFN-γ MAb on the day of infection (Fig. 2A and B). On day 4 or 7 p.i., CFU were determined to assess the consequences of IFN-γ neutralization on early or late resistance, respectively. IFN-γ neutralization exacerbated early listeriosis in heterozygous controls and TCR-$\beta^{-/-}$ mutants. In contrast, anti-IFN-γ MAb treatment had no demonstrable effect on early resistance in RAG-$1^{-/-}$ and TCR-$\delta^{-/-}$ mice. The IFN-γ neutralization impaired resistance to *L. monocytogenes* not only in TCR-$\beta^{-/-}$ and heterozygous mice but also in TCR-$\delta^{-/-}$ mutants. No effect was

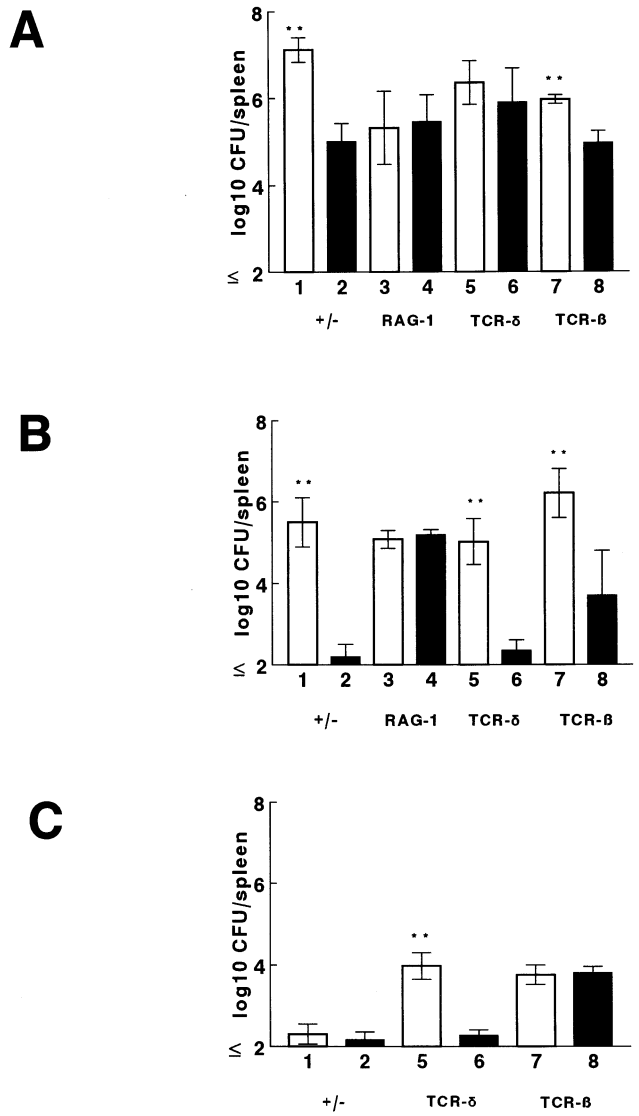

FIG. 2. In vivo neutralization of IFN-γ at different time points of *L. monocytogenes* infection. (A) Mice were treated intraperitoneally with anti-IFN-γ MAb (clone R4-6A2; 0.5 mg intraperitoneally) (open columns) or with rat immunoglobulin (Immunotech) (solid columns) on the day of infection (day zero) with 5×10^3 live *L. monocytogenes* organisms, and CFU were determined 4 days later. 1 and 2, in +/− control mice, a significant ($P < 0.05$) difference between anti-IFN-γ MAb-treated and rat immunoglobulin-treated mice was detected ($\Delta\log_{10}$ CFU, 2.1). 3 and 4, in RAG-$1^{-/-}$ mice, no difference was observed. 5 and 6, in the TCR-$\delta^{-/-}$ mice, the difference was not significant ($\Delta\log_{10}$ CFU, 0.4). 7 and 8, in TCR-$\beta^{-/-}$ mice, the difference was significant ($P < 0.05$) ($\Delta\log_{10}$ CFU, 1.0). (B and C) Mice were infected with live *L. monocytogenes* (5×10^3 live bacteria intravenously), treated with anti-IFN-γ MAb (open columns) or rat immunoglobulin (solid columns) intraperitoneally on day 0 (B) or day 3 (C) p.i., and CFU were determined on day 7 p.i. Significant differences ($P < 0.01$) for treatment on day 0: TCR-$\beta^{-/-}$, $\Delta\log_{10}$ CFU, 2.5; TCR-$\delta^{-/-}$, $\Delta\log_{10}$ CFU, 2.7; +/− controls, $\Delta\log_{10}$ CFU, 3.3. IFN-γ neutralization on day 3 p.i. (C) caused a significant increase ($P < 0.01$) in the bacterial load ($\Delta\log_{10}$ CFU, 1.7) only in TCR-$\delta^{-/-}$ mice. In all panels, results are mean ± SD of five mice per group; ∗ ∗, significant differences (Student's *t* test). Representative data from one of three independent experiments are shown.

observed in RAG-$1^{-/-}$ mutants. Finally, *L. monocytogenes*-infected mutants were treated with anti-IFN-γ MAb on day 3 p.i. and CFU were determined on day 7 p.i. It has been shown previously that IFN-γ neutralization at this later stage of infection does not affect protection in immunocompetent mice (26). Only TCR-$\delta^{-/-}$ mutants, not TCR-$\beta^{-/-}$ mutants or heterozygous controls, suffered from late IFN-γ neutralization (Fig. 2C). We conclude that the failure of anti-IFN-γ MAb to interfere with innate resistance in TCR-$\delta^{-/-}$ and RAG-$1^{-/-}$ mice was due to impaired IFN-γ production in these mutants. In contrast, acquired resistance in TCR-$\delta^{-/-}$ mice was affected by IFN-γ neutralization, suggesting delayed development of IFN-γ-producing α/β T cells, which then contributed to acquired resistance. Inhibitory effects of IFN-γ neutralization on resistance in TCR-$\beta^{-/-}$ mutants are consistent with potent IFN-γ production by both NK cells and γ/δ T lymphocytes in these mice. Consistent with deficient IFN-γ production by RAG-$1^{-/-}$ spleen cells, in vivo neutralization of this cytokine did not affect the CFU values.

Cytofluorimetric analysis of IFN-γ producing lymphocyte subsets. Because both NK cells and γ/δ T cells produce IFN-γ, we wanted to determine which cell was responsible for early IFN-γ production in listeriosis (3, 7, 18, 23, 29). To this end, we determined the presence of intracellular IFN-γ in γ/δ T cells and NK cells by two-color cytofluorometry (1, 7, 12). IFN-γ-expressing cells were not detected in noninfected mice ($f <$ 1/200,000). Similarly, at 1 to 8 h p.i. IFN-γ synthesis was not detected by FCM analysis ($f <$ 1/200,000). On day 1 of listeriosis, intracellular IFN-γ was identified in NK1.1^+ cells from heterozygous controls at a frequency of 1/9,200 cells (Fig. 3A) whereas virtually no IFN-γ was detected in NK1.1^+ cells from *L. monocytogenes*-infected TCR-$\delta^{-/-}$ mutants ($f <$ 1/200,000) (Fig. 3B). IFN-γ expression was minimal in γ/δ T cells from *L. monocytogenes*-infected heterozygous controls at this time point ($f =$ 1/107,000). IFN-γ producing αβ T cells were detectable on day 1 p.i. at a frequency of 1/26,000. The percentages of the lymphoid populations expressing TCR-α/β, TCR-γ/δ, or NK1.1 were 30.5, 1.7, and 10.2%, respectively, for the heterozygous controls and 29.7%, not detectable, and 9.7%, respectively, for the TCR-$\delta^{-/-}$ mutant mice. We conclude that a high proportion of NK1.1^+ cells produce IFN-γ at early time points during sublethal listeriosis and that these cells are controlled by γδ T cells. IFN-γ producing NK1.1^+ cells were, however, detected at a frequency of 1/10.500 in TCR-$\delta^{-/-}$ mutant mice in response to infection with a 50% lethal dose of 1 to 2. These findings emphasize overall activation of the host immune system under such a high listerial burden.

DISCUSSION

The data reported here demonstrate increased susceptibility of γδ T-cell-deficient mutant mice to early listeriosis and, in parallel, compromised early IFN-γ production in these mutants. In contrast, acquired cellular immunity to listeriosis was not affected in TCR-$\delta^{-/-}$ mutants, suggesting that the reduced IFN-γ secretion apparently did not influence subsequent development of specific α/β T cells. Experimental listeriosis of mice has been instrumental in elucidating immune mechanisms operative in acquired resistance to intracellular bacteria (14). Sublethal inocula of *L. monocytogenes* are efficiently restricted by the host immune system. In contrast, the microorganisms evade control by the host immune system when given at high inocula, and bacterial virulence factors and toxins directly cause death of animals within few days. The course of infection, therefore, is best monitored by CFU determination in organs of mice infected with sublethal inocula of *L. monocytogenes*. Accordingly, we considered it important to use low doses of *L. monocytogenes* for our analysis of immunoregulatory mechanisms operative in the early host response.

Early IFN-γ is considered central to immunity against intracellular bacteria for two reasons: (i) control of initial bacterial

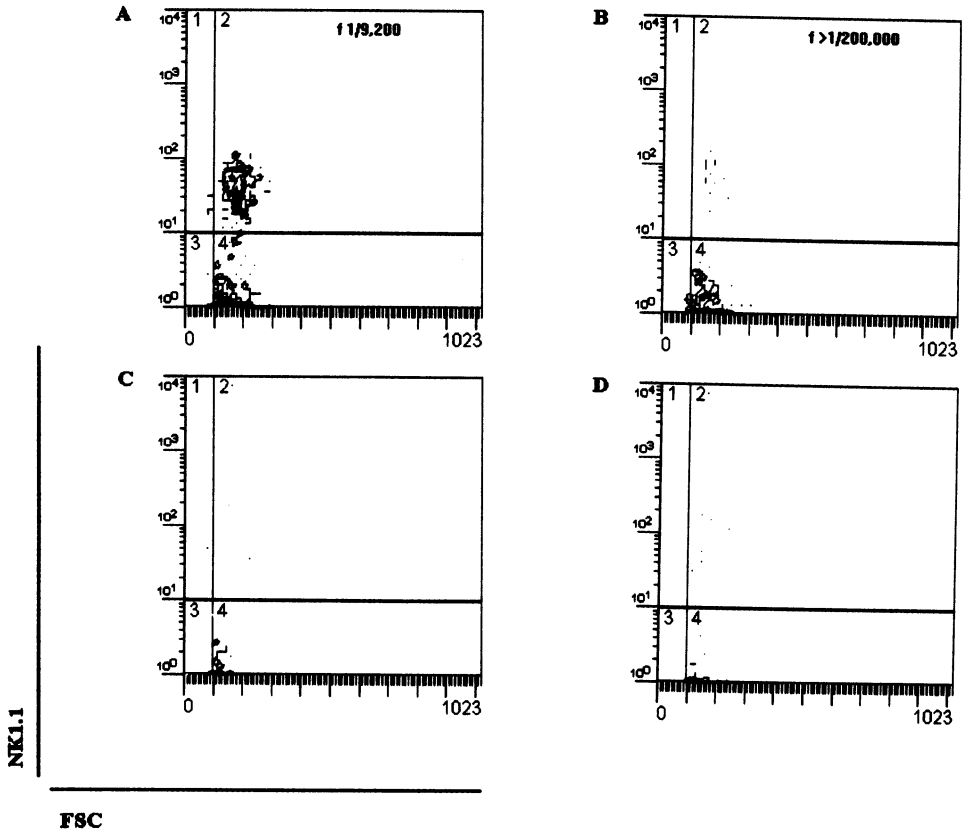

FIG. 3. Identification of IFN-γ-producing spleen cells by cytofluorimetry. Spleen cells were stained for intracellular accumulation of IFN-γ ex vivo after permeabilization of cells and for cell surface NK1.1 expression. The proportion of $NK1.1^+$ cells positively gated on IFN-γ producers is shown. Note that in both mice, other cells were also stained for IFN-γ. (A) Double staining for NK1.1 and IFN-γ on day 1 p.i. in infected +/− controls. (B) Staining in TCR-$\delta^{-/-}$ mice on day 1 p.i. (C and D) The respective controls exclude nonspecific staining. FSC, forward scatter.

replication and (ii) promotion of protective Th1 cell responses (10, 14, 20). Neutralization of IFN-γ by specific MAb treatment exacerbates listeriosis when administered in the early phase of infection (days 0 to 2) but is ineffective at later time points (18). The RAG-$1^{-/-}$ and TCR-$\delta^{-/-}$ mutant mice did not show this initial time dependence of IFN-γ neutralization, and exacerbation was observed in TCR-$\delta^{-/-}$ mutants at later time points only. We conclude that the early IFN-γ-dependent host response against listeriosis is impaired in the absence of γ/δ T lymphocytes.

Thus far, the role of NK cells in listeriosis has been elucidated primarily in the model of immunodeficient SCID and *nu/nu* mice (2, 3, 18, 28). Although NK cells control listerial replication for a considerable time in these T-cell-deficient animals, the mice eventually succumb to infection. The data obtained with the RAG-$1^{-/-}$ mutant mice confirm previous findings from this laboratory (22). In this study, RAG-$1^{-/-}$ mice were unable to control listeriosis and died after day 20 p.i. after being given inocula which were well tolerated by immunocompetent mice. The TCR-$\beta^{-/-}$ mutants were more resistant but also suffered more severely from late listeriosis (after day 21) than their control littermates did (22). In contrast, TCR-$\delta^{-/-}$ mutants suffered from exacerbated listeriosis only transiently, because the development of protective α/β T cells was apparently unaffected (22). IL-12 is considered essential for the development of NK cells and Th1 cells (10). This cytokine was apparently not affected in the TCR-$\delta^{-/-}$ mutants. Our findings therefore suggest that IL-12 was sufficient for promotion of protective α/β T cells of the Th1 type but insufficient for induction of IFN-γ-producing NK cells. In contrast, reduced TNF levels in parallel with transiently exacerbated listeriosis in TCR-$\delta^{-/-}$ mutants point to this proinflammatory cytokine as a link between reduced innate immunity and compromised NK activation. Consistent with our findings, disturbed TNF secretion has been observed in *Salmonella choleraesuis*-infected and lipopolysaccharide-treated TCR-$\delta^{-/-}$ mutant mice (6, 27). Although reduced TNF production in TCR-$\delta^{-/-}$ mutants shows that γ/δ T lymphocytes play a role in TNF production, our studies do not allow conclusion about the cellular source of this cytokine.

To define the lymphoid population(s) responsible for IFN-γ production, we used the method of double staining for intracellular cytokines and cell surface phenotype (1, 12). This approach revealed that $NK1.1^+$ cells rather than γ/δ T cells were the major source of early IFN-γ in immunocompetent animals and, moreover, that IFN-γ production by NK1.1 cells was impaired in TCR-$\delta^{-/-}$ mutant mice. Hence, this report provides the first evidence that antibacterial surveillance by NK cells is controlled by γ/δ T lymphocytes. γ/δ T cells could control NK cell activation in listeriosis through two alternate but not mutually exclusive mechanisms: (i) cognate cell interactions and (ii) cytokine secretion. We favor cytokine involvement in this early regulatory step. Recent elegant experiments have revealed that NK cells express receptors which interact with conserved parts of the major histocompatibility complex class I molecules (4, 13, 19, 25). Thus, NK cells are regulated by

complex control mechanisms to avoid autoaggression by false activation of this potent mediator of immune surveillance.

In conclusion, our data shed further light on the role of γ/δ T lymphocytes in protection against bacterial infection. Although γ/δ T lymphocytes are not essentially required for sterile clearance of listeriosis, they play a key regulatory role at the different phases of the antilisterial host response. The γ/δ T cells seem to regulate both NK cells and α/β T cells, which dominate the early and late phases of the antibacterial host response, respectively (9, 22, 26). Moreover, γ/δ T cells seem to provide the protective link between the receding NK cells and newly arriving α/β T cells. Our data add further information about the close interrelationship between innate and acquired resistance. Not only does the innate immune system influence the type of acquired resistance with IFN-γ and IL-12, promoting development of protective Th1 cells in infections with intracellular bacteria, but also, reciprocally, γ/δ T cells have a major impact on the innate host response.

ACKNOWLEDGMENTS

This work received financial support from the Sonderforschungsbereich 322 "Lympho-Hämopoese."

We thank Anja Dreher and Konrad Tell for expert technical assistance, Susumu Tonegawa and Peter Mombaerts for breeding pairs of mutant mice, Kurt Reifenberg and the animal caretakers for their support in breeding the mice, and Rita Mahmoudi for secretarial help.

REFERENCES

1. **Assenmacher, M., J. Schmitz, and A. Radbruch.** 1994. Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-γ and in interleukin-4-expressing cells. Eur. J. Immunol. **24:**1097–1101.
2. **Bancroft, G. J.** 1993. The role of natural killer cells in innate resistance to infection. Curr. Opin. Immunol. **5:**503–510.
3. **Bancroft, G. J., R. D. Schreiber, and E. R. Unanue.** 1991. Natural immunity: a T-cell-independent pathway of macrophage activation defined in the *scid* mouse. Immunol. Rev. **124:**5–24.
4. **Correa, I., and D. H. Raulet.** 1995. Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells. Immunity **2:**61–71.
5. **Dunn, P. L., and R. J. North.** 1991. Resolution of primary murine listeriosis and acquired resistance to lethal secondary infection can be mediated predominantly by Thy-1^+ $CD4^-$ $CD8^-$ cells. J. Infect. Dis. **164:**869–877.
6. **Emoto, M., H. Nishimura, T. Sakai, K. Hiromatsu, H. Gomi, S. Itohara, and Y. Yoshikai.** 1995. Mice deficient in γ/δ T cells are resistant to lethal infection with *Salmonella choleraesuis*. Infect. Immun. **63:**3736–3738.
7. **Ferrick, D. A., M. D. Schrenzel, T. Mulvania, B. Hsieh, W. G. Ferlin, and H. Lepper.** 1995. Differential production of interferon-γ and interleukin-4 in response to Th1- and Th2-stimulating pathogens by γ/δT cells in vivo. Nature (London) **373:**255–257.
8. **Flesch, I. E. A., J. H. Hess, S. Huang, M. Aguet, J. Rothe, H. Bluethmann, and S. H. E. Kaufmann.** 1995. Early interleukin-12 production by macrophages in response to mycobacterial infection depends on interferon-γ and tumor necrosis factor-α. J. Exp. Med. **181:**1615–1622.
9. **Hiromatsu, K., Y. Yoshikai, G. Matsuzaki, S. Ohga, K. Muramori, K. Matsumoto, J. A. Bluestone, and K. Nomoto.** 1992. A protective role of γ/δT cells in primary infection with *Listeria monocytogenes* in mice. J. Exp. Med. **175:** 49–56.
10. **Hsieh, C.-S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy.** 1993. Development of TH1 CD4 T cells through IL-12 produced by Listeria-induced macrophages. Science **260:**547–549.
11. **Itohara, S., P. Mombaerts, J. Lafaille, J. Iacomini, A. Nelson, A. R. Clarke, M. L. Hooper, A. Farr, and S. Tonegawa.** 1993. T cell receptor δ gene mutant mice: independent generation of α/βT cells and programmed rearrangements of γ/δTCR genes. Cell **72:**337–348.
12. **Jung, T., U. Schauer, C. Heusser, C. Neumann, and C. Rieger.** 1993. Detection of intracellular cytokines by flow cytometry. J. Immunol. Methods **159:** 197–207.
13. **Kärre, K.** 1995. Express yourself or die: peptides, MHC molecules, and NK cells. Science **267:**978–979.
14. **Kaufmann, S. H. E.** 1993. Immunity to intracellular bacteria, p. 1251–1286. In W. E. Paul (ed.), Fundamental immunology, 3rd ed. Raven Press, New York.
15. **Kaufmann, S. H. E.** 1993. Immunity to intracellular bacteria. Annu. Rev. Immunol. **11:**129–163.
16. **Kaufmann, S. H. E., and C. H. Ladel.** 1994. Application of knock-out mice to the experimental analysis of the infections with bacteria and protozoa. Trends Microbiol. **2:**235–242.
17. **Ladel, C. H., I. E. A. Flesch, J. Arnoldi, and S. H. E. Kaufmann.** 1994. Studies with MHC deficient knock-out mice reveal impact of both MHC I and MHC II dependent T cell responses in *Listeria monocytogenes* infection. J. Immunol. **153:**3116–3122.
18. **Leist, T. P., A. Meager, T. Exley, and R. M. Zinkernagel.** 1991. Evidence for a role of IFN-γ in control of *Listeria monocytogenes* in T cell deficient mice. Experientia **47:**630–632.
19. **Malnati, M. S., M. Peruzzi, K. C. Parker, W. E. Biddison, E. Ciccone, A. Moretta, and E. O. Long.** 1995. Peptide specificity in the recognition of MHC class I by natural killer cell clones. Science **267:**1016–1018.
20. **McKnight, A. J., G. J. Zimmer, I. Fogelman, S. F. Wolf, and A. K. Abbas.** 1994. Effects of IL-12 on helper T cell-dependent immune responses in vivo. J. Immunol. **152:**2172–2179.
21. **Meager, A., H. Leung, and J. Wooley.** 1989. Assays for tumor necrosis factor and related cytokines. J. Immunol. **116:**1–23.
22. **Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S. H. E. Kaufmann.** 1993. Different roles of α/β and γ/δT cells in immunity against an intracellular bacterial pathogen. Nature (London) **365:**53–56.
23. **Mombaerts, P., A. R. Clarke, M. A. Rudnicki, J. Iacomini, S. Itohara, J. J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M. L. Hooper, and S. Tonegawa.** 1992. Mutations in T-cell antigen receptor genes δ and β block thymocyte development at different stages. Nature (London) **360:**225–231.
24. **Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou.** 1992. RAG-1-deficient mice have no mature B and T lymphocytes. Cell **68:**869–877.
25. **Moretta, A., M. Vitale, S. Sivori, C. Bottino, L. Morelli, R. Augugliaro, M. Barbaresi, D. Pende, E. Ciccone, M. Lopez-Botet, and L. Moretta.** 1994. Human natural killer cell receptors for HLA-class I molecules. Evidence that the Kp43 (CD94) molecule functions as receptor for HLA-B alleles. J. Exp. Med. **180:**545–555.
26. **Nakane, A., T. Minagawa, M. Kohanawa, Y. Chen, H. Sato, M. Moriyama, and N. Tsuruoka.** 1989. Interactions between endogenous gamma interferon and tumor necrosis factor in host resistance against primary and secondary *Listeria monocytogenes* infection. Infect. Immun. **57:**3331–3337.
27. **Nishimura, H., M. Emoto, K. Hiromatsu, S. Yamamoto, K. Matsuura, H. Gomi, T. Ikeda, S. Itohara, and Y. Yoshikai.** 1995. The role of γ/δ T cells in priming macrophages to produce tumor necrosis factor α. Eur. J. Immunol. **25:**1465–1468.
28. **Petrini, J. H.-J., A. M. Carroll, and M. J. Bosma.** 1990. T-cell receptor gene rearrangements in functional T-cell clones from severe combined immunodeficient (SCID) mice: reversion of the scid phenotype in individual lymphocyte progenitors. Proc. Natl. Acad. Sci. USA **87:**3450–3453.
29. **Skeen, M. J., and H. K. Ziegler.** 1993. Induction of murine peritoneal γ/δ T cells and their role in resistance to bacterial infection. J. Exp. Med. **178:**971–984.
30. **Spitalny, G. L., and E. A. Havell.** 1984. Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. J. Exp. Med. **159:**1560–1565.

Editor: V. A. Fischetti