Chapter 5

Role of endogenous IL-12 in the immune response to staphylococcal enterotoxin B in mice

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Abstract

Staphylococcal enterotoxin B (SEB) is a bacterial superantigen (SAg) produced by *Staphylococcus aureus*. Administration of SEB to mice results in rapid release of cytokines into the circulation, followed by a clonal expansion and subsequent deletion of SEB responsive Vβ8+ T cells. To study the role of IL-12 in the immune response to SEB, IL-12p40 gene deficient (IL-12p40−/−) and wild-type (WT) mice were intraperitoneally injected with SEB (100 μg). SEB induced transient increases in the plasma concentrations of IL-12p70 and IL-12p40 peaking at 4-8 h after injection, accompanied by enhanced expression of IL-12p35 and IL-12p40 mRNA in spleens. The production of TNF, IL-2 and IL-10 did not differ between IL-12p40−/− and WT mice, while IFN-γ concentrations were strongly reduced in IL-12p40−/− mice from 4 h onwards. Although IL-12 is known to potently stimulate T cell proliferation in vitro, the proliferation and subsequent deletion of SEB-reactive Vβ8+ T cells did not differ between IL-12p40−/− and WT mice. Anti-IL-18 did not influence IFN-γ release in either WT or IL-12p40−/− mice. These data suggest that the role of IL-12 in SEB-induced immunopathology is limited to sustaining IFN-γ release by an IL-18 independent mechanism.
Introduction

Bacterial superantigens (SAgs) are a unique group of proteins that activate both antigen presenting cells (APCs) and T cells (1). After binding to MHC class II molecules, SAgs interact with specific Vβ segments of the T cell receptor, resulting in the stimulation of a large proportion of T cells (2). SAgs have been implicated in the pathogenesis of food poisoning syndrome and the development of septic shock in humans (3). Staphylococcal enterotoxin B (SEB) is a product of Staphylococcus aureus and is one of the best characterized and most studied SAgs. Injection of SEB in mice induces rapid activation and proliferation of Vβ8-specific T cells in vivo, resulting in the release of cytokines including TNF, IL-2, IL-10 and IFN-γ, which is followed by an anergic phase (4-6). Several factors have been shown to play an important role in SEB-induced pathology. TNF is released shortly after SEB injection, and has been demonstrated to be a significant mediator of SEB-induced toxicity (7). Also, priming for high IFN-γ production resulted in increased SEB-induced lethality (8). In contrast, the anti-inflammatory cytokine IL-10 has a protective effect, presumably by downregulating the proinflammatory cytokine response, and by inhibiting the function of APCs (9, 10). In addition, endogenously produced nitric oxide (NO) and glucocorticoids protect against SEB-induced shock (11, 12).

IL-12 is a proinflammatory heterodimeric cytokine formed by a p35 and a p40 subunit, which is mainly produced by APCs (13, 14). IL-12 is a potent stimulator of T cell functions, including proliferation, cytotoxicity, and the release of cytokines, IFN-γ in particular. In addition, IL-12 plays an important role in the T helper (Th)1/Th2 balance by promoting the differentiation of naive CD4+ T cells into Th1 type cells, and stimulating the release of Th1 type cytokines like IFN-γ and IL-2. Endogenously produced IL-12 has been shown to play an essential role in the pathogenesis of endotoxin-induced shock, i.e. neutralization of IL-12 profoundly inhibits IFN-γ release, and protects against lethality during experimental endotoxemia in mice (15, 16). Little is known about the role of IL-12 during SAg-induced pathology. A recent study reported on the regulation of IL-12 production in response to SEB, and on the role of IL-12 for IFN-γ production upon primary and secondary challenge with SEB (17).

Like IL-12, IL-18 is a proinflammatory cytokine derived from activated monocytes/macrophages, which is an important co-stimulus for optimal IFN-γ production, especially in the presence of IL-12 (18, 19). The importance of IL-18 for IFN-γ synthesis has been demonstrated in IL-18 gene deficient mice, and mice lacking IL-1β- converting enzyme (ICE), which is required to convert precursor IL-18 into the active IL-18 protein (20-23). In addition, IL-18 has many other stimulatory activities on T and NK cells, including enhancement of proliferation and cytotoxicity, increasing anti-tumor activity, and activation of Th1 cells (19, 24). During experimental endotoxemia in mice, neutralization
of IL-18 protected against LPS-induced liver injury (18). The role of IL-18 in SAg-induced shock is unknown.

In the present study, we investigated the role of IL-12 in SAg-induced responses by comparing the effects of SEB in IL-12p40 gene deficient (IL-12p40"~") and normal wild-type (WT) mice. In addition, to study whether IL-18 plays a role in SEB-induced IFN-γ production and/or acts as a co-stimulus for IL-12-stimulated IFN-γ production, WT and IL-12p40"~" mice were injected with SEB in combination with anti-IL-18 or control serum.

Materials and methods

Mice
All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center. IL-12p40"~" mice were obtained from the Jackson Laboratory (Bar Harbor, ME). IL-12p40"~" mice were on a Balb/c background. Normal Balb/c WT mice were obtained from Harlan Spague Dawley Inc. (Horst, the Netherlands). Sex and age matched 8-10 weeks old mice, 3-8 mice per group per timepoint, were used in all experiments.

Reagents
SEB was obtained from Sigma (St. Louis, MO). Rabbit anti-murine IL-18 antiserum was prepared as described previously, and kindly donated by Dr. C. A. Dinarello (University of Colorado Health Sciences Center, Denver, CO) (20). This antiserum strongly inhibits LPS- or IL-12-induced IFN-γ production by splenocytes in vitro (20, 25), and reduces IFN-γ release and protects against lethality during experimental endotoxemia in mice (26). Rabbit serum was used as a control (Sigma). Sheep anti-murine IL-12 was prepared as described previously (27), and was kindly supplied by the Bioanalytical Sciences Department of Genetics Institute, Inc. (Cambridge, Mass.). Sheep IgG (Sigma) was used as a control.

In vivo experimental studies
Mice were challenged intraperitoneally with a single injection of SEB at dose of 100 μg dissolved in 200 μl saline. Control mice received saline only. In some experiments, mice were pretreated with anti-IL-18 antiserum or control serum (200 μl) 1 h prior to SEB administration. Mice were anesthetized at different time points after challenge with SEB by FFM (fentanyl citrate 0.079 mg/ml, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H2O; of this mixture 7.0 ml/kg intraperitoneally), and sacrificed by bleeding by cardiac puncture. Blood was collected in EDTA-containing tubes, and centrifuged at 1400 x g for 20 min. Supernatants were stored at -20° until assays were performed.
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Reverse transcription PCR (RT-PCR) for cytokine message

Spleens were harvested after injection with saline and at several time points after SEB injection, snap-frozen in liquid nitrogen and stored at -70°C. To extract total cellular RNA, spleens from 3 mice per time point were pooled and homogenised in 1 ml of Trizol Reagent (GibcoBRL, Life Technologies, Grand Island, NY). Then, total RNA was isolated using chloroform extraction and isopropanol precipitation. The RNA pellet was dissolved in 100 μl diethylpyrocarbonate (DEPC)-treated water and quantified by spectrophotometry. Reverse transcription (RT) was performed by mixing 2 μg of total cellular RNA with 0.5 μg oligo(dT) (GibcoBRL) in a total volume of 12 μl. The mixture was incubated at 72°C for 10 min. Thereafter, 8 μl of a solution containing 4 μl 5x First Strand Buffer (GibcoBRL), 10 mM dithiothreitol (DTT; GibcoBRL), 1.25 mM dNTP’s (Amersham Pharmacia, Biotech, UK), and 100 U Superscript Reverse Transcriptase (GibcoBRL), was added, and incubated at 42°C for 1 h. Finally, the tubes were heated to 72°C for 10 min. after which 180 μl H₂O was added to the reaction mixture. Samples were stored at -20°C until further use. For PCR, 5 μl of cDNA solution was mixed with 20 μl of a solution containing, 1x PCR buffer (67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl₂, 10 mM β-mercaptoethanol, 0.67 μg EDTA, 16.6 mM (NH₄)₂SO₄), 2% DMSO (Merck, München, Germany), 1.25 μg BSA (Biolabs Inc., New England), 0.5 U AmpliTaq DNA polymerase (Perkin Elmer Corp., Branchburg, NJ, USA) and 75 ng sense- and anti-sense oligonucleotide primers specific for IL-12p35, IL-12p40 and β-actin (internal standard). The PCR reactions were performed in a thermocycler (Gene Amp. PCR System 9700, Perkin-Elmer Corp.) using the following conditions: 94°C for 5 min (1 cycle), followed immediately by 95°C for 1 min, 55°C (IL-12p35 and IL-12p40) or 58°C (β-actin) for 1 min, 72°C for 1 min (with variable numbers of cycles) and a final extension phase of 72°C for 10 min. For semiquantitative assessment of IL-12p35 and IL-12p40 mRNA, variable numbers of cycles were used to ensure that amplification occurred in the linear phase. To exclude the possibility of finding differences between tubes due to unequal concentrations of cDNA in the PCR-reaction, a PCR using β-actin as the internal standard was performed on each sample. β-actin was found to be linear at 27 amplification cycles, IL-12p35 and IL-12p40 at 29 amplification cycles. The primers used for IL-12p35 (520 bp) were 5'-AAACCTGCTGAAGACCAC-3' (sense) and 5'-AGCTCAGATAGCCCATCAC-3' (antisense), for IL-12p40 (277 bp) 5'-ACTCACATCTGTCTCCAC-3' (sense) and 5'-CCTCTGTCTCCTCATCTTTTC-3' (antisense), and for β-actin (617 bp) 5'-GTCAGAAGGACTCTATGTG-3' (sense) and 5'-GCTCGTTGCAATAGTGATG-3' (antisense). PCR products were visualized by agarose gel electrophoresis.
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Cell isolation procedure
Single cell suspensions were obtained from spleens and mesenteric lymph nodes, by crushing through a 40 μm filter (Costar, Cambridge, MA) with sterile RPMI 1640 supplemented with L-glutamine (Bio Whittaker, Verviers, Belgium), containing 10% fetal calf serum, and 1% Antibiotic-Antimycotic (GibcoBRL). Erythrocytes were lysed with sterile ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4) for 10 minutes. Cells were centrifuged at 600 x g for 5 minutes at room temperature, washed twice with RPMI, and brought to the appropriate cell concentrations.

FACScan analysis
Incubations for FACScan analysis were performed in 96-well V-shaped microplates (Greiner B.V., Alphen a/d Rijn, the Netherlands). For staining, 5 x 10⁵ cells/well were incubated with the following rat anti-mouse monoclonal antibodies (mAbs): FITC-labeled CD4 or CD8 (1:100, both PharMingen), in combination with phycoerythrin-labeled Vβ6 or Vβ8 (1:80, both PharMingen). The appropriate isotype controls (PharMingen) were included in all experiments. Cells were incubated on ice for 30 min., and washed twice with cold FACS buffer (PBS supplemented with 0.01% NaN₃, 0.5 % BSA, and 0.3 mM EDTA) and resuspended in FACS buffer. Lymphocytes were gated by forward and side scatter using a FACScan (Becton Dickinson) and 5,000 cells were counted. Results are expressed as the percentage of gated cells positive for the Abs used.

Assays
Cytokine concentrations were measured by specific ELISAs according to the instructions of the manufacturers. TNF, IFN-γ, IL-2, IL-4, IL-5, IL-12p70 and IL-18 reagents were obtained from R&D Systems (Abingdon, UK), IL-10 and total IL-12p40 reagents were from PharMingen. The lower detection limits of the assays were 14 pg/ml (TNF, IL-2, IL-10 and IL-12p70), 31 pg/ml (IFN-γ, IL-4, IL-5, IL-12p40 and IL-18).

Statistical analysis
Data are expressed as mean ± SE. Differences between groups were analyzed by Mann-Whitney U test. P < 0.05 was considered to represent a statistically significant difference.
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Results

Induction of IL-12
To determine whether IL-12 is produced after i.p. injection of SEB, RT-PCR was performed on spleen samples obtained from mice 4 h after injection of saline (controls) or at 2, 4 and 8 h after SEB. No detectable IL-12p35 and IL-12p40 mRNA was noted in spleens of mice given saline. Administration of SEB induced the expression of both IL-12p35 and IL-12p40 mRNA in the spleen (Figure 1A). Furthermore, SEB injection resulted in transient increases in plasma concentrations of IL-12p70 and total IL-12p40, peaking after 4-8 h (Figure 1B). Plasma concentrations of IL-12p70 were not detectable in control mice, while levels of IL-12p40 were 2769 ± 101 pg/ml.

![Figure 1](image.jpg)

Figure 1. Injection of SEB i.p. (100 µg) induces the expression of IL-12p35 and IL-12p40 mRNA in spleens and systemic release of IL-12p70 and IL-12p40. A. Spleens of 3 mice at each time point were pooled for IL-12 mRNA and β-actin mRNA expression as determined by RT-PCR. B. Plasma concentrations of IL-12p70 and total IL-12p40 (mean ± SE of 6-8 mice per time point) were measured at the indicated time points after injection of SEB. Control mice received saline only.

Cytokine response to SEB
Administration of SEB results in the release of proinflammatory and anti-inflammatory cytokines (4). Indeed, TNF and IL-2 plasma concentrations rapidly increased after SEB injection, reaching peak levels after 2 h (Figure 2). IFN-γ and IL-10 increased gradually after SEB challenge, peaking at 4-8 h. Concentrations of TNF and IL-2 did not differ between IL-12p40−/− and WT mice. The initial increase in IFN-γ concentrations was similar in IL-12p40−/− and WT mice; however, IFN-γ concentrations quickly decreased in IL-
12p40−/− mice after 4 h. Also, concentrations of IL-10 were slightly decreased in IL-12p40−/− mice, although this difference was not significant.

![Plots of TNF, IL-10, IL-2, and IFN-γ over time](image)

**Figure 2.** Plasma concentrations of TNF, IL-2, IL-10 and IFN-γ in IL-12p40−/− mice and WT mice after i.p. injection of 100 μg SEB. Data are mean ± SE of 6-8 mice at each time point. * P < 0.05 vs. WT mice.

**Proliferation of CD4+ and CD8+ cells to SEB in vivo**

Injection of SEB in vivo is characterized by proliferation of Vβ8+ T cells in peripheral lymphoid organs (6). To determine whether IL-12 contributes to the expansion of SEB-reactive T cells, the percentage of CD4+ Vβ8+ and CD8+ Vβ8+ cells in splenocytes and mesenteric lymph node cells was assessed in IL-12p40−/− and WT mice. As shown in Figure 3, at baseline, the percentage of both CD4+ Vβ8+ and CD8+ Vβ8+ cells in spleen and mesenteric lymph nodes did not differ between IL-12p40−/− and WT mice. At 2 days after SEB injection, the percentage of CD4+ Vβ8+ and CD8+ Vβ8+ cells was almost twofold compared the percentages found in control mice. At 4 days after SEB injection, the number of CD4+ Vβ8+ cells had returned back to, or even below, normal, while the number of CD8+ Vβ8+ cells was still slightly increased. There was no difference in the response between IL-12p40−/− and WT mice. This proliferation was specific for SEB-reactive Vβ8+ positive cells, since the number of Vβ6+ cells did not increase after SEB injection (data not shown), in either mice strain.
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Figure 3. Proliferation of Vβ8+ T cells in spleen and mesenterial lymph node cells in response to SEB in IL-12p40−/− (closed symbols) and WT (open symbols). Cell populations were determined by FACScan analysis. IL-12p40−/− and WT mice were injected i.p. with NaCl (t = 0) or 100 μg SEB i.p. and sacrificed at 2 or 4 days after injection. Values are mean of 3 mice per group. Data are expressed as percentage positive cells within the lymphocyte population of spleen or mesenterial lymph node cells.

Effect of anti-IL-18 on SAg-induced IFN-γ production

IL-18 is an essential cofactor for IFN-γ production after LPS challenge (18). To determine whether IL-18 contributes to SEB-stimulated IFN-γ production, and whether IL-18 interacts with IL-12 to induce IFN-γ release, WT and IL-12p40−/− mice were injected with anti-IL-18 or control serum 1 h prior to SEB administration, and sacrificed after 8 h for IFN-γ measurements. This time point was selected since differences in IFN-γ concentrations between IL-12p40−/− and WT mice were most pronounced at 8 h after SEB injection (see Results and Figure 2). Again, IL-12p40−/− mice had significant lower plasma concentrations of IFN-γ (Figure 4). Administration of anti-IL-18 did not affect SEB-stimulated IFN-γ production in either WT or in IL-12p40−/− mice.

Figure 4. Effect of anti-IL-18 on SEB-induced IFN-γ release in IL-12p40−/− and WT mice. All mice were injected with 100 μg SEB i.p. in combination with anti-IL-18 or control serum (200 μl) administered i.p. 1 h prior to SEB, and sacrificed after 8 h for IFN-γ measurements. Data are mean ± SE of 6 mice per group. * P < 0.05 vs. WT mice.
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Discussion

SEB is a bacterial SAg produced by *S. aureus*, that interacts with both APCs and T cells (1). Injection of SEB in mice induces polyclonal T cell activation, resulting in the production of pro- and anti-inflammatory cytokines, and proliferation of SEB-reactive Vβ8+ T cells (2, 4, 5). We here demonstrate that injection of SEB also induces the release of IL-12, a monocyte/macrophage-derived cytokine with potent T cell stimulatory activities. IL-12 is composed of a p35 and a p40 subunit, which are encoded by two separate and unrelated genes (13). Expression of both chains within the same cell is required for the production of the biologically active IL-12p70 heterodimer. SEB stimulated the systemic release of both IL-12p70 and IL-12p40. Similar results were found in a previous study, which demonstrated that IL-12 release is largely dependent on CD40-CD40 ligand interaction and endogenous IFN-γ release (17).

Next we studied the role of IL-12 in the SEB-induced activation of the cytokine network. Injection of SEB results in the early release of TNF, IL-2 and IL-10, which is followed by the secretion of IFN-γ. Both TNF and IFN-γ have been demonstrated to importantly contribute to SEB-induced lethality (7, 8). IL-12 does not influence the early release of cytokines within 4 h after SEB injection, considering that IL-12p40−/− and WT mice displayed no differences in TNF, IL-2, IL-10 and also in IFN-γ release. However, IL-12 contributes to the sustained increase in SEB-induced IFN-γ production, since IFN-γ concentrations were strongly decreased in IL-12p40−/− mice after 4 h. Our results on SEB-stimulated IFN-γ secretion are in contrast with results reported after injection of LPS, where IFN-γ production is largely dependent on IL-12 and neutralization of IL-12 protects against LPS-induced lethality (15, 16, 28). This illustrates the different mechanisms involved in SEB stimulation compared to LPS stimulation, i.e. whereas the former results in polyclonal T cell activation, the latter mainly activates monocytes/macrophages.

Injection of SEB is characterized by the selective proliferation of Vβ8+ T cells in peripheral lymphoid tissues, which involves both CD4+ and CD8+ cells (6). After the initial expansion, the number of Vβ8+ T cells decreases, which is most profound in the CD4+ population. Although the exact mechanism of SEB-induced cell death is unknown, evidence exists for programmed cell death and involvement of Fas-Fas-ligand interaction (29, 30). It has been well established that IL-12 can enhance the proliferation of CD4+ and CD8+ T cells (13). Also, IL-12 has been found to inhibit Fas-mediated apoptosis of CD4+ T cells (31). However, the changes in the number of Vβ8+ cells in response to SEB were not different in IL-12p40−/− mice or in WT mice, suggesting that IL-12 does not play a role in SEB-induced proliferation and death of peripheral T cells.

IL-18 is an important cofactor for IFN-γ production (18, 19). In the presence of IL-12, IL-18 synergistically enhances IFN-γ production (32). The mechanism for synergistic
effects between IL-12 and IL-18 involves the upregulation of IL-18 receptor expression by IL-12 on a variety of cells (33, 34). IL-18 is a produced by activated macrophages, where it is first synthesized as an IL-18 precursor protein, which requires processing by ICE to release the biologically active mature protein (19, 22, 23). The importance of IL-18 for optimal IFN-γ production has been demonstrated in IL-18 and ICE deficient mice, which produce little IFN-γ despite normal IL-12 concentrations (20, 21). In the present study, IL-18 does not contribute to IFN-γ secretion after SAg challenge in both WT and IL-12p40−/− mice, as demonstrated with neutralizing anti-IL-18 antiserum. This anti-serum has previously been demonstrated to strongly inhibit LPS- and IL-12-stimulated IFN-γ production by mouse splenocytes in vitro (20, 25). In addition, during experimental endotoxemia in mice, anti-IL-18 decreased IFN-γ plasma concentrations and protected against lethality after injection with either Escherichia coli or Salmonella typhimurium LPS (26). Therefore, contrary to results found in other infection models, during SAg-induced pathology, IL-12 contributes to the sustained systemic release of IFN-γ independently from IL-18. During in vitro stimulation, IL-18 was found to potentely stimulate the production of TNF from non-CD14+human PBMCs, followed by the release of IL-1β, IL-8 and MIP-1α (35). Also, IL-18 has been found to induce the production of GM-CSF and IL-2 (32, 36). Besides its inhibitory effect on IFN-γ release, injection of anti-IL-18 resulted in decreased production of macrophage inflammatory protein-2 (injection of E. coli LPS) and TNF (S. typhimurium LPS) during murine endotoxemia. We also studied whether IL-18 is involved in SEB-stimulated release of cytokines other than IFN-γ at different time points. Administration of anti-IL-18 did not influence SEB-induced peak levels of TNF and IL-12 (data not shown), suggesting that IL-18 is not a central mediator in SAg-related immune responses.

In conclusion, we demonstrate that IL-12 is strongly induced after in vivo administration of SEB in mice, and that IL-12 contributes to the sustained increase in IFN-γ concentrations, without influencing the release of other cytokines like TNF, IL-2 and IL-10. Although IL-12 is a potent stimulator of T cell proliferation, IL-12 did not play a role in the clonal expansion of SEB-reactive Vβ8+ T cells. Contrary to its function in other infection models, IL-18 did not play a costimulatory role in SEB-induced IFN-γ release.

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