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Effect of Interleukin 12 Neutralization on Host Resistance and Gamma Interferon Production in Mouse Typhoid

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Innately resistant (*Ity*^{r}) A/J mice infected with the virulent *Salmonella typhimurium* C5 strain suppress the early exponential bacterial growth in the reticuloendothelial system toward the end of the first week of infection, with spleen and liver bacterial counts reaching a plateau phase. In vivo administration of neutralizing anti-interleukin-12 (IL-12) antibodies did not affect early bacterial growth in the tissues (days 1 to 3) but impaired the establishment of the plateau, with higher spleen and liver counts by day 7 of the infection in anti-IL-12 treated mice than in untreated controls. Gamma interferon (IFN-γ) was detectable in the sera and spleen homogenates of both control and anti-IL-12-treated mice on days 3 and 7 of the infection. Noticeably, IFN-γ levels were significantly lower in anti-IL-12 treated mice than in control animals. Splenocytes from uninfected A/J mice released IFN-γ in response to concanavalin A (ConA) or to *S. typhimurium* C5. In vitro IL-12 neutralization dramatically impaired the IFNγ response to *S. typhimurium* but not to ConA. Splenocytes harvested from infected anti-IL-12 treated mice on day 7 of the infection produced significantly lower amounts of IFN-γ upon in vitro stimulation with ConA and with a *Salmonella* protein-rich extract than did cells from similarly infected untreated control animals. Spleen cells from infected mice showed lower proliferative (mitogenic) responses to ConA and to a *Salmonella* soluble extract than did cells from uninfected mice. In vivo anti-IL-12 treatment significantly restored the ability of splenocytes from infected mice to proliferate in response to the antigens and ConA. In vivo neutralization of IL-12 in innately susceptible BALB/c mice (*Ity*^{s}) immunized with a live attenuated aromatic-dependent *Salmonella* vaccine reduced host resistance to virulent oral challenge with *S. typhimurium* C5. Thus, in primary *Salmonella* infections, IL-12 mediates the suppression of growth of virulent salmonellae in the reticuloendothelial system, positively modulates IFN-γ production, and is involved in the immunosuppression which accompanies the acute stages of the disease. IL-12 also contributes to host resistance to virulent organisms in secondary infections.

Natural resistance and acquired immunity to *Salmonella* infection are studied primarily in the mouse model by using bacterial strains which in this host cause systemic infections believed to be very similar to human typhoid fever (7). In mice, early growth of salmonellae in the reticuloendothelial system (RES) is controlled by the innate resistance *Ity* gene mediated by resident macrophages (13, 21). In sublethal infections, bacterial growth in the RES is suppressed by a T-cell-independent host response (bacterial counts reaching a plateau) which requires bone marrow-derived cells, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) and coincides with granuloma formation (14, 20, 21, 24, 25, 27). Mice in the plateau phase of a *Salmonella* infection exhibit profoundly depressed responses to T- and B-cell mitogens, suppression being mediated by macrophages with the requirement for IFN-γ (1, 10). Primary infections with some *Salmonella* strains leave long-term resistance to rechallenge (21). Resistance persists long after the primary inoculum has been cleared and involves the specific recall of immunity with the requirement for both $CD4^+$ and $CD8^+$ T cells, opsonizing antibodies, TNF-α, and IFN-γ (22, 23, 33).

IFN-γ production in response to *Salmonella* infection has been documented both in vitro and in vivo. The cytokine is detectable in the circulation of mice infected intraperitoneally or intravenously (i.v.) with *Salmonella typhimurium* (4, 11, 22). Peyer's patches, mesenteric lymph nodes, and spleen cells from mice infected orally with *S. typhimurium* spontaneously express elevated levels of IFN-γ mRNA and produce IFN-γ upon in vitro stimulation with killed bacteria (30). In vitro release of IFN-γ from splenocytes of naive mice upon stimulation with *S. typhimurium* has been reported (28–30); natural killer (NK) cells were found to be the main IFN-γ producers with the requirement of TNF-α and adherent cells (29).

Interleukin-12 (IL-12) is a 70-kDa heterodimeric cytokine produced in response to a variety of stimuli, including products of bacterial origin (6, 9). IL-12 mediates resistance to several intracellular organisms such as *Listeria*, *Toxoplasma*, *Candida*, and *Leishmania* species, *Mycobacterium tuberculosis*, and *Brucella abortus*, and it is required for the establishment of protective Th1 responses (8, 12, 15, 17, 18, 31, 32, 34, 35, 37, 39). IL-12 is a potent IFN-γ inducer by NK cells (8), and its neutralization can impair host resistance to infection (8, 15, 31, 34, 37, 39) and suppress IFN-γ production in vivo and in vitro. IL-12 production in mice infected with live attenuated salmonellae has been recently reported (5); nevertheless, the role of this cytokine in resistance to *Salmonella* infection has not as yet been proved.

In this study, we investigated the effect of in vivo IL-12 neutralization on bacterial growth in the RES and on in vivo and in vitro IFN-γ production in mice undergoing a primary sublethal infection with virulent salmonellae. We also assessed the effect of IL-12 neutralization on the control of bacterial growth in the RES in mice immunized with live attenuated salmonellae and challenged orally with virulent organisms.

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TABLE 1. Effects of in vivo IL-12 or TNF-α neutralization on spleen and liver counts in A/J mice infected with *S. typhimurium* C5[a]

Day	Mean $\log_{10}$ viable count ± SD											
	Expt A				Expt B				Expt C			
	Controls		Anti-IL-12-treated mice		Controls		Anti-IL-12-treated mice		Controls		Anti-TNF-α-treated mice	
	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver
1	2.58 ± 0.21	2.43 ± 0.34	2.31 ± 0.05	2.09 ± 0.17					2.42 ± 0.1	2.6 ± 0.4	2.32 ± 0.23	2.1 ± 0.16
3	3.95 ± 0.14	3.68 ± 0.16	4.00 ± 0.24	3.76 ± 0.36					4.4 ± 0.15	4.7 ± 0.22	4.6 ± 0.1	4.3 ± 0.36
7	4.50 ± 0.32[b]	4.43 ± 0.24[c]	6.16 ± 0.21[b]	6.22 ± 0.17[c]	4.80 ± 0.07[d]	4.83 ± 0.16[e]	6.65 ± 0.20[d]	7.12 ± 0.39[e]	5.1 ± 0.08[f]	4.8 ± 0.3[g]	7.8 ± 0.3[f]	7.6 ± 0.2[g]

[a] Mice were infected i.v. with 10^3 (experiment A, three mice per point; experiment C, six mice per point) or 5×10^3 (experiment B, five mice per point) CFU of *S. typhimurium* C5. For the anti-IL-12 treatment, mice received two i.v. injections of 0.25 mg (sheep anti-IL-12; experiment A) or 1 mg (goat anti-IL-12 IgG; experiment B) of neutralizing IgG 2 h before infection and on day 3. Controls received a similar amount of species-matched IgG. For the anti-TNF-α treatment (experiment C), mice received 1 mg of neutralizing rabbit anti-mouse TNF-α antibodies 2 h before challenge and on day 3 of the infection. Control animals received a similar amount of rabbit normal globulins.

[b] Statistical significance of spleens of control mice versus anti-IL-12-treated mice: $P < 0.001$.

[c] Statistical significance of livers of control mice versus anti-IL-12-treated mice: $P < 0.001$.

[d] Statistical significance of spleens of control mice versus anti-IL-12-treated mice: $P < 0.001$.

[e] Statistical significance of livers of control mice versus anti-IL-12-treated mice: $P < 0.001$.

[f] Statistical significance of spleens of control mice versus anti-TNF-α-treated mice: $P < 0.001$.

[g] Statistical significance of livers of control mice versus anti-TNF-α-treated mice: $P < 0.001$.

MATERIALS AND METHODS

Mice. Female innately *Salmonella*-resistant A/J *Ity*$^{r/r}$ mice were purchased from Harlan OLAC Ltd. (Blackthorn, Bicester, United Kingdom) and used when more than 8 weeks old.

Bacteria. The virulent *S. typhimurium* C5 and the live attenuated *aroA S. typhimurium* SL3261 live vaccine strain have been described elsewhere (13, 22). Aliquots of 37°C stationary overnight cultures in tryptic soy broth (Oxoid) were snap-frozen and stored in liquid nitrogen. For i.v. inoculation, a vial was rapidly thawed and appropriately diluted in phosphate-buffered saline (PBS). Animals were inoculated with 0.2 ml of bacterial suspension in a lateral tail vein, and the inoculum was checked by examining growth on tryptic soy agar pour plates. For oral challenge, bacteria were grown overnight at 37°C in LB broth (Oxoid), harvested by centrifugation at 13,000 × *g*, and resuspended in sterile PBS at the appropriate concentration. Mice were lightly anaesthetized with fluoroalothane and administered 0.2 ml of bacterial suspension intragastrically with a gavage tube (19). For in vitro stimulation of splenocyte cultures, bacteria were grown overnight at 37°C in LB broth (Oxoid), washed once in sterile PBS, and diluted in tissue culture medium (see below) at the appropriate concentration.

Bacterial enumeration in organ homogenates. Mice were killed by cervical dislocation. Spleens and livers were aseptically removed and homogenized in a Colworth Stomacher (Seward) in 5 ml of cold distilled water (13). Viable counts were performed by using pour plates of tryptic soy agar or by the droplet technique, using a Colworth Droplette (Seward) as previously described (13).

Anti-IL-12 antibodies. Neutralizing goat anti-mouse and sheep anti-mouse IL-12 polyclonal immunoglobulin G (IgG) were kindly provided by M. Gately (Hoffmann-La Roche, Nutley, N.J.) and S. Wolf (Genetics Institute Inc., Cambridge, Mass.), respectively.

Mice received i.v. injections of 1 mg of goat anti-IL-12 IgG (1 mg/ml neutralizes 1 ng of recombinant IL-12 per ml) or 0.25 mg of sheep anti-IL-12 (1 mg/ml neutralizes 2 ng of recombinant IL-12 per ml) of neutralizing IgG at times specified below (neutralization was assessed in in vitro assays [6a, 38]). Normal goat or sheep IgG (Sigma, Poole, Dorset, United Kingdom) was used as a control.

Anti-TNF-α antibodies. An anti-TNF-α antiserum was raised in rabbits by immunization with recombinant murine TNF-α, kindly provided by G. R. Adolf, Boehringer Ingelheim, Vienna, Austria, as described previously (24). Whole globulins (2 mg per dose) were used after 40% ammonium sulfate precipitation and dialysis against PBS. Control animals received a similar amount of normal rabbit globulins (Sigma). Two milligrams of globulins neutralized the biological activity of 3×10^4 U of recombinant TNF-α in the L929 cytotoxicity assay as previously described (20).

Collection of sera and organ homogenates for IFN-γ determinations. Mice were bled before being sacrificed for bacterial counting; the sera were collected and stored at −70°C. Spleens were homogenized as described above. Aliquots of spleen homogenates from individual mice were clarified by centrifugation and stored at −70°C until use.

IFN-γ ELISA. IFN-γ was measured by capture enzyme-linked immunosorbent assay (ELISA), using antibody pairs and recombinant IFN-γ purchased from Pharmingen (Cambridge BioScience, Cambridge, United Kingdom). Each well of 96-well ELISA plates (Maxisorp Nunc Immuno plates; Nunc, Roskilde Denmark) was coated at 37°C for 2 h with 50 μl of a capture rat anti-mouse IFN-γ IgG1 monoclonal antibody (clone R4-6A2) in 0.1 M $NaHCO_3$ buffer (pH 8.2) at 2 μg/ml and incubated overnight at 4°C. After blocking with RPMI 1640 supplemented with 10% fetal calf serum (FCS) at 37°C for 1 h, samples (diluted in RPMI 1640–10% FCS) were loaded in 50 μl in triplicate, and the plates were incubated at 37°C for 2 h. Serial twofold dilutions of recombinant IFN-γ ranging from 20 to 0.04 ng/ml were included as standards. To each well was added 100 μl of the biotinylated rat anti-mouse IFN-γ IgG1 monoclonal antibody (clone XMG1.2) at 1 μg/ml in PBS–10% FCS followed by 100 μl of peroxidase-labelled streptavidin (Sigma catalog no. A3151) at 2.5 μg/ml in PBS–10% FCS. *ortho*-Phenylenediamine (1 mg/ml in 0.2 M Na_2HPO_4–0.1 M citrate buffer) in the presence of H_2O_2 was used to develop the plates. The reaction was stopped by addition of 15 μl of 3 M H_2SO_4 per well. Optical density was read at 490 nm. IFN-γ values (picograms per milliliter) were determined by comparison with the standard curve.

Splenocyte cultures. Splenocytes were prepared as described elsewhere (36). Briefly, mice were sacrificed by cervical dislocation, and single-cell suspensions were prepared. Cells were washed once in RPMI 1640 (Sigma) at 300 × *g* and incubated in Gey's solution to lyse the erythrocytes. Leukocytes were washed twice more, resuspended in RPMI 1640 supplemented with 100 U of penicillin per ml, 100 μg of streptomycin (Sigma) per ml, 5 mM glutamine (Sigma), 2×10^{-5} M β-mercaptoethanol (Sigma), 1 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and 10% heat-inactivated FCS (Sigma). For stimulation with whole bacteria (7.5×10^5 or 7.5×10^4 CFU of *S. typhimurium* C5 per ml), cells were dispensed at a concentration of 10^6 per well in flat-bottom 96-well plates (Corning Glass Works, Corning, N.Y.) in 200 μl. For stimulation with an alkali-treated *Salmonella* soluble extract (C5NaOH), the cells were dispensed in round-bottom 96-well plates (Corning) at a concentration of 4×10^5 cells in 200 μl. The plates were incubated in a 95% humidity, 5% CO_2, 37°C atmosphere. The cultures were stimulated with C5NaOH (20 or 2 μg/ml) prepared as described elsewhere (36). For IFN-γ measurements, the supernatants were harvested at 24 and/or 48 h, aliquoted, and stored at −70°C. Results are expressed as picograms per milliliter (mean ± standard deviation) in triplicate cultures.

For proliferation assays, splenocyte cultures stimulated with C5NaOH or with concanavalin A (ConA) (4×10^5 cells in 200 μl in round-bottom wells) were pulsed at 48 h with 1 μCi of [*methyl*-^{3}H]thymidine (Amersham International, Amersham, United Kingdom) for 12 h. The cells were harvested onto glass fiber filters in a semiautomatic cell harvester, and incorporated counts per minute were determined by ^{3}H-sensitive avalanche gas ionization detection on a matrix 96 Direct β counter (Packard, Meriden Conn.). Results are expressed as the mean counts per minute of triplicate cultures ± standard error.

Statistical analysis. Student's *t* test was used to determine the significance of differences between controls and experimental groups.

RESULTS

Effect of in vivo neutralization of IL-12 on *Salmonella* growth in the tissues. Innately resistant A/J mice were sublethally infected i.v. with 10^3 (experiment A) or 5×10^3 (experiment B) CFU of virulent *S. typhimurium* C5. Viable counts were performed at times thereafter. One group of mice in each experiment received two injections of anti-IL-12 neutralizing globulins (0.25 mg of sheep IgG in experiment A; 1 mg of goat IgG in experiment B) 2 h before challenge and on day 3 of the

sublethal infection. Controls received similar amounts of species-matched normal IgG.

The anti-IL-12 treatment did not affect early bacterial growth in the RES (Table 1, experiment A), day 1 and day 3 viable counts being similar in anti-IL-12-treated and control mice. Conversely, day 7 spleen and liver viable counts were higher in anti-IL-12-treated mice than in controls, resulting in a clear exacerbation of the infection (Table 1, experiments A and B). Thus, in vivo IL-12 neutralization does not affect the early stages of a *Salmonella* infection but impairs the suppression of bacterial growth in the RES.

Effect of in vivo IL-12 neutralization on IFN-γ levels in sera and spleen homogenates of infected mice. In experiments A and B, IFN-γ was measured in the sera and spleen homogenates of infected controls and anti-IL-12-treated mice. Samples from age- and sex-matched noninfected (naive) mice were included.

IFN-γ was not detectable in either the circulation or the organs of uninfected mice or of infected mice on day 1 after challenge. In experiment A (lower challenge dose), IFN-γ was present in sera and spleen homogenates of control mice at days 3 and 7 postinfection. Anti-IL-12-treated mice showed a reduction in IFN-γ levels in sera and spleen homogenates which was statistically significant on day 7 but not on day 3 postinfection. In experiment B (higher challenge dose), IFN-γ levels in sera were higher than in experiment A. Anti-IL-12 treatment caused a marked and significant reduction of serum IFN-γ both on day 3 and on day 7 (Table 2). Thus, in vivo IL-12 neutralization induces a decrease in IFN-γ in mice infected with *S. typhimurium* C5.

Effect of in vivo TNF-α neutralization on serum IFN-γ levels in *Salmonella*-infected mice. A/J mice were infected as in experiment A. One group of mice received 1 mg of neutralizing rabbit anti-mouse TNF-α antibodies 2 h before challenge and on day 3 of the infection. Control animals received a similar amount of rabbit normal globulins. Spleen and liver counts in controls and anti-TNF-α-treated mice were similar on days 1 and 3 after infection. Thereafter, TNF-α neutralization induced the expected exacerbation of the infection (21, 24, 26), with higher bacterial loads in the RES of anti-TNF-α-treated mice on day 7 (Table 1).

IFN-γ was not detectable in the sera of the infected mice on day 1 after challenge. Similar IFN-γ levels of the cytokine were detected in the sera of controls and anti-TNF-α-treated mice on day 3 of the infection. Conversely, the sera from anti-TNF-α-treated mice contained significantly higher amounts of IFN-γ on days 5 and 7 after challenge than did sera from control animals (Table 2). Similar results were obtained in similar repeat experiments or when mice were infected with a higher dose of virulent salmonellae (i.e., as in experiment B; data not shown).

Thus, TNF-α neutralization exacerbates the course of a *Salmonella* infection without causing a reduction (but rather causing an increase) in the amount of circulating IFN-γ.

Effect of in vitro IL-12 neutralization on IFN-γ production by splenocytes. Spleen cells from naive A/J mice were stimulated with 7.5×10^5 or 7.5×10^4 CFU of *S. typhimurium* C5 per ml or with 5 μg of ConA per ml in the presence of 2 μg of either goat anti-IL-12 IgG or sheep anti-IL-12 IgG per well. Parallel cultures were stimulated in the presence of similar amounts of species-matched normal IgG or in the absence of antibodies. IFN-γ was measured in the supernatants at 48 h. Previous pilot studies showed that in our experimental conditions, IFN-γ levels in culture supernatants peak at 48 h poststimulation. Figure 1 shows that simulation with ConA and with either dose of bacteria induced high levels of IFN-γ in the

TABLE 2. Effects of in vivo IL-12 and TNF-α neutralization on IFN-γ levels in A/J mice infected with *S. typhimurium* C5[a]

Level in:	Mean IFN-γ (pg/ml) ± SD													
	Expt A				Expt B				Expt C					
	Naive mice	Controls		Anti-IL-12-treated mice		Naive mice	Controls		Anti-IL-12-treated mice		Naive mice	Controls		Anti-TNF-α-treated mice	
		Day 3	Day 7	Day 3	Day 7		Day 3	Day 7	Day 3	Day 7		Day 3	Day 5	Day 3	Day 5	Day 7
Spleen	330 ± 34	1,283 ± 229	2,822 ± 377[b]	760 ± 541	1,368 ± 377[b]											
Serum	<100	183 ± 166	1,231 ± 406[c]	<100	355 ± 404[c]	<400	2,924 ± 1,502[d]	5,316 ± 615[e]	538 ± 142[d]	797 ± 316[e]	<200	500 ± 250	650 ± 85[f]	900 ± 125[g]	750 ± 130	1,750 ± 375[f]	2,135 ± 100[g]

[a] Mice were infected and treated as for Table 1. IFN-γ levels are shown for groups of three (experiment A), five (experiment B), or six (experiment C) mice per point.
[b] Statistical significance of IFN-γ levels in spleen homogenates of control mice versus anti-IL-12-treated mice: $P = 0.026$.
[c] Statistical significance of IFN-γ levels in sera of control mice versus anti-IL-12-treated mice: $P = 0.03$.
[d] Statistical significance of IFN-γ levels in sera of control mice versus anti-IL-12-treated mice on day 3 postinfection: $P = 0.02$.
[e] Statistical significance of IFN-γ levels in sera of control mice versus anti-IL-12-treated mice on day 7 postinfection: $P < 0.001$.
[f] Statistical significance of IFN-γ levels in sera of control mice versus anti-TNF-α-treated mice on day 5 postinfection: $P < 0.001$.
[g] Statistical significance of IFN-γ levels in sera of control mice versus anti-TNF-α-treated mice on day 7 postinfection: $P < 0.001$.

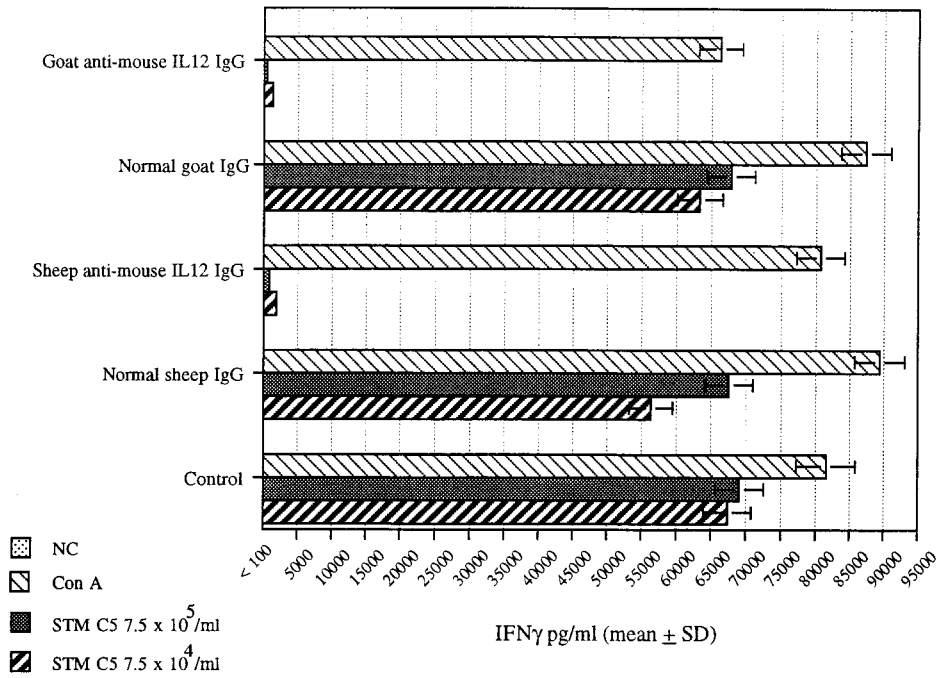

FIG. 1. Effect of in vitro IL-12 neutralization on IFN-γ production by splenocytes of A/J mice stimulated in vitro with ConA or with *S. typhimurium* C5. Spleen cells (10^6 per well) were stimulated with ConA (5 μg/ml) or with *S. typhimurium* C5 (STM C5; 7.5×10^5 or 7.5×10^4 CFU/ml) or were cultured with medium alone (NC) in the presence of either 2 μg of goat anti-IL-12 IgG or 2 μg of sheep anti-IL-12 IgG per ml. Parallel sets of wells were incubated with a similar amount of species-matched normal IgG. One set of wells was stimulated in the absence of antibodies (control). Supernatants for IFN-γ determinations were collected at 48 h. Results are expressed as means ± standard deviations (SD) of IFN-γ levels from quadruplicate cultures.

culture supernatants. In vitro neutralization of IL-12 dramatically reduced the amount of IFN-γ produced upon stimulation with bacteria, but it did not significantly affect IFN-γ production induced by ConA. Thus, in vitro IFN-γ production by mouse spleen cells in response to bacterial stimulation requires IL-12.

Effect of in vivo IL-12 neutralization on in vitro IFN-γ production from splenocytes. A/J mice were infected and treated in vivo as in experiment B. Splenocytes from naive mice, infected controls, and infected anti-IL-12-treated mice were stimulated in vitro with C5NaOH at 20 or 2 μg/ml or with ConA at 1.25 μg/ml. IFN-γ was measured in the supernatants of triplicate cultures at 24 and 48 h. Previous experiments showed that the highest release of IFN-γ from spleen cells in response to C5NaOH is detectable 24 and 48 h after in vitro stimulation (data not shown).

Figure 2 shows that spleen cells from naive (uninfected) mice released IFN-γ upon stimulation with ConA but with neither dose of C5NaOH. Splenocytes harvested on day 7 of the in vivo infection with *S. typhimurium* C5 spontaneously released IFN-γ both at 24 and 48 h, with IFN-γ levels greatly increasing after ConA or C5NaOH stimulation. Spleen cells from mice treated in vivo with anti-IL-12 antibodies also released IFN-γ spontaneously. Nevertheless, the increase of IFN-γ production in response to ConA was significantly lower than in cells from untreated infected control mice both at 24 and 48 h. Spleen cells from anti-IL-12-treated animals did not show a significant increase in IFN-γ production in response to either dose of C5NaOH. Thus, in vivo neutralization of IL-12 during a *Salmonella* infection severely impairs the ability of spleen cells from infected mice to release IFN-γ upon in vitro restimulation.

Effect of in vivo IL-12 neutralization on mitogenic responses by splenocytes. A/J mice were infected and treated in vivo as in experiment B. Splenocytes from naive mice, infected controls, and infected anti-IL-12-treated mice were stimulated in vitro with C5NaOH at 20 or 2 μg/ml or with ConA at 1.25 μg/ml. [^{3}H]thymidine incorporation was measured at 48 h.

Figure 3 shows strong proliferative responses from cells from naive mice stimulated with the higher dose (20 μg/ml) of C5NaOH or ConA. Weaker proliferative responses were observed in response to the lower dose (2 μg/ml) of C5NaOH. Proliferative responses to ConA and to either dose of C5NaOH were severely suppressed in cells from infected control mice. In vivo treatment with anti-IL-12 antibodies significantly restored the ability of splenocytes to respond to the mitogenic stimuli. Thus, IL-12 is involved in the development of unresponsiveness to mitogenic responses observed in mice infected with *S. typhimurium* C5.

Effect of in vivo IL-12 neutralization on bacterial numbers in the RES of immunized mice challenged with virulent salmonellae. BALB/c mice were immunized i.v. with ca. 10^6 CFU of *S. typhimurium* SL3261. Three months after vaccination, mice were challenged orally with 10^9 CFU (ca. 1,000 50% lethal doses) of virulent *S. typhimurium* C5. One group of mice received 1 mg of goat anti-IL-12 IgG at the time of oral challenge and 0.25 mg of sheep anti-IL-12 IgG on day 3 of the secondary infection. Controls received a similar amount of species-matched IgG.

Day 7 spleen and liver viable counts were significantly higher in anti-IL-12-treated mice than in control animals ($\log_{10}$ viable counts of 4.30 ± 0.20 [spleen] and 4.43 ± 0.39 [liver] for anti-IL-12-treated mice and of 2.15 ± 0.3 [spleen] and 1.83 ± 0.25 [liver] for control mice). Thus, IL-12 contributes to the control of bacterial growth of virulent organisms in the RES of mice vaccinated with a live attenuated *Salmonella* vaccine.

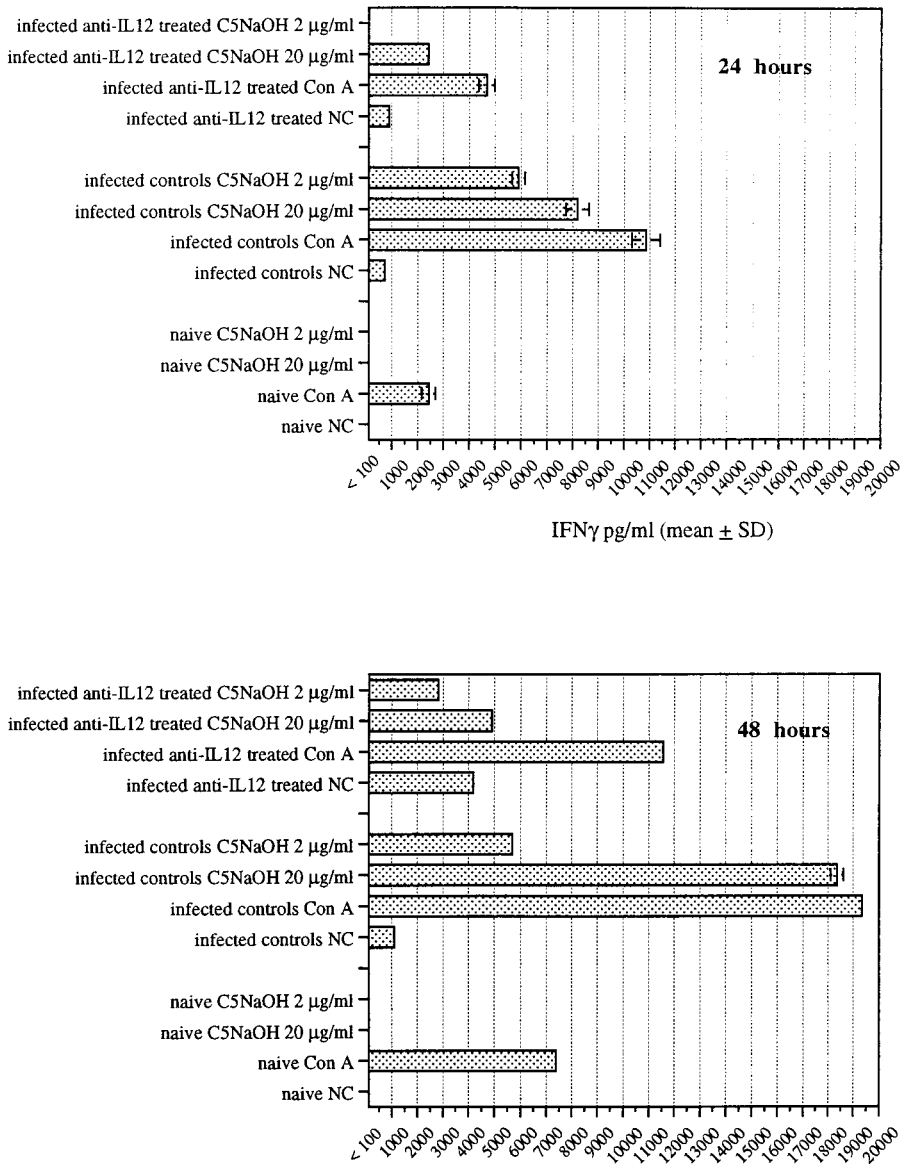

FIG. 2. In vitro IFN-γ production from splenocytes of uninfected A/J mice (naive) and from spleen cells harvested from control and anti-IL-12-treated mice on day 7 of an i.v. infection with 5×10^3 CFU of *S. typhimurium* C5. Infected controls received 1 mg of normal goat IgG 2 h before infection and on day 3 after challenge; anti-IL-12-treated mice were similarly injected with 1 mg of goat anti-mouse IL-12 IgG. Different sets of cells were stimulated in vitro with 1.25 μg of ConA or with C5NaOH (2 or 20 μg/ml). One set of cells was incubated with medium alone (NC). Supernatants were collected 24 and 48 h after in vitro culture. Results are expressed as means ± standard deviations (SD) of IFN-γ levels from triplicate cultures.

DISCUSSION

In this report, we show that in vivo administration of anti-IL-12 antibodies exacerbates the course of a sublethal *Salmonella* infection with virulent organisms in innately resistant mice. The treatment induced a reduction of IFN-γ levels in the sera and in the spleens of the infected mice. In vivo IL-12 neutralization impaired in vitro IFN-γ production from splenocytes upon antigen stimulation and also prevented development of the unresponsiveness to mitogenic stimuli normally observed in splenocytes from mice acutely infected with salmonellae. We also report that IL-12 contributes to the control of bacterial growth of a virulent oral challenge in the RES of mice immunized with a live attenuated *Salmonella* vaccine.

In sublethal *Salmonella* infections, survival requires the suppression of bacterial exponential growth in the RES, leading to a plateau phase (21). Functional T cells are not essential for the establishment of the plateau (14), which conversely requires TNF-α and IFN-γ (20, 24, 25, 27), the former acting as a macrophage recruitment factor (21a) and the latter presumably being a macrophage activator (16). In this report, we show that IL-12 is also required for the establishment of the plateau phase. In our study, IL-12 neutralization did not affect early bacterial growth in the RES (days 1 to 3), which is controlled by resident macrophages. In anti-IL-12-treated mice, bacterial growth proceeded past the point at which an IFN-γ- and TNF-α-dependent host response determines the establishment of the plateau phase. The effector mechanisms involving IL-12 are yet to be elucidated. We found a severe reduction of IFN-γ

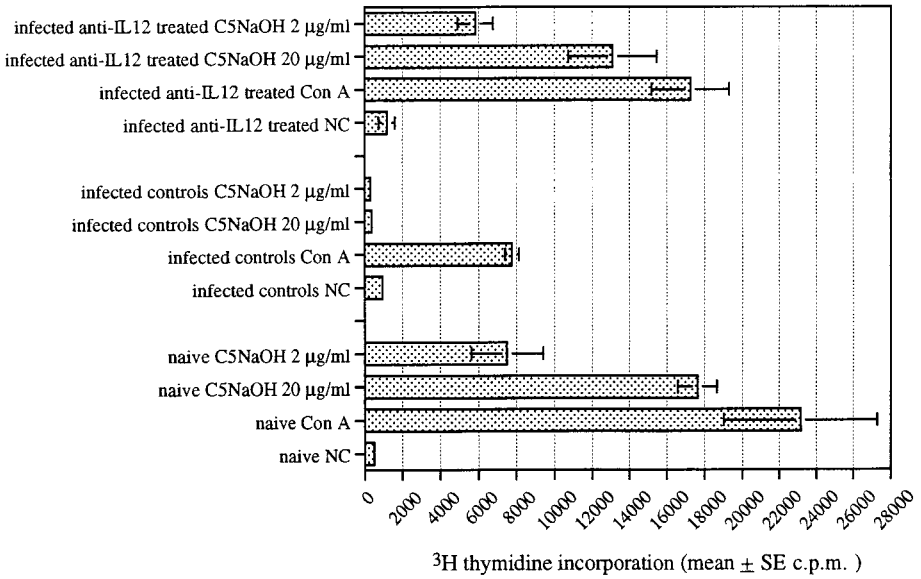

FIG. 3. Proliferative responses ([^{3}H] thymidine incorporation) from splenocytes of naive, infected, and infected anti-IL-12-treated mice in response to C5NaOH and to ConA. The mice were infected and treated as for Fig. 2. The cells were stimulated as for Fig. 2. Results are expressed as means ± standard errors (SE) of triplicate 48-h cultures. NC, incubation with medium alone.

levels in anti-IL-12-treated animals compared with untreated controls, indicating that IL-12 acts as a positive modulator of IFN-γ production in mouse salmonellosis. IFN-γ neutralization alone can impair resistance to *Salmonella* infection in mice, and IFN-γ is known to enhance the bactericidal activity of *Salmonella*-infected macrophages (16). Taken together, these observations suggest that positive modulation of IFN-γ production may be the mechanism by which IL-12 mediates resistance to salmonellosis in primary infections. Evidence from other experimental models also supports the view that IL-12 mediates host resistance to intracellular organisms by positively modulating IFN-γ production. IL-12 neutralization exacerbated *Listeria*, *M. tuberculosis*, *Toxoplasma*, and *Candida* infections in mice, concomitantly impairing IFN-γ production, while treatment with recombinant IL-12 can enhance both IFN-γ production and resistance to infection (8, 12, 15, 31, 32, 34, 37, 39).

The reduction of circulating IFN-γ in anti-IL-12-treated mice cannot be ascribed to the presence of higher bacterial loads (i.e., toxic effect on IFN-γ-producing cells) in the organs of these mice. In fact, in this report we show that administration of an anti-TNF-α antiserum to *Salmonella*-infected mice exacerbates the course of a primary infection without inducing a reduction (but rather causing an increase) in the levels of circulating IFN-γ. We have reported a similar increase of serum IFN-γ levels also in mice immunized with live attenuated aromatic-dependent *Salmonella* vaccines and treated with anti-TNF-α antibodies or with depleting anti-CD4$^+$ and anti-CD8$^+$ monoclonal antibodies prior to challenge with virulent organisms (22). In these T-cell-depleted and anti-TNF-α-treated mice, a severe increase in spleen and liver bacterial counts and higher serum IFN-γ levels could be seen (22). Furthermore, IFN-γ is detectable in the circulation of BALB/c and A/J mice during the late stages of lethal infections (high bacterial numbers in the RES) with *S. typhimurium* C5 (reference 22 and our unpublished observations).

The in vivo increase in circulating IFN-γ in anti-TNF-α-treated mice seems to contradict in vitro data from other laboratories which show that IFN-γ production by mouse splenocytes stimulated with salmonellae can be impaired by neutralization of TNF-α (29). Nevertheless, the same discrepancy has been reported for the *Listeria* model, in which in vitro TNF-α neutralization reduces IFN-γ release by spleen cells (3, 35), whereas in vivo TNF-α neutralization during primary infections does not induce a decrease in serum IFN-γ (26). Taken together, these findings suggest that IL-12 exerts strong positive modulation on in vivo IFN-γ production whereas TNF-α is not essential for IFN-γ production. Noticeably, evidence from the *Toxoplasma* model indicates that TNF-α is essential for IFN-γ production from IL-12-stimulated spleen-derived NK cells but not from bone marrow-derived NK cells, suggesting that IFN-γ production by NK cells does not always require TNF-α (12).

In this report, we show that splenocytes from normal uninfected A/J mice produce IFN-γ when stimulated in vitro with ConA or *S. typhimurium* C5. In vitro IL-12 neutralization dramatically reduced IFN-γ production in response to *S. typhimurium* while only marginally affecting the IFN-γ response to ConA. As shown by others, in vitro IFN-γ production to ConA is dependent on Thy 1.2+ (T cells) but not on asialo-GM1$^+$ or NK1.1 (NK cells), cells whereas IFN-γ production in response to bacteria requires NK cells but not T cells (2, 28). Although it seems likely that IL-12 mediates IFN-γ production by NK cells in response to *S. typhimurium* but not IFN-γ release by T cells in response to ConA, the cells involved in these phenomena remain to be conclusively identified.

In this report, we show that in vitro IL-12 neutralization impairs the ability of mouse splenocytes to produce IFN-γ upon in vitro restimulation with C5NaOH or with ConA. Our results show that splenocytes from *Salmonella*-infected mice release high amounts of IFN-γ upon stimulation with ConA and with C5NaOH, cells from naive mice releasing IFN-γ upon stimulation with ConA but not with C5NaOH. The in vitro response to either stimulus was severely impaired in the splenocytes from anti-IL-12-treated mice. We also show that cells from both untreated controls and anti-IL-12-treated mice, but not from naive mice, spontaneously produce IFN-γ, cytokine levels being somewhat higher in anti-IL-12-treated mice.

Taken together, these results indicate that the in vivo anti-IL-12 treatment does not completely abolish the intrinsic ability of mouse splenocytes to produce IFN-γ, but it decreases the ability of the cells to respond to bacterial or mitogenic stimuli.

We also observed that addition of anti-IL-12 antibodies to the in vitro cultures of splenocytes from *Salmonella*-infected mice completely abolished the spontaneous release of IFN-γ (data not shown), suggesting that continuous IL-12 neutralization is required to downregulate the spontaneous release of IFN-γ by cells of infected mice. The nature of the cell populations from infected mice involved in IFN-γ production to the *S. typhimurium* soluble extract has yet to be investigated. The observation that the *S. typhimurium* soluble extract used by us induced IFN-γ release by spleen cells from infected mice, but not from cells from naive mice, and the fact that in vivo treatment with IL-12 could reduce in vitro IL-12 production upon restimulation undoubtedly indicate that IFN-γ-producing cells undergo IL-12-dependent activation during infection.

Our present data are in line with observations by others showing that IL-12 positively modulates the ability of mouse splenocytes or purified T cells (8, 31, 32, 39) and human peripheral blood leukocytes or T-cell clones (9, 18) to produce IFN-γ in response to bacterial or mitogenic stimuli. Future studies will investigate the relevance of modulation of IFN-γ production by IL-12 on the development of acquired immunity to *Salmonella* infection and on the establishment of the Th1 pattern of T-cell responses observed in mice immunized with live attenuated *aroA Salmonella* vaccines.

In this study, we found that splenocytes from mice acutely infected with *S. typhimurium* C5 show a marked impairment of proliferative responses to mitogens. These findings are in line with observations by others who found that spleen cells from *Salmonella*-infected mice exhibit profoundly depressed responses to B- and T-cell mitogens, suppression being mediated by macrophages and requiring IFN-γ (1, 10). The fact that anti-IL-12-treated mice show reduced in vivo IFN-γ levels, decreased in vitro IFN-γ production in response to mitogens or *Salmonella* antigens, as well as restored proliferative responses suggests that IL-12-induced IFN-γ production may be at least part of the mechanisms responsible for the development of the immunosuppression. Similar evidence comes from the *Toxoplasma* mouse model, in which suppression of T-cell proliferative responses can be partially reversed by neutralization of endogenous IL-12 (15).

In this report, we show that IL-12 contributes to the control bacterial growth upon rechallenge with virulent organisms in immunized mice. The effect of the anti-IL-12 antisera on the course of a secondary infection was not as dramatic as the effect of T-cell depletion, IFN-γ, or TNF-α neutralization observed by us in previous studies (22). This might indicate that IL-12 plays a complementary role in the control of bacterial growth in secondary infections. Similarly, evidence from the tuberculosis mouse model indicates that in vivo neutralization of IL-12 impairs, but does not ablate, specific host resistance to *M. tuberculosis* (8).

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