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Interleukin 12 Is Produced In Vivo during Endotoxemia and Stimulates Synthesis of Gamma Interferon

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Gamma interferon (IFN-γ) is produced in response to circulating lipopolysaccharide (LPS) and contributes to the lethality of endotoxic shock. To address the cellular source of IFN-γ production in vivo, T cells and B cells were magnetically purified from C57BL/6 mouse spleens 5 h following endotoxin injection. IFN-γ RNA was abundant in splenic $CD4^+$ and $CD8^+$ T cells and in a T- and B-cell-depleted population of splenocytes containing 34% $NK1.1^+$ natural killer (NK) cells. Because interleukin 12 (IL-12) is a known inducer of IFN-γ synthesis by cultured T cells and NK cells, we examined whether IL-12 might be involved in IFN-γ release during endotoxemia. mRNA encoding the p40 subunit of IL-12 increased markedly in the spleens of C57BL/6 mice at 2 h after LPS injection, whereas p35 IL-12 mRNA was constitutively expressed at all times. Bioactive IL-12 (p70 heterodimer) was detected in mouse serum at 2 to 4 h after LPS injection. Similar results were obtained using a p40 subunit-specific enzyme-linked immunosorbent assay. Endotoxin-insensitive C3H/HeJ mice generated threefold less IL-12 p70 and IFN-γ at these times than endotoxin-sensitive C3H/HeOuJ mice. Pretreatment of mice with polyclonal anti-mouse IL-12 antibody reduced IFN-γ levels present at 6 h post-LPS nearly sixfold in three separate experiments. These studies support a role for IL-12 as a proximal stimulator of IFN-γ release during endotoxemia.

The role of proinflammatory cytokines in the pathophysiology of septic shock has been well characterized using rodent models of endotoxemia. Interleukin 1 (IL-1) and tumor necrosis factor (TNF-α) generated by macrophages in response to lipopolysaccharide (LPS) have been identified as important mediators of endotoxic lethality and organ dysfunction (8, 34, 35). The lymphokine gamma interferon (IFN-γ) is also produced during endotoxemia and contributes significantly to mortality (4, 15, 19, 22). Indeed, the well-described lethality of TNF-α released in the first 2 h of endotoxin shock may be dependent on synergistic effects mediated by IFN-γ appearing later in the course of disease (9). Although natural killer (NK) cells may be activated directly by endotoxin (24), T cells are not known to be directly endotoxin sensitive. However, both T cells and NK cells have been identified as sources of endotoxin-induced IFN-γ in spleen cell cultures (25). This and the fact that circulating IFN-γ appears 4 to 5 h after macrophage-derived TNF and IL-1 in the course of endotoxemia suggest that IFN-γ synthesis is stimulated by cytokines previously released from macrophages or other endotoxin-sensitive cells. The in vivo source of IFN-γ and the contribution of other factors to IFN-γ synthesis have not been extensively characterized in this important model of clinical pathology. In particular, the contribution of cytokines that are known to induce IFN-γ production in vitro, such as IL-2 and IL-12 (20, 31), has not been examined in vivo.

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MATERIALS AND METHODS

Mice. Four- to six-week-old female C3H/HeJ, C3H/HeOuJ, and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and kept in the Cleveland VA Medical Center animal care facility.

Cytokine reagents and antibodies. Antibodies used for the isolation of $CD4^+$ and $CD8^+$ T cells were GK1.5 (rat immunoglobulin G2B [IgG2b]; American Type Culture Collection [ATCC], Rockville, Md.) and 2.43 (rat IgG2b; ATCC), respectively. Monoclonal antibody (MAb) S4B6 (rat IgG2a; ATCC) was used to neutralize murine IL-2 in vivo. The NK1.1-specific MAb PK136 (mouse IgG2b) was provided by William Seaman (UCSF, San Francisco, Calif.) and was used to assess NK cell numbers in splenocyte fractions. Recombinant mouse IL-12 (specific activity, 2×10^8 U/mg; endotoxin activity, 0.5 to 0.9 EU/mg) used to standardize the IL-12 bioassay was provided by Hoffmann-La Roche, Inc. (Nutley, N.J.). Human recombinant IL-2 (specific activity, 1.8×10^7 U/mg) was obtained from Cetus Corporation (Emeryville, Calif.). Anti-mouse IL-12 MAbs (5C3 and 3F5 [both rat IgG2a]) were provided by Maurice Gately (Hoffmann-La Roche).

Goat anti-mouse IL-12 antisera and IgG isolation. A goat was immunized subcutaneously with 200 μg of purified recombinant mouse IL-12 (Genetics Institute, Cambridge, Mass.) in complete Freund's adjuvant. The goat was reimmunized with 100 μg of IL-12 mixed with incomplete Freund's adjuvant (IFA) on day 28 of immunization and with 50 μg of IL-12 in IFA on day 42, which was followed thereafter by monthly booster immunizations of 10 μg of IL-12 in IFA. Serum was obtained prior to immunization and then at 2-week intervals commencing on day 42. The IgG fraction of the goat antisera was further purified by protein G-Sepharose affinity chromatography according to the instructions of the manufacturer (Pharamacia LKB, Piscataway, N.J.); IgG-enriched fractions were pooled and dialyzed against phosphate-buffered saline

(PBS) overnight at 4°C. One-half microgram of this purified goat anti-IL-12 IgG neutralized 200 pg of recombinant murine IL-12 standard as determined by an IL-12 bioassay.

Selection of splenic lymphocyte subsets. Spleens were minced with scissors and pressed through 200-mesh stainless steel screens in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS; BioWhittaker, Inc., Walkersville, Md.) at 4°C. After three washes in HBSS with supplemental 1% fetal bovine serum (BioWhittaker, Inc.), the cells were incubated for 15 min in HBSS–1% fetal bovine serum with magnetic particles coated with goat antibodies against mouse IgG and IgM (Collaborative Research, Inc., Bedford, Mass.). Labeled B cells were collected using an external magnetic source (Robbins Scientific Corp., Sunnyvale, Calif.). The B-cell-depleted splenocytes were then incubated with biotinylated anti-CD4 MAb (GK1.5 [ATCC]) or anti-CD8 MAb (2.43 [ATCC]) at 20 μg/ml for 30 min, washed three times, and incubated with streptavidin-coated magnetic particles for isolation of labeled cells. Isolated $CD4^+$ and $CD8^+$ T cells were 93 and 85% pure, respectively, after removal of beads and subsequent analysis by fluorescence flow cytometry as described elsewhere (17). Isolated B cells were 98% $B220^+$. Splenocytes subjected to two rounds of combined T- and B-cell-directed magnetic harvest provided a population consisting of 34% $NK1.1^+$ cells, as assessed by flow cytometry, and 25 to 30% mononuclear phagocytes, as assessed by morphology and latex particle ingestion. About 10% of cells in this negatively selected pool were residual $CD4^+$ and $CD8^+$ T cells or B cells; the remaining cells were not further characterized.

Generation of tissue and cellular RNA. Cell pellets or tissues were homogenized in 6 ml of lysis solution (6 M guanidine hydrochloride, 25 mM EDTA [pH 8.0], 0.1 M sodium acetate [pH 5.0], 0.1% Sarkosyl) and RNA precipitated at −20°C by the addition of 3 ml of 100% ethanol. The pellet obtained after a 20-min centrifugation (12,000 × *g*) was redissolved in 2 ml of lysis solution, aspirated 10 times through a 20-gauge needle to shear DNA, and precipitated again with 1 ml of 100% ethanol. The pellet was dissolved in diethylpyrocarbonate-pretreated water containing 0.2% sodium dodecyl sulfate, 100 mM NaCl, 20 mM Tris (pH 8.0), and 10 mM EDTA and then extracted twice with phenol-chloroform-isoamyl alcohol (50:48:2) and once with chloroform-isoamyl alcohol (24:1). The aqueous phase was precipitated at −70°C after the addition of sodium acetate (pH 5.6) to a final concentration of 0.3 M and the addition of 2.5 volumes of 100% ethanol. $Poly(A)^+$ RNA was obtained using oligo(dT) affinity chromatography (1).

Northern (RNA) blots. $Poly(A)^+$ RNA was subjected to electrophoresis in 1% agarose–6% formaldehyde gels and then transferred to nylon membranes (Hybond N; Amersham, Arlington Heights, Ill.). Prehybridized membranes were incubated overnight at 65°C with antisense RNA probes labeled with [^{32}P]CTP (3,000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear, Boston, Mass.) for autoradiography as described elsewhere (17) or with digoxigenin-UTP for chemiluminescence-based detection (Genius Systems; Boehringer Mannheim, Indianapolis, Ind.).

DNA and oligonucleotide reagents. Mouse IFN-γ, glyceraldehyde phosphate dehydrogenase (GAPD), and IL-2 cDNA fragments were subcloned into pGEM-3Z (Promega, Madison, Wis.) and Bluescript II (Stratagene, La Jolla, Calif.) vectors as described elsewhere (16, 17). A 601-bp *Sal*I fragment of the mouse IL-12 p35 cDNA and a 460-bp *Pst*I fragment of the p40 cDNA were subcloned into Bluescript and pGEM-3Z, respectively (32).

IFN-γ enzyme-linked immunosorbent assay (ELISA). Enzyme immunoassay (96-well) plates (Immulon-4; Dynatech Laboratories, Chantilly, Va.) were coated with 5 μg of R46A2 anti-IFN-γ rat MAb per ml in carbonate buffer (pH 9.5) overnight, washed five times with PBS containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.), and incubated with serum samples for 60 min. After five more washes with PBS-Tween, biotinylated XMG1.2 anti-IFN-γ rat MAb was applied for 30 min at a concentration of 2 μg/ml, the plates were washed again, and a 1:3,000 dilution of streptavidin-horseradish peroxidase (Zymed Laboratories, South San Francisco, Calif.) was added for 30 min. After five washes with PBS-Tween, *o*-phenylenediamine (Sigma) was added as chromagen, the optical density at 492 nm was determined, and the concentration of sample IFN-γ was determined with reference to serially diluted IFN-γ standards contained on the same plate (specific activity, 5×10^6 antiviral U/mg; Genentech, Inc., South San Francisco, Calif.).

IL-2 ELISA. The same protocol was used to detect mouse IL-2, except that the MAbs JES6-1A12 and biotinylated JES6-5H4 (Pharmingen, San Diego, Calif.) were substituted for capture and detection of IL-2, respectively, and recombinant mouse IL-2 (specific activity, 4×10^6 U/mg; Genzyme, Boston, Mass.) was used as the standard.

IL-12 p40 ELISA. The same protocol, with the following modifications, was used to measure concentrations of the p40 subunit of IL-12. Enzyme immunoassay plates were coated overnight at 4°C with 40 μl of a 0.1 M carbonate buffer (pH 9.5) containing 2.5 μg each of anti-p40 MAbs 5C3 and 3F5 per ml. After washing and blocking as described above, sequential dilutions of recombinant mouse IL-12 and experimental sera were applied in 40-μl volumes to individual wells and incubated at room temperature for 3 h. After being washed with PBS-Tween, each well was incubated with 50 μl of biotinylated goat anti-mouse IL-12 (2.5 μg/ml in PBS–1% bovine serum albumin [BSA]) for 1 h at room temperature. The plates were then washed, incubated with streptavidin-horseradish peroxidase, and developed for colorimetric reactions as described above. The colorimetric response was reproducibly linear between 0.5 and 20 ng of recombinant heterodimeric IL-12 per ml (each ng of p70 = 0.53 ng of p40).

IL-12 bioassay. Levels of IL-12 in serum or conditioned culture medium were measured by immunoabsorption onto anti-IL-12-coated ELISA plates and then by bioassaying of the captured cytokine (11). Enzyme immunoassay plates (96 well [Immulon-4; Dynatech Laboratories]) were coated overnight at 4°C with 5C3 anti-mouse IL-12 MAb in a concentration of 5 μg/ml in carbonate buffer (pH 9.5). The plates were washed three times with sterile PBS and blocked for 1 h at 37°C with PBS containing 1% BSA with supplemental penicillin (100 U/ml) and streptomycin (100 μg/ml). Experimental samples and recombinant IL-12 standards were diluted in TCM culture medium (Dulbecco modified Eagle medium with supplemental 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 0.1 mM nonessential amino acids, 60 μg of arginine HCl per ml, 2 mM glutamine, 0.05 mM 2-mercaptoethanol, and 5% fetal bovine serum) and added in 100-μl aliquots to antibody-precoated plates. Samples were incubated for 3 h at room temperature, and the plates were washed three times with PBS. Freshly harvested C57BL/6 splenocytes were suspended at 5×10^6 cells per ml in TCM–5% fetal bovine serum containing 12.5 U of recombinant human IL-2 per ml and applied in aliquots of 200 μl per well to the assay plates. Conditioned media were harvested 48 h later and assayed for IFN-γ concentration by IFN-γ ELISA. The MAb capture bioassay was not affected by the presence of up to 1 μg of LPS per ml in the sample. Serial dilutions of recombinant IL-12 resulted in a sigmoidal IFN-γ response curve, with a near-

linear increase from 60 to 1000 pg of applied IL-12 per ml. The specificity of the responder cell bioassay for IL-12 was assessed by the addition of neutralizing goat anti-mouse IL-12 IgG (1/5,000 dilution) to the culture conditions.

Statistics. Analysis of differences in the levels of cytokines in serum used the Mann-Whitney rank sum test as described elsewhere (12).

RESULTS

$CD4^+$ and $CD8^+$ T cells and NK cells express IFN-γ mRNA in vivo during endotoxemia. Previous studies have detected IFN-γ in the sera of mice at 6 to 12 h following exposure to LPS, with IFN-γ mRNA becoming apparent in the spleen at 4 to 6 h after exposure (15). To identify cellular sources of IFN-γ in vivo, we isolated $CD4^+$, $CD8^+$, and surface Ig^+ lymphocytes from the spleens of C57BL/6 mice that had been challenged 5 h previously with 300 μg of *Salmonella enteriditis* LPS and from control mice injected with endotoxin-free saline. Splenic cell populations were obtained by magnetic separation of cells labeled with subset-specific MAbs linked via biotin-avidin interactions to ferrous microparticles. This technique resulted in 85 to 93% pure $CD8^+$ and $CD4^+$ T cells and approximately 98% pure B cells (17). Negative depletion of CD4, CD8, and surface Ig-positive lymphocytes by two rounds of combined magnetic bead selection was used to obtain a T- and B-cell-depleted splenocyte fraction that contained approximately 34% $NK1.1^+$ lymphocytes, 25% mononuclear phagocytes, and approximately 10% contaminating T and B cells.

Hybridization of RNA obtained from these cells with antisense RNA probes specific for mouse IFN-γ detected no IFN-γ mRNA in splenocyte fractions from control mice. However, abundant IFN-γ message was present within $CD4^+$ and $CD8^+$ T-cell- and NK-cell-enriched fractions that had been exposed in vivo to endotoxin (Fig. 1). IL-2 mRNA expression was also examined to determine if lymphokine expression was generalized, which would occur if T cells were activated during endotoxemia. However, antisense RNA probes specific for mouse IL-2 hybridized only with a 1.2-kb transcript contained in B cells. As has been previously reported, this message is also expressed by normal splenic B cells not exposed to endotoxin (17).

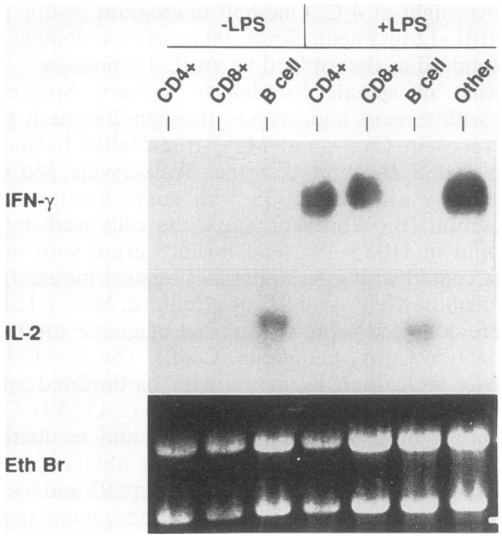

FIG. 1. The induction of IFN-γ RNA in distinct splenocyte populations 5 h after i.p. injection of LPS. Spleens from control (−LPS) and LPS-treated (+LPS) C57BL/6 mice were harvested as described and fractionated into $CD4^+$, $CD8^+$, and surface Ig^+ (B-cell)-enriched pools using magnetic beads bound to subset-specific antibody ligands. Other, splenocytes depleted of T and B cells utilizing two rounds of magnetic bead selection and that were enriched in NK cells and macrophages. RNA was obtained from the fractionated cells, subjected to electrophoresis, blotted onto nylon membranes, and hybridized with antisense probes specific for mouse IFN-γ and IL-2. The gel was stained with ethidium bromide (Eth Br) to compare loadings of RNA in each lane (20 μg per sample).

IL-12 mRNA expression precedes IFN-γ production. Because IL-12 is capable of eliciting IFN-γ production by T cells and NK lymphocytes in a pattern reminiscent of that observed in Fig. 1, we determined the expression of IL-12 mRNA in vivo at times prior to the appearance of IFN-γ during endotoxemia. C57BL/6 mice were challenged with 300 μg of *S. enteriditis* LPS and poly(A)$^+$ splenic RNA obtained at 0, 2, 6, 12, and 24 h after injection. Since IL-12 is a heterodimer composed of p35 and p40 subunits that must be coproduced to generate functional cytokine, the expression of both p35 and p40 subunit mRNAs was measured (13, 32). The p35 chain of IL-12 was constitutively expressed throughout the study period, although increased expression was transiently apparent at 12 h (Fig. 2). This was partially matched by an increased abundance of mRNA encoding the housekeeping gene GAPD. In contrast, p40 mRNA was markedly increased at 2 h of endotoxemia; lower levels of expression were also apparent at 6 h. The predominant p40 RNA species observed was approximately 2.6 kb in size, consistent with previous descriptions (32), although prolonged exposures also showed corresponding changes in an approximately 1.9-kb transcript (not shown). IFN-γ mRNA first became apparent at 6 h and was present through 12 h of endotoxemia.

IL-12 is detectable in the sera of endotoxemic mice. To prove that the dual expression of p35 and p40 IL-12 mRNA observed in vivo resulted in functional IL-12 production, mouse serum was assayed for IL-12 bioactivity at 0, 2, 4, and 6 h after endotoxemia (Table 1). By a MAb capture bioassay technique that is specific for IL-12 and sensitive to approximately 60 pg of recombinant IL-12 standard per ml, C57BL/6 mice had no detectable IL-12 p70 in their sera prior to injection with LPS. Increased levels of IL-12 in sera were apparent from 2 to 4 h after endotoxin exposure; IL-12 bioactivity declined thereafter (6 h). Similar changes were apparent when these same sera were assayed using a p40 subunit-specific ELISA that detects both IL-12 heterodimers and p40 homodimers. In contrast to the failure to detect basal levels of IL-12 bioactivity in serum, total p40 immunoreactivity was present in C57BL/6 serum prior to endotoxin exposure, and these values increased by 10- and 50-fold at 2 and 4 h, respectively, after endotoxin injection. The appearance and peak activity of IFN-γ in the serum followed that of IL-12 bioactivity by approximately 2 h.

In another experiment, we compared circulating IL-12 p70 bioactivity and p40 immunoreactivity after endotoxin was administered to LPS-sensitive C3H/HeOuJ and LPS-resistant C3H/HeJ mice (Table 2). Although detectable IL-12 bioactivity was elicited by endotoxin in both strains of mice, peak serum activity was threefold greater in the C3H/HeOuJ mice. Again, total levels of circulating p40 were much greater than those of bioactive p70. Concentrations of circulating IFN-γ at 4 and 6 h after endotoxin challenge were markedly increased in the endotoxin-sensitive C3H/HeOuJ strain compared with endotoxin-insensitive mice. Again, increased levels of IFN-γ in the serum followed the appearance of IL-12 by approximately 2 h.

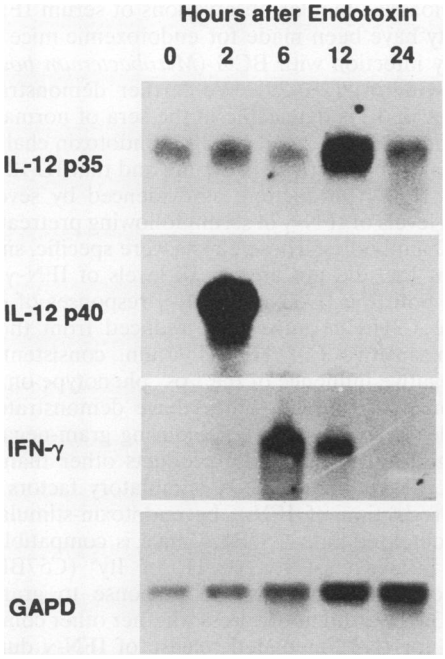

FIG. 2. Expression of IL-12 p40 and p35 subunit mRNAs and IFN-γ mRNA in C57BL/6 spleens following injection of LPS. Spleens were harvested at the indicated times following LPS challenge, and 7.5-μg aliquots of poly(A)$^+$ RNA from each sample were sequentially hybridized with antisense RNA probes specific for the indicated genes. Analysis of the housekeeping gene GAPD is included to assess RNA loading.

Although IL-2 probes hybridized with a 1.2-kb transcript present in mouse spleen RNA, IL-2 protein was not detected in the sera of endotoxemic mice at any of these time points by an ELISA protocol sensitive to approximately 0.1 ng of recombinant IL-2 standard per ml (data not shown).

Anti-IL-12 blocks IFN-γ production in response to endotoxin. To prove that IL-12 production was causally linked to IFN-γ generation in vivo during endotoxemia, C57BL/6 mice were pretreated with 4.4 mg of neutralizing goat anti-mouse IL-12 IgG, and these and untreated control mice were challenged with 100 μg of *S. enteriditis* LPS given by intraperitoneal (i.p.) injection 18 h subsequently. Circulating IFN-γ was decreased approximately fivefold at 6 h after LPS injection (Table 3). Similar results were obtained in two separate experiments in which pretreatment with anti-IL-12 antibodies reduced peak IFN-γ levels by six- to sevenfold, whereas pretreatment with 2.6 mg of preimmune goat IgG did not block the appearance of circulating IFN-γ. Despite the presence of IL-2 mRNA in the spleens of these mice, pretreatment with 2.0 mg of anti-IL-2 MAb S4B6 only modestly reduced IFN-γ levels by 34% at 4 h and 9% at 6 h after endotoxin challenge (Table 4).

TABLE 1. Levels of IL-12 p70 and p40 and IFN-γ in serum after injection with LPS[a]

Cytokine	Concn in serum at the following h after endotoxin treatment (ng/ml ± SEM):			
	0	2	4	6
p70	≤0.06	0.11 ± 0.01	0.38 ± 0.017	0.06 ± 0.04
p40	0.3 ± 0.04	2.9 ± 0.5	15.8 ± 1.9	20.5 ± 0.4
IFN-γ[b]	≤0.7	≤0.7	3.1 ± 0.6	13.5 ± 1.2

[a] C57BL/6 mice were injected with 100 μg of *S. enteriditis* by i.p. injection, and sera were collected at the designated times for cytokine analysis. IL-12 p70 levels were measured by a MAb capture bioassay, and IL-12 p40 and IFN-γ levels were measured directly by specific ELISA. *n*, four mice per group per time point.

[b] These sera are those that were assayed as control values for Table 3, experiment 3.

TABLE 2. Levels of IL-12 p70 and p40 and IFN-γ in serum induced by LPS in endotoxin-resistant and -sensitive strains of mice[a]

Mouse type and cytokine	Concn in serum at the following h after endotoxin treatment (ng/ml ± SEM):			
	0	2	4	6
C3H/HeJ (LPS^r)				
p70	≤0.06	0.52 ± 0.13	0.43 ± 0.14	0.20 ± 0.04
p40	0.9[b]	7.4 ± 0.5	7.4 ± 0.8	7.4 ± 1.3
IFN-γ	≤0.7	1.6 ± 0.8	6.0 ± 1.0	3.2 ± 2.0
C3H/HeOuJ (LPS^s)				
p70	≤0.06	1.01[b]	1.43 ± 0.03	0.68 ± 0.44
p40	0.9[b]	12.6[b]	15.9 ± 1.7	15.4 ± 3.2
IFN-γ	≤0.7	≤0.7	20.6 ± 4.3	113 ± 32

[a] Mice were injected with 300 μg of *S. enteriditis* by i.p. injection, and sera were collected at the designated times for cytokine measurements. IL-12 p70 levels were determined by a MAb capture assay; total IL-12 p40 and IFN-γ levels were determined by ELISA as described in Materials and Methods. *n* = three mice per group per time point.

[b] *n* = two mice per group per time point.

DISCUSSION

Our findings implicate IL-12 as a mediator of IFN-γ release in vivo during experimental endotoxemia. Specifically, IFN-γ mRNA is expressed by T cells and NK cells of endotoxin-challenged mice in a pattern consistent with the known effects of IL-12; splenic IL-12 mRNA and serum IL-12 are detected shortly after injection of LPS; bioactive IL-12 circulates in the serum before the appearance of IFN-γ; and pretreatment with

TABLE 3. Effect of pretreatment with polyclonal goat anti-mouse IL-12 IgG on IFN-γ production during experimental endotoxemia[a]

Expt no. and pretreatment	Concn of IFN-γ in serum at the following h after endotoxin treatment (ng/ml ± SEM):		
	0	4	6
Expt 1			
Control	ND	1.9 ± 0.1	10.9 ± 1.6
Anti-IL-12 (4.4 mg)	ND	0.7 ± 0.1	2.4 ± 0.2
P value[b]	ND	0.21	0.02
Expt 2			
Preimmune goat IgG (2.6 mg)	≤0.7	5.0 ± 0.6	7.9 ± 1.1
Anti-IL-12 (2.6 mg)	≤0.7	1.7 ± 0.1	1.3 ± 0.8
P value[b]	NS	0.04	0.02
Expt 3			
Control	≤0.7	3.1 ± 0.6	13.5 ± 1.2
Anti-IL-12 (4 mg)	≤0.7	1.4 ± 0.4	2.7 ± 1.1
P value[b]	NS	0.12	0.03

[a] C57BL/6 mice were injected with 100 μg of *S. enteriditis* LPS by i.p. injection (*n* = four mice per group per time point). The mice received the indicated amounts of antibody by i.p. injection 18 h before LPS challenge. NS, not significant; ND, not done.

[b] Mann-Whitney rank sum test.

TABLE 4. Effect of pretreatment with anti-IL-2 MAb (S4B6) on IFN-γ production during experimental endotoxemia[a]

Pretreatment	Concn of IFN-γ in serum at the following h after endotoxin treatment		
	0	4	6
Control	≤0.2	2.9 ± 0.9	6.5 ± 0.3
Anti-IL-2 (2.0 mg)	≤0.2	1.9 ± 0.7	5.9 ± 0.7
P value[b]	NA	0.51	0.66

[a] C57BL/6 mice were injected with 300 μg of *S. enteriditis* LPS by i.p. injection 18 h after i.p. injection with anti-IL-2 MAb. Results are from experiment 1. *n* = four mice per group per time point. NA, not applicable.
[b] Mann-Whitney rank sum test.

anti-IL-12 antibody blocks production of IFN-γ in response to endotoxin challenge. The expression of IFN-γ mRNA in vivo by both NK cells and $CD4^+$ and $CD8^+$ T cells is consistent with conclusions obtained from studies of cultured mouse splenocytes challenged with LPS (5, 25). This pattern of IFN-γ stimulation can also be observed after treatment of T cells and NK cells with IL-2 and/or IL-12 (6, 14, 21). A role for T-cell-derived IL-2 in the in vitro generation of IFN-γ in response to LPS had been previously suggested (5, 23). However, we found that T cells did not contain detectable amounts of IL-2 transcript following endotoxemia, suggesting that the release of T-cell IFN-γ was not part of a broader activation of lymphokines. Furthermore, the administration of neutralizing anti-IL-2 antibodies did not significantly influence levels of IFN-γ in the serum in our studies. Although IL-2 nucleic acid probes hybridized with a 1.2-kb mRNA present in the B cells of both normal and endotoxin-challenged mice, cultured B cells are not known to release bioactive IL-2 unless they are activated by mitogens or cross-linking of surface immunoglobulin (33). Consequently, we focused on the possible involvement of IL-12 in the induction of IFN-γ during endotoxemia.

As had been described in previous studies, mRNA for the p35 subunit of IL-12 was constitutively expressed in mouse spleens (32), and dramatic changes were not evident following exposure to LPS. The transient increase in expression of p35 at 12 h was not further examined in these studies, although this may represent delayed upregulation of p35 transcription in response to LPS or to cytokines previously elicited by endotoxemia. In contrast, p40 expression at 2 h of endotoxemia was markedly increased above basal levels, consistent with the production of active heterodimeric IL-12 at a time prior to the appearance of IFN-γ. Similar increases in p40 expression were evident in the peritoneal cells of mice 2 h after endotoxin injection (data not shown). We did not further address the cellular source of IL-12 synthesis in these in vivo studies. Other reports have demonstrated that B- and T-cell-deficient SCID mice generate IFN-γ in response to LPS, thereby implicating macrophage products in this response (31). The ability of A20 mouse B-cell lines to release IL-12 upon exposure to endotoxin suggests that these cells might also contribute to in vivo IL-12 release during inflammatory responses (27). Indeed, recent studies of human peripheral mononuclear blood cells have shown that both B cells and adherent monocytes are sources of IL-12 in response to LPS (7).

Data from the present study and a previous study (18) identified bioactive IL-12 in the sera of endotoxemic mice. C57BL/6 mice infected with *Leishmania major* for 4 weeks and then challenged with endotoxin produced greatly increased amounts of circulating IFN-γ (18). This was preceded by the appearance within the serum of a factor with strong IFN-γ-inducing activity determined to be IL-12 by a anti-IL-12 MAb capture bioassay. Similar observations of serum IFN-γ-inducing activity have been made for endotoxemic mice previously primed by infection with BCG (*Mycobacterium bovis* bacillus Calmette-Guérin) (28, 29). We further demonstrate in this report that IL-12 is detectable in the sera of normal C57BL/6 mice, commencing at 2 and 4 h after endotoxin challenge, well before peak IFN-γ activity in serum, and that IL-12 is causally linked to IFN-γ production, as evidenced by sevenfold decreases in levels of IFN-γ in serum following pretreatment with anti-IL-12 antibodies. These effects were specific, since preimmune goat IgG did not alter peak levels of IFN-γ in serum. Similarly, both the IL-12 and IFN-γ responses of endotoxin-insensitive C3H/HeJ mice were reduced from those of the endotoxin-sensitive C3H/HeOuJ strain, consistent with the known negative influence of the Lps^d phenotype on monokine production (3). However, others have demonstrated normal IFN-γ release by C3H/HeJ mice during gram-negative infection, suggesting that bacterial products other than LPS may induce IL-12 or similar IFN-γ stimulatory factors (10). The greater production of IFN-γ by endotoxin-stimulated C3H/HeOuJ compared with C57BL/6 mice is compatible with the reported influence of Ity^r (C3H) or Ity^s (C57BL/6) backgrounds on IFN-γ synthesis in response to gram-negative bacteria (30). We did not address whether other cofactors were necessary for IL-12-mediated release of IFN-γ during endotoxemia; a requirement for TNF-α as a coinducer of IFN-γ has been previously suggested (2, 31).

Circulating p40 is also detectable in the sera of endotoxemic mice in concentrations exceeding that of bioactive IL-12, a phenomenon described in studies on stimulated human peripheral blood mononuclear cells (7). Detectable amounts of p40 also were present in normal sera and in media conditioned by culture with unstimulated mouse spleen cells (data not shown). Whether this represents constitutive production of p40 molecules or delayed clearance of p40 homodimers requires further study. A recent report has described inhibition of IL-12 bioactivities by free p40 molecules, consistent with a role for p40 in serum in maintaining inflammatory homeostasis (26). However, we found that the excess p40 present during endotoxemia was not sufficient to prevent IL-12-dependent IFN-γ release in vivo.

These data demonstrate that the in vivo production of IFN-γ by NK cells and T cells during endotoxemia is dependent on the earlier synthesis and release of bioactive IL-12. It will be of interest to determine if anti-monokine antibodies and monokine receptor antagonists presently being studied as therapy for septic shock are able to alter IL-12 and IFN-γ production. If not, then the use of specific IL-12 or IFN-γ antagonists such as IL-10 (7) may provide additional clinical benefit by downregulating destructive inflammatory responses during endotoxic shock.

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