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Interleukin-12 Is Critical for Induction of Nitric Oxide-Mediated Immunosuppression following Vaccination of Mice with Attenuated *Salmonella typhimurium*

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Studies from our laboratory have shown that infection of mice with an attenuated strain of *Salmonella typhimurium* causes a marked suppression in the capacity of splenocytes to generate an in vitro plaque-forming cell (PFC) response to sheep erythrocytes. The suppression has been shown to be mediated by mature, adherent macrophages (Mϕs) and nonadherent, precursor Mϕs. Nitric oxide has been identified as the suppressor factor. The present study investigated the role of interleukin-12 (IL-12) in the generation of nitric oxide-mediated immunosuppression in this model. *Salmonella* inoculation resulted in marked suppression of PFC responses and high levels of nitrite production. When mice were treated with anti-IL-12 prior to inoculation, nitrite levels in splenocyte cultures were reduced by 75% and the suppression of PFC responses was prevented. The nonadherent splenocyte fraction from *Salmonella*-inoculated mice, which contains precursor Mϕs and is weakly immunosuppressive, was treated with IL-12 in vitro. IL-12 augmented the capacity of this fraction to suppress PFC responses by normal splenocytes in a coculture system. Additionally, IL-12 induced nitrite and gamma interferon (IFN-γ) production in a dose-dependent manner. Treatment with anti-IFN-γ blocked nitrite production and suppression, indicating that IFN-γ is an important intermediary in the pathway of IL-12-induced immunosuppression. These results indicate that IL-12 is critical for the induction of nitric oxide-mediated immunosuppression following *S. typhimurium* inoculation and, through its ability to stimulate IFN-γ production, can induce nitric oxide-producing suppressor Mϕs.

Macrophage (Mϕ)-mediated immunosuppression has been found after infection with a variety of microbes and several microbial extracts (15). Previous studies by our laboratory have shown that inoculation of mice with an *aroA* mutant of *Salmonella typhimurium*, strain SL3235, is capable of inducing long-term protection against infection with virulent *Salmonella* or *Listeria monocytogenes* challenge (19, 37). This attenuated strain confers protection against virulent *Salmonella* in both genetically hypersusceptible and resistant mouse strains of the C3H lineage (19, 28, 37). Concomitant with the ability of SL3235 to confer protection, selective immunosuppression was observed, as evidenced by the inability of immunized mice to respond to B- and T-cell mitogens (42) or to heterologous antigens such as sheep erythrocytes (SRBC) or tetanus toxoid (4, 5, 17). At the time of onset of maximal suppression, SL3235-inoculated mice also exhibit enhanced cell-mediated immunity, as evidenced by the induction of tumoricidal and leishmaniacidal activity in peritoneal Mϕs and cross-protection against challenge with *L. monocytogenes* (19, 37, 57). Our laboratory has demonstrated that Mϕs are responsible for the suppression of lymphocyte function following SL3235 inoculation in that removal of the adherent Mϕ population restored lymphocyte activity (4, 42). Two distinct populations of Mϕs have been identified in the spleens of infected mice: a mature adherent cell which displays characteristics associated with mature Mϕ such as vacuolation, phagocytic activity, and nonspecific esterase activity; and a nonadherent precursor Mϕ which is MAC-1 positive but does not display other Mϕ or lymphocyte characteristics (4). The mature, adherent Mϕ population is highly suppressive, while the nonadherent spleen cell population, which contains precursor Mϕs as well as other cell types, is only weakly suppressive (4). Nitric oxide has been established as the factor responsible for the suppression by the mature Mϕ population based on the observation that the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (NMMA) blocks the suppression (6), but the mechanism by which the nonadherent immune cell fraction suppresses has not been investigated. The concept that nitric oxide can mediate immunosuppression was first proposed in 1991 in a system which did not involve a living infectious agent (3). The ability of nitric oxide to suppress lymphocyte function following infection has been demonstrated by our laboratory, as well as by investigators using *Trypanosoma brucei* (62) or *Toxoplasma gondii* (9).

The suppressive capacity of splenocytes taken from *Salmonella*-inoculated mice was prevented by the addition of antibodies against gamma interferon (IFN-γ) into in vitro cultures (6), which suggests that IFN-γ is important in the pathway leading to nitric oxide-mediated immunosuppression. It has been previously shown that IFN-γ production is induced following *Salmonella* infection (49, 52, 53). Further, IFN-γ in conjunction with lipopolysaccharide (LPS) induces expression of the inducible nitric oxide synthase (iNOS) in mouse Mϕs (14, 18, 46). A potent inducer of IFN-γ production by NK and T cells is interleukin-12 (IL-12) (8, 40, 45, 52). Recent studies have shown that IL-12 is important in resistance to *Salmonella* infection (39, 48) as well as infection by other intracellular pathogens of Mϕs (21, 33, 45, 55, 59, 61, 63). The study presented here tested the hypothesis that the induction of nitric

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oxide-mediated immunosuppression following *Salmonella* infection occurs via an IL-12-dependent pathway and that IL-12 induces the differentiation of precursor Mφs into nitric oxide-producing suppressor cells.

MATERIALS AND METHODS

Mice. Six-week-old female C3HeB/FeJ mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and housed in sterilized cages and bedding, with mouse chow and water provided ad libitum. All mice were acclimatized for a minimum of 1 week prior to experimentation.

Bacterial strain and infection model. An avirulent strain of *S. typhimurium*, SL3235, was used for all experiments. SL3235 is an *aroA* mutant which is deficient in aromatic synthesis and has a 50% lethal dose of greater than 10^7 bacteria when given intraperitoneally (i.p.). Infection was accomplished by injecting 5×10^5 log-phase bacteria i.p. as previously described (4). Control animals were injected i.p. with a comparable volume of sterile, endotoxin-free, isotonic saline (Abbott Laboratories, Chicago, Ill.).

αIL-12 treatment in vivo. Polyclonal sheep anti-mouse IL-12 antiserum (αIL-12) was kindly provided by Genetics Institute (Cambridge, Mass.). The immunoglobulin G (IgG) fraction from normal sheep (Sigma Chemical, St. Louis, Mo.) was used as a control. Contaminating endotoxin was removed from αIL-12 preparations by phase separation using Triton X-114 (Sigma) (2). Endotoxin levels in αIL-12 preparations were less than 0.1 endotoxin unit per ml as determined by the E-TOXATE assay (Sigma) following Triton X-114 treatment. Five hours prior to infection with SL3235, mice were given a single i.p. inoculation of either 500 μg of αIL-12 or sheep IgG in 0.5 ml of sterile phosphate-buffered saline.

Cell isolation and preparation. Seven days after infection, the mice were sacrificed by cervical dislocation and the spleens were removed aseptically. Single spleen cell suspensions were prepared as previously described (6). Splenocyte populations were fractionated into nonadherent and adherent fractions by 2 h of adherence to plastic followed by passage over Sephadex G-10 (Sigma) as described elsewhere (4). Cellular composition of the splenocyte suspensions was determined by light microscopy and differential staining techniques using Diff-Quik (Sigma).

In vitro treatment. In selected wells in each experiment, the nitric oxide synthase inhibitor NMMA (1.25 or 1.6 mM; Sigma), IL-12 (0.005 to 5.0 U/ml; Genetics Institute), or hamster anti-murine IFN-γ (500 ng/ml; Genzyme, Cambridge, Mass.) was added during the culture period.

Nitrite production. Splenocytes were suspended in RPMI 1640 containing 5% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), 50 U of penicillin-streptomycin (Gibco) per ml, 2 mM L-glutamine (Sigma), and 50 μM β-mercaptoethanol (Sigma). The cells were cultured for 48 h at a concentration of 10^7 cells per ml in 96-well plates (Costar, Cambridge, Mass.). Cell-free culture supernatants were collected and assayed for nitrite, a stable degradation product of nitric oxide, using the colorimetric Griess reaction (14).

Primary in vitro antibody response. Antibody-producing cells were generated in vitro by the method of Mishell and Dutton (48a), with modification, as previously described (4). In selected wells, the nitric oxide synthase inhibitor NMMA (Sigma) was present during the culture period. The plaque-forming cell (PFC) response was determined by the Cunningham modification of the Jerne hemolytic plaque assay (13). Data are expressed as PFC per 10^7 splenocytes. Coculture experiments using cell fractions from infected mice and normal splenocytes were conducted with the target (normal) cells at a concentration of 10^7 cells per ml and the effector (immune) cells at a concentration of 2.5×10^6 cells per ml, yielding a final cell concentration of 1.25×10^7 cells per ml. Control assays using 1.25×10^7 normal splenocytes were conducted to account for any effects due to cell crowding.

IFN-γ ELISA. Splenocytes were cultured at a concentration of 10^7 cells per ml for 48 h, and cell-free supernatants were harvested and frozen at −70°C until determination of IFN-γ levels by sandwich enzyme-linked immunosorbent assay (ELISA) using a matched pair of monoclonal antibodies as instructed by the manufacturer (PharMingen, San Diego, Calif.). Briefly, samples were incubated overnight at 4°C on plates coated with anti-IFN-γ (4 μg/ml). The plates were washed with phosphate-buffered saline containing Tween 20 prior to the addition of biotinylated anti-IFN-γ (4 mg/ml) for 45 min. The plates were washed, and a 1:500 dilution of strepavidin-alkaline phosphatase (Calbiochem, San Diego) was added for 30 min. Following a thorough washing, *p*-nitrophenyl phosphate (1 mg/ml; Sigma) was added, and the plates were incubated at room temperature for approximately 90 min. Optical density at 405 nm was determined by using an automated microplate reader (Bio-Rad, Richmond, Calif.). Murine IFN-γ (PharMingen) was used to construct a standard curve.

Statistical analysis. All measurements were made by using a minimum of triplicate samples per variable for each experiment. Data are expressed as mean ± standard deviation (SD) for a representative experiment. Comparisons were analyzed by Student's *t* test. Differences were considered significant when *P* was ≤0.05.

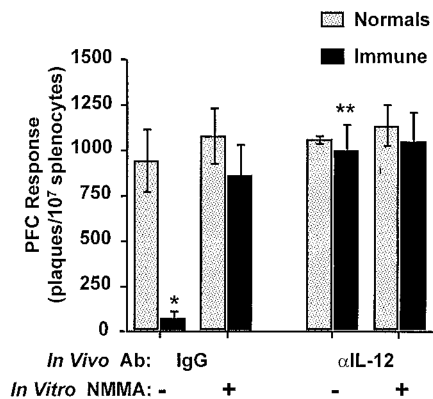

FIG. 1. Effect of in vivo αIL-12 treatment on SL3235-induced suppression of the in vitro PFC response. Splenocytes from saline-inoculated and SL3235-inoculated mice pretreated with sheep IgG or αIL-12 were isolated 7 days after inoculation and cultured for 5 days with SRBC. PFC responses were assessed as described in Materials and Methods. Selected wells were treated with NMMA (1.25 mM). The experiment was done three times. Data are the mean ± SD of triplicate samples from a representative experiment. *, $P < 0.01$ compared to IgG-treated normal group; **, $P < 0.01$ compared to IgG-treated immune group and not significant compared to IgG-treated normal group. Ab, antibody.

RESULTS

IL-12 is required for the induction of SL3235-mediated suppression of PFC responses. To assess the effect of SL3235 inoculation on immunocompetence, the primary antibody response to SRBC was assessed in vitro, using a PFC assay. The PFC response by splenocytes from SL3235-inoculated (immune) mice was suppressed by greater than 90% compared with splenocytes from saline-inoculated (normal) mice pretreated with IgG. αIL-12 pretreatment of the SL3235-inoculated mice completely abrogated the suppression (Fig. 1). αIL-12 did not alter the PFC response by spleen cells from saline-inoculated mice. When the nitric oxide synthase inhibitor NMMA (1.25 mM) was present in the PFC cultures, no suppression in the PFC response of splenocytes from infected mice was observed. The ability of NMMA to abrogate the suppression of the PFC response is consistent with our previously published results that the suppression induced by attenuated *Salmonella* is nitric oxide mediated (6).

The effect of αIL-12 pretreatment on nitrite production was also assessed as shown in Fig. 2. Splenocytes from normal (saline-inoculated) mice produced negligible nitrite when pretreated with IgG or αIL-12. However, SL3235 inoculation induced spleen cells to produce high levels of nitrite in vitro. IgG pretreatment did not significantly alter nitrite production compared with animals receiving SL3235 inoculation alone (data not shown). αIL-12 pretreatment of infected mice markedly reduced splenocyte nitrite production by 77%.

Seven days after SL3235 inoculation, spleen size was increased 4.5-fold. Splenomegaly is a major feature of *Salmonella* infection (29, 51), and we have previously shown that the increase in spleen size is due to the increase in the number of neutrophils, mature Mφs, and precursor Mφs (4, 37). αIL-12 pretreatment reduced the splenomegaly only marginally (to an approximately fourfold increase). Based on differential counts, SL3235 inoculation increased the percentage of Mφ in the spleen from 7.6% ± 1.3% to 29.0% ± 1.9% (mean ± standard error of the mean for three experiments) and increased the percentage of neutrophils from 9.5% ± 0.9% to 34.7% ± 3.9% (mean ± standard error of the mean for three experiments). In vivo treatment with αIL-12 had no statistically significant effect

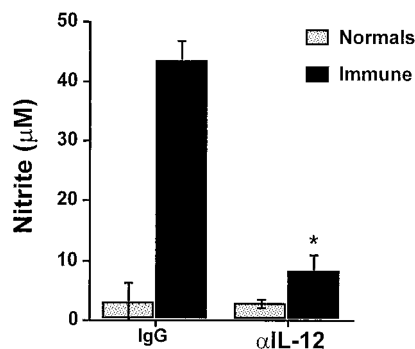

FIG. 2. Effect of in vivo αIL-12 treatment on SL3235-induced splenocyte nitrite production in vitro. Splenocytes from saline-inoculated mice and SL3235-inoculated mice pretreated with sheep IgG or αIL-12 were isolated 7 days after inoculation and cultured for 48 h in vitro. Nitrite concentrations of cell-free supernatants were assessed as described in Materials and Methods. The experiment was done four times. Data are the mean ± SD of triplicate samples from a representative experiment. *, $P < 0.01$ for αIL-12-treated immune group compared to IgG-treated immune group.

on these SL3235-induced changes in splenocyte cellular composition.

IL-12 augments immunosuppression by immune nonadherent splenocytes in coculture. PFC responses by normal splenocytes were suppressed by approximately 40% when cocultured with nonadherent immune cells (Fig. 3). Treatment of the cocultures with IL-12 (5 U/ml) augmented the suppression to approximately 80%, a statistically significant increase. When αIFN-γ (500 ng/ml) was included in the IL-12-treated cocultures, no suppression was observed (Fig. 3), indicating that the ability of IL-12 to induce immunosuppressive capacity in nonadherent immune cells is mediated through its ability to stimulate IFN-γ secretion. In addition, the ability of IFN-γ (50 U/ml) to augment the suppression was comparable to that of IL-12 (Fig. 3). The augmentation of immunosuppression by IL-12 was completely abrogated by treatment with NMMA (1.6 mM), indicating that nitric oxide is the suppressor factor (Table 1). Additionally, the augmentation of immunosuppressive capacity by IL-12 was comparable to that of IFN-γ, and immunosuppression induced by IFN-γ was also blocked by NMMA. The nonadherent immune cells most likely caused the immunosuppression, as IL-12 did not induce nitric oxide production by normal spleen cells as it did with the nonadherent immune cells (Fig. 4). IL-12 treatment of normal splenocytes caused a slight reduction in the PFC response. Unfractionated immune splenocytes and adherent immune splenocytes also markedly suppressed PFC responses by normal splenocytes, and this suppression was inhibitable by NMMA (data not shown).

IL-12 modulates nitric oxide production by nonadherent immune splenocytes. Spleen cells or cell fractions were treated with IL-12 to determine the ability of this cytokine to regulate nitrite production. Spleen cells from saline-inoculated mice produced negligible amounts of nitrite, and stimulation with IL-12 had little effect. Further, the nonadherent immune spleen cell fraction also produced very little nitrite compared with unfractionated immune cells (Fig. 4), which is consistent with our previous observations (6). However, IL-12 stimulation of the nonadherent immune cells induced nitrite production in a dose-dependent manner (Fig. 4), which was inhibitable by NMMA (1.6 mM) (Fig. 5). IL-12 did not significantly alter nitrite production by unfractionated immune cells. The ability of IFN-γ to induce nitrite production was also tested (Fig. 5). IFN-γ (500 U/ml) and IL-12 (5 U/ml) were found to be equally effective in inducing high levels of nitrite by nonadherent immune splenocytes. More importantly, concurrent treatment with αIFN-γ (500 ng/ml) almost completely blocked the IL-12-induced nitrite production by the nonadherent immune cells, and nitrite production by the unfractionated immune cells was partially blocked (Fig. 5). These results indicate that IL-12 is capable of inducing nitric oxide production by nonadherent immune spleen cells through the intermediate stimulation of IFN-γ production and are consistent with the observed effect of IL-12 in augmenting the suppressive capacity of the nonadherent immune splenocytes in the PFC assay as shown in Fig. 3.

IL-12 regulation of IFN-γ production. Analysis of the supernatants from unfractionated immune spleen cells showed that SL3235 inoculation induced significant IFN-γ production compared with normal cells (Table 2). Removal of adherent splenocytes (immune nonadherent cell fraction) blocked the production of IFN-γ, suggesting a possible role for mature Mϕs in IFN-γ production following SL3235 inoculation. IL-12 was an extremely potent stimulus for IFN-γ production by normal spleen cells, with greater than 400 ng of IFN-γ per ml being secreted into the supernatants. IL-12 was also capable of inducing a significant increase in the level of IFN-γ production by nonadherent immune cells and a smaller increase in IFN-γ production by unfractionated immune cells. However, levels of IFN-γ production by both immune cell groups were surprisingly lower (approximately 20-fold) than those generated by normal splenocytes under IL-12 stimulation.

DISCUSSION

Consistent with our previous findings, inoculation of mice with SL3235 induced a profound suppression in the ability of splenocytes to generate an in vitro antibody response to SRBC (4, 5), and the suppression was mediated by nitric oxide (6). In the present study, IL-12 was shown to be essential for the induction of the immunosuppression. Pretreatment of the mice with polyclonal antibodies against IL-12 prior to SL3235 inoculation completely abrogated the immunosuppression and splenocyte nitric oxide production. These results indicate that the availability of bioactive IL-12 in vivo following SL3235

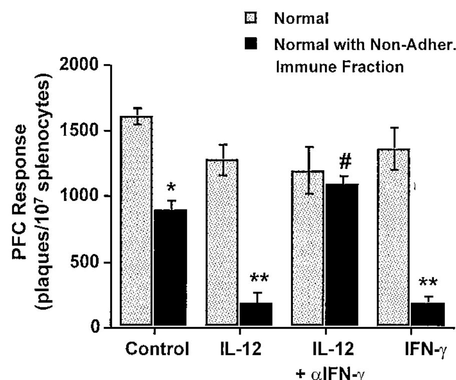

FIG. 3. Splenocytes were isolated from mice 7 days after SL3235 inoculation, and the nonadherent immune cell fraction was isolated as described in Materials and Methods. The cells (2.5×10^6) were cocultured with normal splenocytes (10^7) and SRBC for 5 days, and PFC responses were assessed. The experiment was done twice. Data are the mean ± SD of triplicate samples from a representative experiment. *, $P < 0.01$ compared to normal control group; **, $P < 0.001$ compared to normal group with nonadherent immune fraction control; #, $P < 0.001$ compared to normal group with nonadherent immune fraction stimulated with IL-12.

TABLE 1. Effect of NMMA on PFC responses

Expt	In vitro treatment	Mean PFC/10^7 splenocytes ± SD			
		Normal splenocytes		Coculture of normal splenocytes and nonadherent immune splenocytes	
		−NMMA	+NMMA[a]	−NMMA	+NMMA
1	None	1,610 ± 62	ND[b]	893 ± 72[c]	ND
	IL-12 (5 U/ml)	1,277 ± 318[c]	ND	185 ± 83[d,e]	1,015 ± 261[f,g]
	IFN-γ (50 U/ml)	1,362 ± 163	ND	185 ± 44[d,e]	1,078 ± 77[f,g]
2	None	1,985 ± 242	ND	1,138 ± 240[c]	ND
	IL-12 (5 U/ml)	1,418 ± 78[c]	1,668 ± 81	341 ± 104[d,e]	1,466 ± 245[f,g]
	IFN-γ (50 U/ml)	1,737 ± 318	1,901 ± 104	388 ± 104[d,e]	1,332 ± 127[d,f]

[a] NMMA was added at the initiation of the PFC culture at a concentration of 1.6 mM.
[b] ND, not done.
[c] $P < 0.05$ compared with normal splenocytes receiving no treatment.
[d] $P < 0.01$ compared with respective normal group.
[e] $P < 0.01$ compared with coculture receiving no treatment.
[f] $P < 0.01$ compared with respective coculture without NMMA addition.
[g] Not significant compared with normal splenocyte groups.

inoculation is critical for the subsequent induction of nitric oxide-mediated immunosuppression. In vitro studies were also carried out to substantiate this conclusion. We have previously demonstrated that in the spleens of SL3235-inoculated mice, in addition to adherent Mϕs, there is an immature nonadherent precursor Mϕ population. Nonadherent spleen cells from immune mice display weak suppressive capacity toward PFC responses (4). In the present study, we showed that in vitro treatment of nonadherent immune spleen cells with IL-12 markedly enhanced their immunosuppressive capacity. Nitric oxide was shown to be the suppressor factor, as concurrent treatment of these cells with NMMA blocked suppression.

A major biological action of IL-12 is the induction of IFN-γ production by NK and T cells (10, 11, 20, 26, 40, 67, 68). The results presented here have shown that the mechanism by which IL-12 induces immunosuppression following *Salmonella* inoculation is probably through the induction of IFN-γ. Several lines of evidence support this position. First, IL-12 stimulation of nonadherent immune splenocytes induced IFN-γ production; second, anti-IFN-γ prevented IL-12-induced nitric oxide-mediated immunosuppression by nonadherent immune cells; and last, the effects of IFN-γ on nitric oxide production and immunosuppression were comparable to those of IL-12. These results support the conclusion that IL-12 exerts its effect through its ability to stimulate IFN-γ secretion, which stimulates nitric oxide production by the Mϕ populations, with resultant immunosuppression. This conclusion is consistent with our previously published results showing that in vitro αIFN-γ treatment of immune splenocytes blocked SL3235-induced immunosuppression (6).

The ability of IL-12 to stimulate IFN-γ production in our system was not surprising; however, the 20-fold-greater production by normal splenocytes than by immune cells was unexpected. We hypothesize that the reason for the immune cell refractoriness to IL-12 stimulation is related to the suppression of T-cell function following *Salmonella* infection. We have previously proposed that the immunosuppression observed after *Salmonella* infection is due to bystander autotoxicity to lymphocytes in the vicinity of nitric oxide-producing Mϕs (18). The relative inability of the immune T and/or NK cells to respond to IL-12 (as assessed by IFN-γ production) may reflect nitric oxide-mediated toxicity to these lymphocytes. Previously, we have observed that NK cell activity is enhanced 2 to 4 days after *Salmonella* inoculation and returned to baseline levels by 10 days postinoculation (56). Nitric oxide production is maximal at 7 days after SL3235 inoculation (6). These findings would be consistent with the nitric oxide-mediated down-regulation of IFN-γ production. Further studies will be required to determine the mechanism(s) behind this rather intriguing observation.

A proposed model of Mϕ differentiation and activation following *Salmonella* infection could include IL-12. Following SL3235 inoculation, mature Mϕs would be stimulated to produce and secrete IL-12 by *Salmonella*, either directly (12) or through LPS, a potent activator of IL-12 p40 production (25, 26). However, it has been shown that viable *Salmonella* induces threefold-greater levels of p40 mRNA expression than killed *Salmonella* (12), suggesting that signals in addition to LPS are involved in *Salmonella*-induced IL-12 production. IL-12 would then induce IFN-γ production by NK and/or T cells, and the lymphocyte-derived IFN-γ would feed back to stimulate the Mϕ populations (both mature and precursor Mϕs). The initial stimulus of *Salmonella* along with the IL-12-induced IFN-γ release would induce the expression of iNOS, nitric oxide production, and immunosuppression. We have shown that IFN-γ can induce iNOS mRNA expression in the nonadherent immune cell population (31). This model is consistent with our

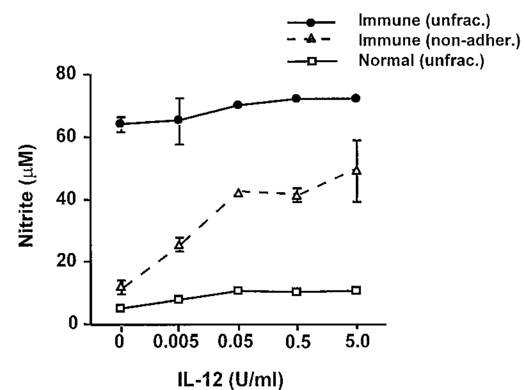

FIG. 4. Effect of IL-12 on nitrite production by splenocyte fractions from saline-inoculated and SL3235-inoculated mice. Splenocytes (unfractionated and nonadherent cell fraction) were isolated from mice 7 days after SL3235 or saline inoculation as described in Materials and Methods and were cultured at a concentration of 10^7 cells per ml for 48 h. Nitrite concentrations in cell-free supernatants were assessed. The experiment was done three times. Data are the mean ± SD of triplicate samples from a representative experiment.

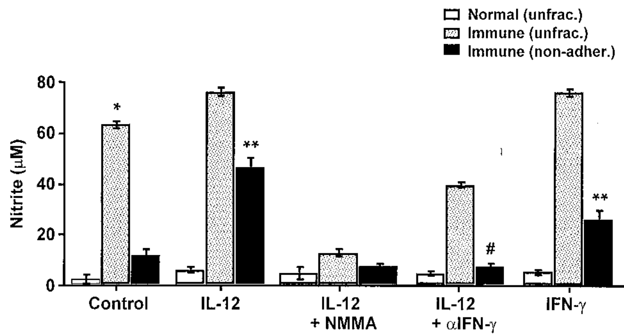

FIG. 5. Splenocytes (unfractionated and nonadherent cell fraction) were isolated from mice 7 days after SL3235 or saline inoculation as described in Materials and Methods and were cultured at a concentration of 10^7 cells per ml for 48 h. Nitrite concentrations in cell-free supernatants were assessed. Cells were stimulated with IL-12 (5 U/ml) or IFN-γ (50 U/ml) with or without the addition of NMMA (1.6 mM) or αIFN-γ (500 ng/ml). The experiment was done three times. Data are the mean ± SD of triplicate samples from a representative experiment. *, $P < 0.001$ compared to normal control; **, $P < 0.05$ compared to nonadherent immune fraction control; #, $P < 0.01$ compared to nonadherent immune fraction stimulated with IL-12.

previously published work showing that NK cells are activated early after SL3235 inoculation, prior to the manifestation of immunosuppression (56), and our preliminary studies showing that NK cells and IFN-γ are critical for SL3235-induced nitric oxide production (60). It has been shown that IL-1β is required for optimal stimulation of NK cell IFN-γ release by IL-12 (34), and we have shown that SL3235 induced IL-1β expression (70), thereby supporting NK cells as the source of IFN-γ in our system. The apparent requirement for IFN-γ as an intermediary in IL-12-stimulated nitric oxide production is consistent with the observations of Zidek et al. showing that IL-12 in conjunction with LPS stimulated nitrite production by murine peritoneal cells that was dependent on IFN-γ production (75).

While αIL-12 treatment was effective in preventing immunosuppression following *Salmonella* infection, surprisingly little change was observed in the magnitude of the inflammatory response in the spleen associated with the infection. Seven days after SL3235 inoculation, spleen size was increased 4.5-fold. We have previously shown that splenomegaly is a major feature of *Salmonella* infection and that the increase in spleen size is due to the increase in the number of neutrophils, mature Mϕs, and precursor Mϕs (4, 30). αIL-12 pretreatment only marginally reduced the splenomegaly. SL3235 inoculation increased the percentage of Mϕ and neutrophils in the spleen, and αIL-12 had no effect on these SL3235-induced changes in splenocyte cellular composition. These data indicate that the immunosuppression and nitric oxide production are independent of the inflammatory response to SL3235 infection.

A majority of the previous studies with IL-12 have focused on its beneficial effects in inducing cellular immune responses (1, 22, 45, 54, 67, 71). Two studies have demonstrated that IL-12 is important in resistance to *Salmonella* infection (39, 48). IL-12 is also critical in resistance to a range of other microbes, including *L. monocytogenes* (7, 68), *Leishmania major* (1, 58), *Brucella abortus* (73), mycobacteria (21, 55), plasmodia (61, 63), *Histoplasma capsulatum* (74), and *Schistosoma mansoni* (71). A common characteristic among most of these microbes is that they are intracellular pathogens of Mϕs and require a Th1 type of immune response involving Mϕs and lymphocytes for adequate host defense. The role of IL-12 in the induction of cell-mediated immunity has been well documented (8, 45, 66) and has led to the proposal to use IL-12 as an adjuvant to augment Th1-type responses to antigens used as vaccines for protection against these organisms (1, 24, 54). IL-12 has also been shown to be important in host defense against tumors (22, 50, 54, 69). Our results indicate a potentially adverse effect of IL-12 production following *Salmonella* infection, namely, a selectively immunosuppressed state in which a strong cellular immune response is adversely affecting the ability of the host to respond to mitogenic stimulation or to other antigenic challenges. Other animal models that also suggest potentially detrimental effects of IL-12 include potentiation of endotoxic shock (72) and aggravation of several autoimmune diseases (23, 32, 43, 65). The common factor with all of these conditions is the induction of activated Mϕ populations. These hyperactive cells appear to have a potentially deleterious effect on the host. The significance of the immunosuppression for the well-being of the host is difficult to assess in mice receiving *Salmonella* vaccine, as they are highly protected against virulent *Salmonella* and *Listeria* infections (19, 37) as well as exhibiting resistance to a transplanted tumor (16). Following immunization of mice with SL3235, the nonspecific immunity to *Listeria* wanes by 21 days, but specific immunity to *Salmonella* persists for at least 6 months (37). Furthermore, during the first 3 weeks after immunization, no delayed-type hypersensitivity response is evident to *Salmonella* antigens (36, 38), nor can responsive T cells be detected in proliferative assays using *Salmonella* antigens (37). However, by 1 month postimmunization, peritoneal exudate T cells which respond to *Salmonella* protein can be found (37). We have suggested that the paradox of immunosuppression concomitant with superior protection to virulent *Salmonella* and *Listeria* can be resolved by the hypothesis that both phenomena are mediated by the same mechanism, namely, Mϕ-derived nitric oxide (18). The observation that specific T-cell-mediated immune responses to *Salmonella* are not detectable until the primary inoculation with SL3235 has been resolved would be consistent with this hypothesis. Potentially antigen responsive cells would be inactivated by the mechanism of nitric oxide-mediated bystander autotoxicity (18). The mechanism(s) by which nitric oxide suppresses lymphocyte function is unknown but likely to be related to its ability to inhibit ribonucleotide reductase activity (41, 44) and/or mitochondrial respiration (27, 35, 64).

In assessing the implications of the observation that a vaccine strain of *Salmonella* can induce nitric oxide-mediated im-

TABLE 2. IL-12 stimulation of IFN-γ production by splenocytes from normal and immune mice

IL-12 (U/ml)	Mean IFN-γ (ng/ml) ± SD[a]		
	Normal (unfractionated)	Immune (unfractionated)	Immune (nonadherent fraction)
0	0.6 ± 0.2	9.1 ± 0.3[b]	1.4 ± 0.2
0.05	126.4 ± 6.5[c]	11.7 ± 0.5[d]	11.2 ± 1.1[e]
0.5	358.8 ± 24.8[c]	15.8 ± 2.0[d]	16.6 ± 1.7[e]
5	438.7 ± 10.3[c]	14.0 ± 1.3[d]	23.1 ± 1.7[e]

[a] Splenocytes were cultured at a concentration of 10^7 cells per ml for 48 h. IFN-γ levels were assessed in cell-free supernatants by ELISA as described in Materials and Methods. Data are from a representative experiment of three.

[b] $P < 0.001$ compared with normal unfractionated cells and nonadherent immune cells not receiving IL-12 stimulation.

[c] $P < 0.001$ compared with normals not stimulated with IL-12 and all immune groups.

[d] $P < 0.01$ compared with unfractionated immune cells not stimulated with IL-12.

[e] $P < 0.001$ compared with nonadherent immune cells not stimulated with IL-12.

munosuppression, an important consideration is the route of administration. In the studies presented here, SL3235 was given i.p. Attenuated strains of *Salmonella* such as SL3235 were originally developed as oral vaccines. Whether oral administration of SL3235 would result in immunosuppression is unclear. However, a recent study has shown that oral administration of attenuated *Salmonella* induced p40 mRNA expression at a distal site (peritoneal Mφs) (12). This observation suggests that the route of vaccine administration may not be critical to the induction of IL-12 and subsequent nitric oxide-mediated immunosuppression. Another consideration is the degree to which observations in the mouse are applicable to human responses to attenuated *Salmonella*. While it is well established that rodent Mφs produce nitric oxide, controversy over nitric oxide production by human Mφs exists. While human macrophages have been difficult to activate for nitric oxide production with cytokines in vitro, mononuclear phagocytes taken from patients with inflammatory or infectious diseases have consistently been shown to be capable of expressing iNOS and producing nitric oxide (47). The ultimate effect of IL-12, whether beneficial or detrimental, may depend upon the degree to which it activates Mφ nitric oxide production and the anatomical locale of the activation.

In summary, the results presented in this report indicate that IL-12 is critical for the induction of Mφ-derived nitric oxide and immunosuppression following *Salmonella* infection. Furthermore, IL-12, through its ability to stimulate IFN-γ production, can induce precursor Mφs to become nitric oxide-producing suppressor cells. These results indicate that IL-12 is important in the generation of immunosuppression following *Salmonella* infection in mice. Further analysis using this model following oral inoculation of *Salmonella* should lead to a more complete understanding of the potential adverse consequences of nitric oxide-mediated immunosuppression consequent to vaccination with attenuated strains.

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