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Transient Control of Interleukin-4-Producing Natural Killer T Cells in the Livers of *Listeria monocytogenes*-Infected Mice by Interleukin-12

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Unconstrained development of gamma interferon (IFN-γ)-secreting natural killer (NK) cells and T helper (Th) 1 cells is central to protection against *Listeria monocytogenes*. In contrast, interleukin 4 (IL-4) is considered harmful. IL-12 produced by infected macrophages promotes, and IL-4 interferes with, protective antilisterial immunity. The liver NK T lymphocytes, which are a potent source of IL-4, are downregulated at an intermediate stage of listeriosis. Here we demonstrate that endogenous IL-12 participates in the control of IL-4-producing liver NK T lymphocytes during listeriosis. The effects of *L. monocytogenes* infection on IL-4-producing liver NK T lymphocytes were reversed by antibody neutralization of IL-12 but not of IFN-γ or tumor necrosis factor alpha (TNF-α). IL-4 production by liver NK T lymphocytes was virtually unaffected by heat-killed *L. monocytogenes* (HKL). Viable *L. monocytogenes* markedly increased the numbers of IL-12 producers in livers in parallel with an increase in macrophage numbers, whereas HKL failed to do so with similar efficiency. These results indicate that in the liver endogenous IL-12 improves protective immunity against listeriosis by downregulating IL-4-producing NK T lymphocytes. Moreover, our findings that HKL have a low level of IL-12-inducing activity and fail to control IL-4-producing NK T lymphocytes in the liver are consistent with the lesser protective capacity of HKL compared to that of live listeriae.

Listeria monocytogenes is an intracellular pathogen which replicates in professional and nonprofessional phagocytes (6). The liver is the major target organ of systemic listeriosis, and both Kupffer cells and hepatocytes are inhabited (5, 9, 11, 16, 19, 21, 22). Effective protection against *L. monocytogenes* depends on activation of gamma interferon (IFN-γ)-producing natural killer (NK) and T helper (Th) 1 cells (6). In contrast, interleukin 4 (IL-4) is generally considered harmful during listeriosis (10, 28, 33). Principally, the induction of NK cells and Th1 cells is promoted by IL-12, whereas IL-4 favors development of Th2 cells (2, 13, 17, 18, 23, 24, 30). It is generally agreed that viable *L. monocytogenes* induces superior protection compared to heat-killed *L. monocytogenes* (HKL) (27, 32). The superior efficacy of viable over killed listeriae seems related to the greater IL-12-inducing capacity of viable microorganisms (26). Evidence has been presented that NK T lymphocytes are a major source of the rapid IL-4 burst which promotes Th2 cell development after T-cell receptor (TCR) ligation (1, 3, 4, 7, 8, 12, 35). Recently, we have reported that $CD4^+$ $NK1^+$ T cells represent a prominent population of IL-4-secreting lymphocytes in the liver (7, 8). Moreover, we found that this IL-4 production is downregulated during intermediate stages of listeriosis (8).

The present study shows that this control of IL-4-producing NK T lymphocytes in the livers of listeria-infected mice is transient and reversible. In contrast to viable *L. monocytogenes* organisms, HKL failed to downregulate these cells. Moreover, we show that locally produced IL-12 is involved in the downregulation of IL-4-secreting NK T lymphocytes in the liver. In contrast, IFN-γ and tumor necrosis factor alpha (TNF-α) apparently did not participate in the downregulation of this cell population. Our data further support the notion that active downregulation of IL-4-producing liver NK T lymphocytes participates in the unconstrained development of protective immunity against *L. monocytogenes* in vivo. The involvement of IL-12 suggests that control of these IL-4 producers represents a general safeguard mechanism of Th1 immune responses which counteracts default Th2 cell development.

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MATERIALS AND METHODS

Mice. Breeding pairs of IFN-γ receptor (R) gene-disrupted mutant mice were kindly provided by M. Aguet (Genentech, Inc., San Francisco, Calif.) (14) and bred on a C57BL/6 background (beyond the second generation). Mutants as well as C57BL/6 mice were maintained under specific pathogen-free conditions at the central animal facilities of the University of Ulm. Mutants and C57BL/6 mice of either sex were used at 8 to 12 weeks of age.

Microorganisms and infection. A sample of *L. monocytogenes* EGD-infected liver homogenate was grown in tryptic soy broth for 18 h at 37°C, and aliquots were frozen at −70°C until used. The final concentration of viable bacteria was determined by plate counts. Mice were infected intravenously (i.v.) with viable bacteria as indicated in the figures and tables. HKL were prepared by heating bacteria in a water bath at 80°C for 3 h. HKL were washed three times with phosphate-buffered saline and frozen at −20°C until use. Bacterial survival after heat treatment was excluded by negative culture (threshold: 10^{13} organisms/ml). Mice received various numbers of HKL i.v.

MAbs. The following monoclonal antibodies (MAbs) were purified from hybridoma culture supernatants by ammonium sulfate precipitation and affinity chromatography on protein A or G Sepharose (Pharmacia, Freiburg, Germany): anti-CD3ε MAb (145-2C11; hamster immunoglobulin G [IgG]), anti-TCRα/β MAb (H57-597; hamster IgG), anti-TCRγ/δ MAb (GL3; hamster IgG), anti-NK1.1 MAb (PK136; mouse IgG2a), anti-IFN-γ MAb (R4-6A2 and AN-18.17.24; rat IgG1), anti-IL-4 MAb (BVD6-24G2; rat IgG1), anti-FcγR MAb (2.4G2; rat IgG2b), and anti-IL-12 (p40) MAb (C15.6.7, rat IgG1; C17.8, rat IgG2a). Anti-TCRα/β MAb, anti-IFN-γ MAb (AN18.17.24), anti-IL-4 MAb, anti-NK1.1 MAb, and anti-IL-12 MAb (C.15.6.7) were biotinylated. Anti-NK1.1 MAb and anti-TCRα/β MAb were conjugated with fluorescein isothiocyanate

TABLE 1. Influence of endogenous IFN-γ and TNF-α on frequencies of IL-4 producers among liver mononuclear cells during listeriosis[a]

In vitro stimulation	Mean no. of IL-4-producing cells/10^6 cells ± SD					
	C57BL/6 mice				IFN-γ $R^{-/-}$ mice	
	Uninfected	Infected	Infected + anti-IFN-γ MAb	Infected + anti-TNF-α serum	Uninfected	Infected
Anti-CD3 MAb	26,500 ± 4,720	3,200 ± 580	3,520 ± 380	3,410 ± 580	14,800 ± 2,240	3,860 ± 310
Anti-TCRα/β MAb	4,800 ± 740	950 ± 140	1,020 ± 210	1,100 ± 140	4,950 ± 650	1,110 ± 200
Anti-TCRγ/δ MAb	350 ± 40	390 ± 50	320 ± 40	290 ± 30	200 ± 30	210 ± 10
Hamster IgG	210 ± 30	300 ± 20	290 ± 40	320 ± 30	190 ± 20	190 ± 20
P815 alone	200 ± 10	330 ± 20	330 ± 30	300 ± 20	240 ± 20	230 ± 20
Nil	210 ± 10	330 ± 30	340 ± 40	340 ± 20	180 ± 30	200 ± 20

[a] C57BL/6 mice were left untreated or were treated i.p. with 500 μg of anti-IFN-γ MAb or 50 μl of anti-TNF-α antiserum and infected i.v. with 2×10^3 *L. monocytogenes* bacteria immediately thereafter. IFN-γ $R^{-/-}$ mice were infected i.v. with 2×10^3 *L. monocytogenes* bacteria. Liver mononuclear cells were prepared on day 0 and day 4 p.i., and numbers of IL-4-secreting cells were determined by the ELISPOT technique. Recovery numbers of liver mononuclear cells: uninfected mice (C57BL/6), 2.3×10^6; infected mice (C57BL/6), 6.8×10^6; infected mice with anti-IFN-γ MAb treatment (C57BL/6), 6.0×10^6; infected mice with anti-TNF-α serum treatment (C57BL/6), 6.1×10^6; uninfected mice (IFN-γ $R^{-/-}$), 2.0×10^6; infected mice (IFN-γ $R^{-/-}$), 8.2×10^6. Estimated absolute numbers of $CD4^+$ $NK1^+$ liver T lymphocytes: uninfected mice (C57BL/6), 3.5×10^5; infected mice (C57BL/6), 4.1×10^4; infected mice with anti-IFN-γ MAb treatment (C57BL/6), 5.4×10^4; infected mice with anti-TNF-α serum treatment (C57BL/6), 4.9×10^4; uninfected mice (IFN-γ $R^{-/-}$), 1.6×10^5; infected mice (IFN-γ $R^{-/-}$), 5.7×10^4. Representative results from one of three different experiments from triplicate cultures are shown.

(FITC) by conventional methods. Normal hamster IgG, anti-IL-4 MAb (BVD4-1D11; rat IgG2a), and FITC-conjugated anti-Mac-1 MAb (Bear1; mouse IgG1) were obtained from Dianova (Hamburg, Germany). Phycoerythrin (PE)-conjugated anti-CD4 MAb (H129.19; rat IgG2b) and streptavidin (SA)-conjugated Red 670 were purchased from Life Technologies (Gaithersburg, Md.).

In vivo treatments. Mice received anti-IL-12 MAb (C.15.6.7) (500 μg), anti-IFN-γ MAb (R4-6A2) (500 μg), or anti-TNF-α rabbit serum (50 μl) (Genzyme) intraperitoneally (i.p.) immediately before *L. monocytogenes* infection, and liver mononuclear cells were prepared on day 4 postinfection (p.i.).

Cell preparation and flow cytometry. Liver mononuclear cells were prepared as described previously (7). Purified liver mononuclear cells (40/70% interphase of a discontinuous Percoll density gradient) were stained with appropriate dilutions of the MAbs. Biotinylated MAbs were visualized by SA-conjugated Red 670. Before being stained with a specific MAb, cells were incubated with 50 μg of anti-FcγR MAb and 50 μg of normal hamster IgG per ml in order to block FcγR-mediated binding of the MAb. Each staining step was performed at 4°C for 30 min; each washing step was performed with phosphate-buffered saline containing 2.5% normal human serum and 0.1% sodium azide. After being stained, cells were washed twice and then fixed with 1% paraformaldehyde (Serva). Stained cells (10^4) were analyzed by a FACScan (Becton Dickinson) cell sorter equipped with Lysis II or Cell Quest software. Unless otherwise stated, small lymphoid cells were gated by forward and side scatter.

ELISPOT assay. MAb-facilitated IFN-γ or IL-4 production by liver mononuclear cells was measured by the enzyme-linked immunospot (ELISPOT) method as described previously (7, 8). For detection of IL-12 (p40) production by liver mononuclear cells, the C17.8 MAb was used for coating and the biotinylated C15.6.7 MAb was employed for IL-12 detection.

RESULTS

Effects of endogenous IFN-γ, TNF-α, and IL-12 on the downregulation of IL-4-producing NK T lymphocytes in the livers of *L. monocytogenes*-infected mice. We have recently reported that *L. monocytogenes* downregulates IL-4-producing liver NK T lymphocytes (8). We speculated that this downregulation favors development of protective immune mechanisms. IL-12, TNF-α, and IFN-γ, on the one hand, and IL-4, on the other hand, counterregulate each other, and the balance between Th1-promoting cytokines IL-12, TNF-α, and IFN-γ and the Th2-promoting cytokine IL-4 determines Th polarization (13, 17, 18, 23, 24, 30). IL-12, TNF-α, and IFN-γ are produced soon after *L. monocytogenes* infection (6). We therefore wondered whether these cytokines downregulated IL-4 production and liver NK T lymphocytes. Mice were treated with a neutralizing antibody against IFN-γ, TNF-α, or IL-12 and infected with *L. monocytogenes* immediately thereafter. Liver mononuclear cells were prepared before and after infection, and numbers of IFN-γ- and IL-4-producing cells after TCR or CD3 ligation were determined. In parallel, the proportion of $CD4^+$ $NK1^+$ T lymphocytes among these liver mononuclear cells was assessed by microfluorimetry.

Consistent with our previous findings (8), frequencies of IL-4 producers (Table 1) and proportions of $CD4^+$ $NK1^+$ T lymphocytes (Fig. 1A) in the livers of listeria-infected mice were markedly reduced. In contrast, numbers of IFN-γ producers increased after *L. monocytogenes* infection (Table 2). The *L. monocytogenes*-induced reductions of IL-4 production and of the $CD4^+$ $NK1^+$ liver T-lymphocyte population were not reversed by IFN-γ or TNF-α neutralization (Fig. 1A, Table 1). Similarly, after *L. monocytogenes* infection, frequencies of IL-4 producers were drastically reduced and those of IFN-γ producers were markedly increased in IFN-γ $R^{-/-}$ mice (Tables 1 and 2). The proportion of $CD4^+$ $NK1^+$ liver T lymphocytes was profoundly diminished in *L. monocytogenes*-infected IFN-γ $R^{-/-}$ mutants, although the percentage of liver NK T lymphocytes in naive IFN-γ $R^{-/-}$ mice was slightly lower than that in naive C57BL/6 mice (Fig. 1B). These results suggest that *L. monocytogenes*-induced downregulation of IL-4-producing liver NK T lymphocytes occurred independently of endogenous IFN-γ and TNF-α.

We then assessed the involvement of endogenous IL-12 in the downregulation of IL-4-producing liver NK T lymphocytes. In naive mice neither the frequencies of IL-4 and IFN-γ producers (Table 3) nor the proportion of $CD4^+$ $NK1^+$ T lymphocytes in the liver was affected by anti-IL-12 MAb treatment (Fig. 2). In contrast, the *L. monocytogenes*-induced reduction of IL-4 producers and the downregulation of $CD4^+$ $NK1^+$ liver T lymphocytes were partially reversed by IL-12 neutralization (Fig. 2, Table 3). Conversely, frequencies of IFN-γ producers among liver mononuclear cells were decreased (Table 3). These data demonstrate that endogenous IL-12 is involved in the downregulation of IL-4-producing liver NK T lymphocytes during listeriosis. However, our data do not exclude the possibility that IL-4 produced by other cells is modulated in addition.

Control of $CD4^+$ $NK1^+$ liver T lymphocytes during listeriosis is transient. C57BL/6 mice were infected i.v. with 5×10^3 *L. monocytogenes* bacteria, and liver mononuclear cells were prepared on days 0, 4, 8, and 12 of infection. Consistent with our previous findings (8), a high proportion of $CD4^+$ $NK1^+$ T lymphocytes was identified in livers from naive mice and most of them had disappeared on day 4 p.i. (Fig. 3). The recovery of

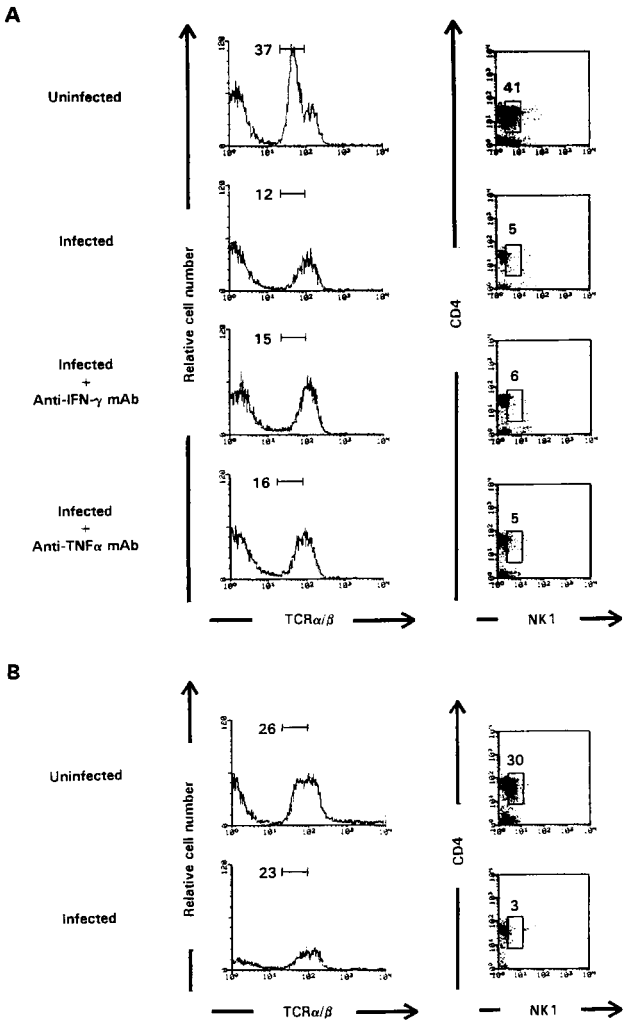

FIG. 1. Influence of endogenous IFN-γ and TNF-α on the downregulation of $CD4^+$ $NK1^+$ liver T lymphocytes by *L. monocytogenes*. Liver mononuclear cells were triple stained with FITC-conjugated anti-NK1.1 MAb, PE-conjugated anti-CD4 MAb, and biotinylated anti-TCRα/β MAb followed by SA-conjugated Red 670. The profiles of TCRα/β are displayed as histograms after gating on small lymphoid cells. Numbers in histograms are percentages of small lymphoid cells. The data for NK1.1/CD4 are displayed as dot plots after gating on $TCR\alpha/\beta^{int}$ small lymphoid cells. Numbers in dot plots are percentages of $TCR\alpha/\beta^{int}$ small lymphoid cells. (A) C57BL/6 mice. (B) IFN-γ $R^{-/-}$ mice. Data are from the same mice used for Table 1. Representative results from a total of six mice per group are shown. For further details see Table 1.

$CD4^+$ $NK1^+$ liver T lymphocytes commenced by day 8 p.i. and was complete on day 12 p.i. In agreement with published data (19), high numbers of bacteria were detected in liver on day 4 p.i. ($[4.4 \pm 2.1] \times 10^5$ CFU/liver) and bacteria had been cleared by day 8 p.i. (<25 CFU/liver). These results reveal transient and reversible downregulatory effects of *L. monocytogenes* infection on $CD4^+$ $NK1^+$ liver T lymphocytes.

Effects of HKL on IL-4 production by liver NK T lymphocytes. Activation of protective immunity against intracellular bacteria including *L. monocytogenes* is generally best achieved by viable microbes (32). In part, this could be due to the failure of HKL to efficiently stimulate IL-12 and to downregulate IL-4 production (26). We therefore assessed the influence of HKL administration on the fate of IL-4-producing liver NK T lymphocytes. As shown in Fig. 4, the single administration of 10^9 HKL did not affect $CD4^+$ $NK1^+$ liver T lymphocytes on day 4 after administration. Similarly, $CD4^+$ $NK1^+$ liver T lymphocytes were not affected by administration of 10^5, 10^6, 10^7, or 10^8 HKL (data not shown), and no effect was detected on days 8, 12, and 16 after a single administration of 10^9 HKL (data not shown). Even repeated HKL administration of 10^9 HKL failed to affect $CD4^+$ $NK1^+$ liver T lymphocytes significantly (Fig. 4). Although the relative proportion of $CD4^+$ $NK1^+$ liver T lymphocytes was reduced after HKL administration (Fig. 4), recovery numbers of liver mononuclear cells were increased twofold compared to those for naive mice. Accordingly, no difference in the absolute numbers of $CD4^+$ $NK1^+$ liver T lymphocytes between the control group and the repeated-HKL administration group was identified (see the legend to Fig. 4). It is possible that this reduction in the proportion of $CD4^+$ $NK1^+$ liver T lymphocytes was caused by a dilution effect. We also assessed the influences of HKL administration on cytokine producers among liver mononuclear cells. Single administration of HKL did not affect frequencies of IFN-γ- and IL-4-producing cells, and repeated HKL administration diminished the numbers of IL-4 producers in accordance with the reduction in the relative proportion of $CD4^+$ $NK1^+$ T lymphocytes (Table 4). Conversely, numbers of IFN-γ-producing cells were modestly increased by repeated HKL administration (Table 4).

Effects of viable *L. monocytogenes* and HKL on IL-12-producing cells in the liver. We wondered whether the different effects of *L. monocytogenes* infection and HKL administration on IL-4-producing liver NK T lymphocytes were related to differential IL-12 production in the liver. Mice received 5×10^3 viable *L. monocytogenes* bacteria or, alternatively, 10^9 HKL once or three times. Liver mononuclear cells were prepared 1 day after the last administration, and frequencies of IL-12

TABLE 2. Influence of endogenous IFN-γ and TNF-α on frequencies of IFN-γ producers among liver mononuclear cells during listeriosis[a]

In vitro stimulation	Mean no. of IFN-γ-producing cells/10^6 cells ± SD					
	C57BL/6 mice				IFN-γ $R^{-/-}$ mice	
	Uninfected	Infected	Infected + anti-IFN-γ MAb	Infected + anti-TNF-α serum	Uninfected	Infected
Anti-CD3 MAb	6,400 ± 490	13,200 ± 2,050	12,900 ± 2,100	11,900 ± 1,200	21,100 ± 1,500	42,800 ± 7,500
Anti-TCRα/β MAb	2,080 ± 370	3,870 ± 630	3,440 ± 540	3,700 ± 530	5,820 ± 650	20,700 ± 3,400
Anti-TCRγ/δ MAb	160 ± 30	140 ± 10	190 ± 20	150 ± 10	140 ± 30	13,200 ± 2,600
Hamster IgG	190 ± 30	150 ± 20	120 ± 30	190 ± 20	110 ± 20	13,800 ± 1,400
P815 alone	80 ± 20	100 ± 20	150 ± 30	170 ± 10	100 ± 30	12,900 ± 2,100
Nil	80 ± 20	110 ± 20	110 ± 30	120 ± 30	110 ± 10	13,100 ± 1,500

[a] C57BL/6 mice were left untreated or were treated i.p. with 500 μg of anti-IFN-γ MAb or 50 μl of anti-TNF-α antiserum and infected i.v. with 2×10^3 *L. monocytogenes* bacteria immediately thereafter. IFN-γ $R^{-/-}$ mice were infected i.v. with 2×10^3 *L. monocytogenes* bacteria. Liver mononuclear cells were prepared on day 0 and day 4 p.i., and numbers of IFN-γ-secreting cells were determined by the ELISPOT technique. Representative results from one of three different experiments from triplicate cultures are shown. Data are from the same mice as those used for Table 1. For further details see Table 1.

TABLE 3. Influence of endogenous IL-12 on frequencies of IL-4 and IFN-γ producers among liver mononuclear cells during listeriosis[a]

In vitro stimulation	Mean no. of IL-4-producing cells/10^6 cells ± SD				Mean no. of IFN-γ-producing cells/10^6 cells ± SD			
	Uninfected	Uninfected + anti-IL-12 MAb	Infected	Infected + anti-IL-12 MAb	Uninfected	Uninfected + anti-IL-12 MAb	Infected	Infected + anti-IL-12 MAb
Anti-CD3 MAb	26,200 ± 3,800	22,400 ± 2,050	2,540 ± 160	8,500 ± 1,050	6,900 ± 580	6,800 ± 740	14,200 ± 1,900	9,620 ± 910
Anti-TCRα/β MAb	5,760 ± 780	5,540 ± 380	780 ± 110	2,480 ± 190	1,280 ± 210	1,020 ± 300	3,960 ± 750	2,060 ± 190
Anti-TCRγ/δ MAb	220 ± 30	240 ± 30	320 ± 50	300 ± 40	140 ± 40	180 ± 20	840 ± 150	400 ± 50
Hamster IgG	210 ± 30	200 ± 30	330 ± 40	280 ± 30	100 ± 30	140 ± 20	780 ± 210	420 ± 140
P815 alone	150 ± 30	180 ± 20	300 ± 60	200 ± 50	80 ± 10	160 ± 20	880 ± 70	380 ± 70
Nil	190 ± 10	200 ± 40	310 ± 20	220 ± 50	80 ± 20	180 ± 20	880 ± 190	360 ± 110

[a] C57BL/6 mice were either left untreated or treated i.p. with 500 μg of anti-IL-12 MAb and infected i.v. with 5×10^3 *L. monocytogenes* bacteria immediately thereafter. Liver mononuclear cells were prepared on day 0 and day 4 p.i., and numbers of IL-4- and IFN-γ-secreting cells were determined by the ELISPOT technique. Recovery numbers of liver mononuclear cells: uninfected mice, 2.2×10^6; uninfected mice with anti-IL-12 MAb treatment, 2.4×10^6; infected mice, 7.4×10^6; infected mice with anti-IL-12 MAb treatment, 5.9×10^6. Estimated absolute numbers of $CD4^+$ $NK1^+$ liver T lymphocytes: uninfected mice, 3.4×10^5; uninfected mice with anti-IL-12 MAb treatment, 3.3×10^5; infected mice, 4.7×10^4; infected mice with anti-IL-12 MAb treatment, 4.3×10^5. Representative results from one of three different experiments from triplicate cultures are shown.

producers were estimated. As shown in Table 5, numbers of IL-12 producers among liver mononuclear cells were drastically increased after *L. monocytogenes* infection. Although frequencies of spontaneous IL-12 producers were already elevated after *L. monocytogenes* infection, in vitro stimulation with HKL caused a further increase. In contrast, such an increase was not observed with liver mononuclear cells from mice treated with HKL once. Repeated administration of HKL increased numbers of IL-12-producing cells only slightly. As shown in Fig. 5, *L. monocytogenes* infection markedly increased the proportion of mononuclear phagocytes in the liver. In contrast, numbers of mononuclear phagocytes were not altered by a single HKL treatment and were only slightly elevated after repeated HKL administration. These results suggest that downregulation of IL-4-secreting liver NK T lymphocytes by viable *L. monocytogenes*, but not by HKL, is caused by the attraction of mononuclear phagocytes to the liver and increased local IL-12 secretion.

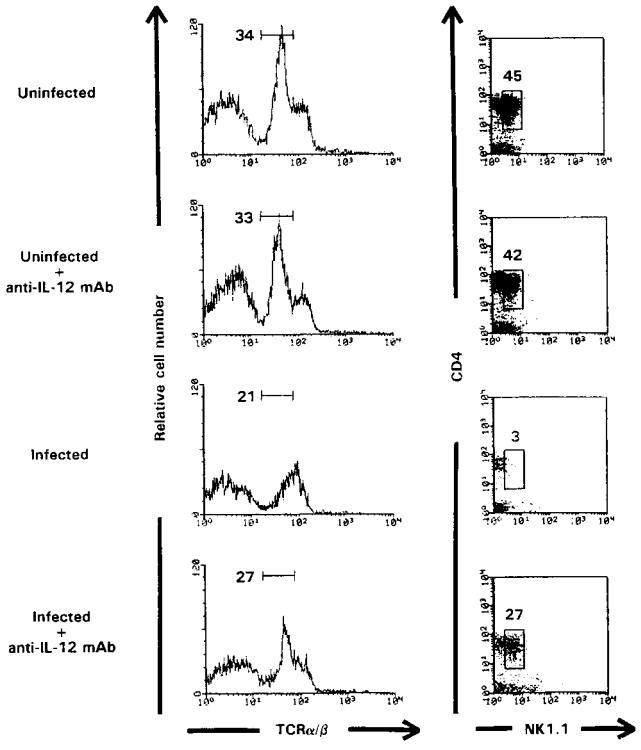

FIG. 2. Influence of endogenous IL-12 on the downregulation of $CD4^+$ $NK1^+$ liver T lymphocytes by *L. monocytogenes*. Data are from the same mice used for Table 3. Representative results from a total of six mice per group are shown. For further details see the legend for Fig. 1 and Table 3.

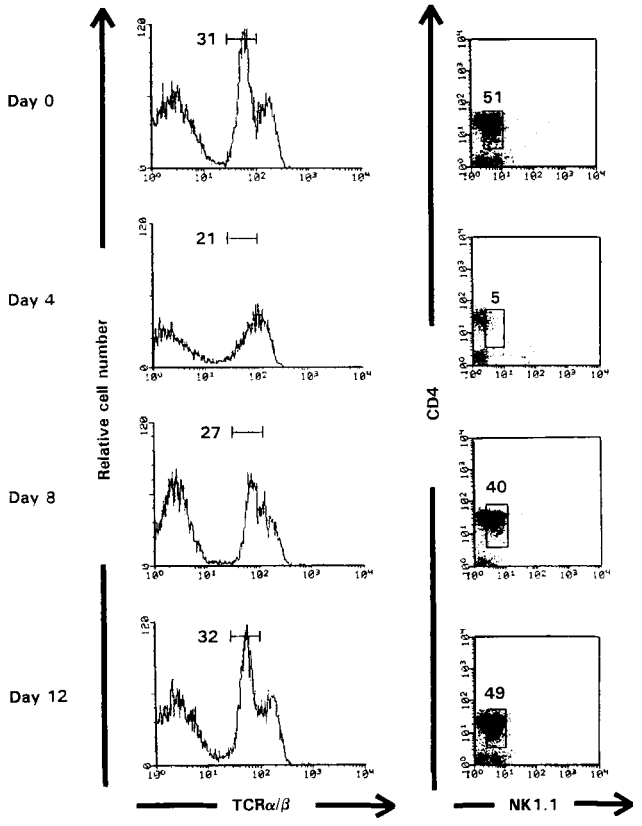

FIG. 3. Transient downregulation of $CD4^+$ $NK1^+$ liver T lymphocytes during listeriosis. C57BL/6 mice were infected i.v. with 5×10^3 *L. monocytogenes* bacteria, and liver mononuclear cells were prepared on days 0, 4, 8, and 12 p.i. Representative results from a total of six mice per group are shown. For further details see the legend for Fig. 1.

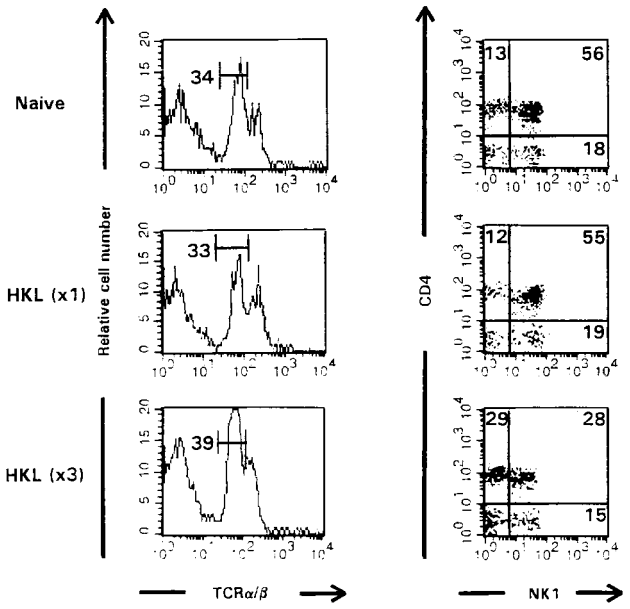

FIG. 4. Cell surface phenotypes of liver lymphocytes before and after HKL immunization. C57BL/6 mice were immunized i.v. with 10^9 HKL once (x1) or three times at 5-day intervals (x3), and liver mononuclear cells were prepared on day 0 and day 4 after the last administration. Cells were triple stained with FITC-conjugated anti-TCRα/β MAb, PE-conjugated anti-CD4 MAb, and biotinylated anti-NK1.1 MAb followed by SA-conjugated Red 670. Recovery numbers of liver mononuclear cells: naive, 2.2×10^6; HKL (x1), 2.3×10^6; HKL (x3), 4.0×10^6. Estimated absolute numbers of $CD4^+$ $NK1^+$ liver T lymphocytes: naive, 4.2×10^5; HKL (x1), 4.2×10^5; HKL (x3), 4.4×10^5. Representative results from a total of six mice per group are shown. For further details see the legend for Fig. 1.

DISCUSSION

IFN-γ is central to protection against *L. monocytogenes*, whereas IL-4 exacerbates listeriosis (6, 10, 33). IL-12 promotes the development of Th1 cells, whereas IL-4 favors development of a Th2-cell response (13, 17, 18, 23, 24, 30). The prominent IFN-γ and small-to-absent IL-4 response during listeriosis therefore is probably promoted by profound IL-12 and TNF-α secretion by infected macrophages, which stimulates NK cells to produce IFN-γ (6). IFN-γ and IL-12 promote the subsequent development of Th1 cells. Recently, we found that IL-4-producing liver NK T lymphocytes were downregulated by *L. monocytogenes* infection (8) and speculated that this mechanism further favors protection and averts default polarization towards disease exacerbation.

Our data raise the question about the fate of liver NK T lymphocytes during *L. monocytogenes* infection. Several possibilities exist. First, it is possible that the NK T cells egress from the liver. Yet we did not observe increased levels of NK T cells in lymphoid organs such as the spleen and lymph nodes (8). Second, it could be argued that accumulation of inflammatory cells in the liver during listeriosis reduces the relative proportion of this population. Although this dilution effect indeed occurs, absolute numbers of NK T cells were still diminished at least sevenfold. Finally, the possibility needs to be considered that these cells change their phenotype and/or density during infection and thus evade detection in the liver by the methods employed. Current efforts are aimed at resolving these issues.

The present report shows that downregulation of IL-4-secreting NK T lymphocytes in the livers of listeria-infected mice is transient. Moreover, we identified IL-12 as an endogenous mediator of *L. monocytogenes*-induced downregulation. IL-12 is a heterodimeric cytokine which enhances NK cell-mediated cytotoxicity and induces IFN-γ production by NK cells and Th1 cells (13, 17, 18, 23, 24, 30). In contrast, downregulation of IL-4-producing liver NK T lymphocytes was not affected by anti-IFN-γ MAb or anti-TNF-α antiserum and also occurred in *L. monocytogenes*-infected IFN-γ $R^{-/-}$ mutant mice. These data reveal that IFN-γ and TNF-α are not essential for control of IL-4 secretion in the liver. Our results demonstrating that IL-12 produced at the outset of listeriosis is responsible for active downregulation of IL-4-producing liver NK T lymphocytes further support the central role of this cytokine in protection.

The findings reported here reveal differential effects on IL-4-producing liver NK T lymphocytes during viable *L. monocytogenes* infection and HKL treatment. Listeriosis downregulated IL-4-producing liver NK T lymphocytes (8), whereas HKL failed to do so. Although our group has recently reported successful vaccination against listeriosis by repeated HKL administration (27), we do not question that protective immunity against intracellular bacteria is best induced by viable bacteria (32). Although IL-12 stimulation by HKL in vitro has been demonstrated (13), differences between viable and killed bacteria have been described for IL-12 and IL-1 induction in vivo (20, 26). Similarly, in our experiments, IL-12 induction by viable listeriae was markedly higher than that by HKL. Evidence that IL-1 and IL-12 costimulate IFN-γ production in NK cells and that IL-1 is involved in IL-12-mediated resistance against intracellular pathogens, including *L. monocytogenes*, has been presented (15, 25). Because (i) NK T lymphocytes promptly produce IL-4 after TCR engagement (1, 3, 4, 7, 8, 12, 34, 35) and (ii) IL-4 favors maturation of Th2 cells and counteracts Th1 cell differentiation (13, 18, 24), we assume that control of IL-4 production by $CD4^+$ $NK1^+$ liver T lymphocytes supported the development of protective T-cell responses.

IL-12 directly activates IFN-γ-secreting NK cells and Th1 cells, and we do not question the importance of this IL-12 effect. Rather, we assume that, in addition, IL-12 induced

TABLE 4. Frequencies of IL-4 and IFN-γ producers among liver mononuclear cells before and after HKL immunization[a]

In vitro stimulation	Mean no. of IL-4-producing cells/10^6 cells ± SD			Mean no. of IFN-γ-producing cells/10^6 cells ± SD		
	Naive	HKL (x1)	HKL (x3)	Naive	HKL (x1)	HKL (x3)
Anti-CD3 MAb	28,900 ± 1,800	27,900 ± 2,900	16,200 ± 3,200	6,980 ± 1,080	7,160 ± 1,210	8,160 ± 860
Anti-TCRα/β MAb	6,240 ± 1,090	5,980 ± 730	3,980 ± 530	1,420 ± 230	1,400 ± 290	1,980 ± 310
Anti-TCRγ/δ MAb	190 ± 10	200 ± 50	220 ± 20	100 ± 10	140 ± 20	180 ± 10
Hamster IgG	190 ± 40	200 ± 60	240 ± 60	110 ± 10	130 ± 20	120 ± 10
P815 alone	140 ± 10	190 ± 30	170 ± 20	140 ± 10	130 ± 10	190 ± 20
Nil	180 ± 40	140 ± 50	190 ± 70	100 ± 20	130 ± 10	200 ± 10

[a] Representative results from one of three different experiments from triplicate cultures are shown. Data are from the same mice as those used for Fig. 4. For further details see the legend for Fig. 4 and Table 1. x1 and x3 are defined in the legend for Fig. 4.

TABLE 5. Influence of viable *L. monocytogenes* and HKL on frequencies of IL-12 producers among liver mononuclear cells[a]

In vitro stimulation	Mean no. of IL-12-producing cells/10^6 cells ± SD			
	Naive	Viable *L. monocytogenes*	HKL (x1)	HKL (x3)
Nil	2,150 ± 450	8,580 ± 720	2,300 ± 280	2,970 ± 400
HKL	3,220 ± 330	35,700 ± 2,800	3,410 ± 430	4,860 ± 350

[a] Liver mononuclear cells were incubated in the presence or absence of 10^6 HKL overnight in ELISPOT plates coated with anti-IL-12 MAb. Data are from the same mice as those used for Fig. 5. Representative results from one of three different experiments from triplicate cultures are shown. x1 and x3 are defined in the legend for Fig. 4.

downregulation of IL-4-producing NK T lymphocytes and in this way provided an auxiliary mechanism which further supported protective immunity. Moreover, IL-12 has been shown to influence IL-4 and IL-10 secretion in a negative way and IFN-γ and IL-12 production in a positive way in several infection systems including listeriosis (29, 31). Because indirect evidence suggests that IL-4 counteracts IL-12 (13) and because IL-4 has detrimental effects in infections with intracellular pathogens (10, 28, 33), such an additional mechanism should facilitate optimum control of infection.

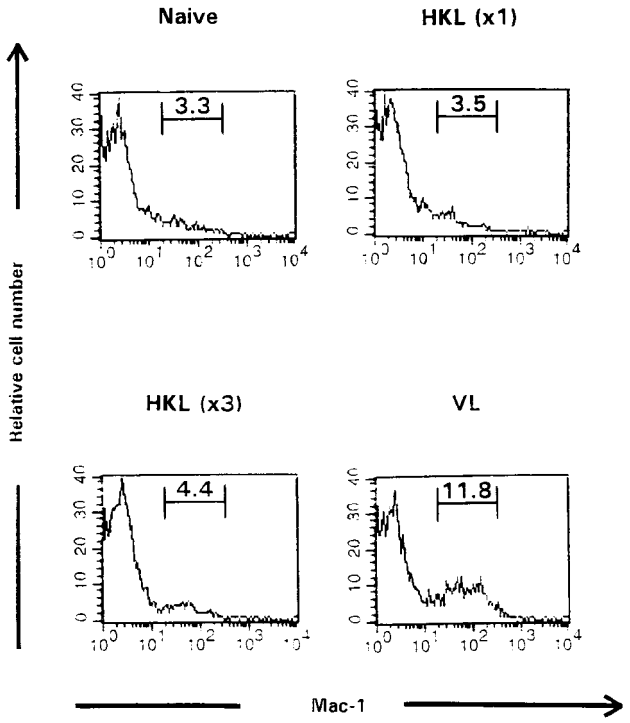

FIG. 5. Influence of viable *L. monocytogenes* and HKL on mononuclear phagocytes in the liver. C57BL/6 mice received i.v. 5×10^3 viable *L. monocytogenes* cells (VL). Alternatively, mice received i.v. 10^9 HKL once (x1) or three times at 5-day intervals (x3). Liver mononuclear cells were prepared 1 day after the last administration. Cells were stained with FITC-conjugated anti-Mac-1 MAb. The profiles of Mac-1 are displayed as histograms without gating. Numbers in histograms are the percentages of total liver mononuclear cells. Recovery numbers of liver mononuclear cells: naive, 2.0×10^6; HKL (x1), 2.1×10^6; HKL (x3), 3.9×10^6; viable *L. monocytogenes*, 7.2×10^6. Estimated absolute numbers of Mac-1^+ cells: naive, 6.6×10^4; HKL (x1), 7.4×10^4; HKL (x3), 1.7×10^5; viable *L. monocytogenes*, 8.5×10^5. Representative results from a total of six mice per group are shown.

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