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Altered Immune Responses and Susceptibility to *Leishmania major* and *Staphylococcus aureus* Infection in IL-18-Deficient Mice

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IL-18, formerly designated IFN-inducing factor, is a novel cytokine produced by activated macrophages. It synergizes with IL-12 in the induction of the development of Th1 cells and NK cells. To define the biological role of IL-18 in vivo, we have constructed a strain of mice lacking IL-18. Homozygous IL-18 knockout (−/−) mice are viable, fertile, and without evident histopathologic abnormalities. However, in contrast to the heterozygous (+/−) or wild-type (+/+), mice, which are highly resistant to the infection of the protozoan parasite *Leishmania major*, the IL-18−/− mice are uniformly susceptible. The infected IL-18−/− mice produced significantly lower levels of IFN-γ and larger amounts of IL-4 compared with similarly infected +/+ and +/− mice. In contrast, when infected with the extracellular Gram-positive bacteria *Staphylococcus aureus*, the IL-18−/− mice developed markedly less septicaemia than similarly infected wild-type (+/+). However, the mutant mice developed significantly more severe septic arthritis than the control wild-type mice. This was accompanied by a reduction in the levels of Ag-induced splenic T cell proliferation, decreased IFN-γ and TNF-α synthesis, but increased IL-4 production by the mutant mice compared with the wild-type mice. These results therefore provide direct evidence that IL-18 is not only essential for the host defense against intracellular infection, but it also plays a critical role in regulating the synthesis of inflammatory cytokines, and therefore could be an important target for therapeutic intervention. *The Journal of Immunology*, 1999, 163: 2821–2828.

Interleukin 18 is a cytokine produced by monocyctic cells capable of promoting proliferation and IFN-γ production by Th1, CD8+ T, and NK cells in mice and in humans (1). It shares some of the biological activities with IL-12, but without significant structural homology, and serves as a costimulatory factor in the activation of Th1, but not Th2, cells (2). It appears not to drive Th1 cell development, but synergizes with IL-12 for IFN-γ production (3). IL-18 and IL-12 are both produced by activated macrophages. However, unlike IL-12, IL-18 is also expressed in many other cell types, including dendritic cells, keratinocytes, osteoblasts, pituitary gland cells, adrenal cortical cells, intestinal epithelial cells, skin cells, and brain cells (4–10). Recombinant murine IL-18 administration was reported to protect mice against *Crytococcal neoformans* and *Yersinia enterocolitica* infection (11, 12). To determine the direct and definitive biological role of IL-18 in vivo, we constructed a strain of IL-18−/− mice and investigated their phenotype in response to infections with an intracellular protozoan parasite, *Leishmania major*, and an extracellular Gram-negative strain of bacteria, *Staphylococcus aureus*.

Murine leishmaniasis is the functional prototype of polarized Th1 and Th2 cell responses. Most strains of mice infected with *L. major* develop an eventually self-healing lesion. The disease progression can be modified and resistance increased by a variety of immunological manipulations (for review, see Ref. 13). Disease progression and resistance are associated with the preferential development of Th2 cells or Th1 cells, respectively (for review, see Ref. 14). It is generally accepted that in cutaneous leishmaniasis, the preferential induction of IL-12 leads to the expansion of Th1 cells, which activate macrophages to produce large amounts of nitric oxide that mediates the destruction of the parasites in resistant individuals. In contrast, in susceptible patients, the preferential induction of IL-4 leads to the development of Th2 cells secreting IL-4, which inhibits the expression of inducible nitric oxide synthase, thus leading to the unchecked multiplication of the parasites (for reviews, see Refs. 15 and 16). We demonstrate here that in contrast to the heterozygous and wild-type mice, which were resistant to *L. major* infection, the IL-18−/− mice were uniformly susceptible. This was associated with a shift from a Th1 to a Th2 response.

The i.v. injection of mice with an exotoxin-producing strain of *S. aureus* leads to development of severe septic arthritis (17, 18). The articular lesion exhibits synovial hyperplasia, containing neutrophils, macrophages, and a prominent T cell infiltrate, with associated erosion of underlying cartilage and bone. Anti-CD4 or anti-Vβ11 Ab administration to delete superantigen-responsive T cell subsets improves disease outcome following infection (19, 20), confirming a central role of T cell activation in pathogenesis. Moreover, amplification of this T cell response by infection of Vβ3 transgenic mice with a strain of toxic shock syndrome toxin-1 (TSST-1)-producing *S. aureus* lead to significantly enhanced disease severity, with systemic involvement and increased mortality (21). This indicates that regulatory pathways normally operate to
limit immunologically mediated systemic complications following S. aureus infection. Such pathways include the production of glucocorticoids and anti-inflammatory cytokines. We demonstrate here that mice deficient in IL-18 developed markedly reduced sepsicaemia, but with enhanced septic arthritis. This, again, was accompanied by a shift from the Th1 to the Th2 immune response. Together these results show directly the critical role of IL-18 in the host resistance against intracellular parasitic infections and in the modulation of inflammatory responses, suggesting that IL-18 may be an important target for therapeutic interventions.

Materials and Methods

Generation of IL-18−/− mice

IL-18 genomic DNA was screened from a 129/sv mouse genomic library (Stratagene, Cambridge, U.K.), subcloned into pBluescript vector (Stratagene, La Jolla, CA), and characterized by restriction enzyme mapping and DNA sequencing. A targeting construct was made to replace exons 4, 5, and 6 with a bacterial β-galactosidase- and neomycin-resistant genes. Southern blot analysis of tail DNA of offspring from the heterozygous intercross with primer P1 (B) or primer P2 (C), as indicated in A, was performed. D. IL-18 mRNA was not detected in the peritoneal macrophages from homozygous mice by RT-PCR. The RT-PCR product was 397 bp. R, EcoRI restriction sites.

L. major infection in vivo

Groups of homozygous and heterozygous mice (female, 6–8 wk old) were infected in the right hind footpad with 1 × 10^7 stationary phase promastigotes of L. major (LV39). Lesion development was measured at regular intervals with a constant pressure, spring-loaded, dial caliper (Kroeplin, Munich, Germany) and was expressed as the footpad thickness increase in the infected right hind foot compared with that in the uninfected left hind foot. At the end of experiments, mice were sacrificed, and footpads were removed. The number of parasites present in the infected footpad was

FIGURE 1. Disruption of the IL-18 gene. A. The structure of the IL-18 locus, the targeting vector, and the predicted homologous recombination events are shown. A targeting vector was designed to replace about 5 kb of the IL-18 gene, which contains exons 4, 5, and 6 and part of exons 3 and 7. The start codon for IL-18 was also deleted by the replacement with bacterial β-galactosidase- and neomycin-resistant genes. Southern blot analysis of tail DNA of offspring from the heterozygous intercross with primer P1 (B) or primer P2 (C), as indicated in A, was performed. D. IL-18 mRNA was not detected in the peritoneal macrophages from homozygous mice by RT-PCR. The RT-PCR product was 397 bp. R, EcoRI restriction sites.
FIGURE 2. Mice lacking IL-18 failed to control L. major infection. A. Groups of mice were infected in the footpads with 1 \times 10^6 parasites, and lesion development was followed at regular intervals with a dial caliper and expressed as the difference in the thickness of the infected and the uninfected contralateral footpads. Homozygous mice developed progressive and uncontrolled lesions and have significantly higher parasite load at the end of the experiment (day 40) compared with heterozygous control mice. Data are the mean \pm SEM (n = 5). **, p < 0.01 vs heterozygous controls (experiments were terminated on day 40 when the lesion began to ulcerate, as required by Home Office U.K. guidelines). B. Sera were collected from the sacrificed mice and assayed for IL-12 (p40 plus p70), IFN-\gamma, and IL-4 by ELISA. C. Spleen cells were cultured with soluble L. major Ags, and T cell proliferation (3 days culture), IFN-\gamma, and IL-4 in the supernatants (2-day culture) were determined. Data are the mean \pm SEM (n = 5). *, p < 0.05; **, p < 0.01 (compared with heterozygous control). Similar results were obtained with draining lymph node cells.

quantified using a limiting dilution method previously described (23). Draining lymph nodes and spleens were harvested at various times during the infection for determination of in vitro proliferation and cytokine production in response to killed L. major Ags (23). Cytokines in the culture supernatant or in the serum were determined by ELISA using paired Ab (PharMingen, San Diego, CA) as previously described (24). The lower limit of detection in each assay was as follows: TNF-\alpha, 10 pg/ml; IFN-\gamma, 30 pg/ml; IL-4, 40 pg/ml; and IL-12 (p40 and p70), 20 pg/ml.

S. aureus infection

S. aureus LS-1 was originally isolated from a swollen joint of a New Zealand Black/White mouse with spontaneous arthritis (a gift from Dr. T. Bremell, University of Lund, Lund, Sweden) (25). This bacterial strain produces large amounts of TSST-1, coagulase, and catalase. The bacteria were kept frozen at \(-20^\circ\)C in a PBS solution (0.13 M NaCl, 10 mM sodium phosphate (pH 7.4), 5% BSA, and 10% DMSO) until use. Before use, the bacterial solution was thawed and washed in PBS. Viable counting was used to check the number of bacteria as colony-forming units per milliliter by blood agar plate culture. Groups of male homozygous or wild-type mice were injected i.v. with 1 \times 10^7 live bacteria in PBS. Mice were observed daily for up to 7 days; the number of limbs involved was determined, and footpad/intemalleolar diameters were measured with a dial caliper (Kroeplin). An articular index was derived (three or fewer points per limb): 1, erythema alone; 2, swelling and erythema; and 3, erythema, swelling, and extension/loss of function. The severity of septicemia was judged clinically by characteristic changes (24) in coat appearance, posture, presence of spontaneous movement, and mucocutaneous abscess formation, from which a septic index (severity score; maximum score, 12) was derived for each animal by two observers in blinded fashion. Viable S. aureus tissue distribution was estimated by whole organ culture as described previously (21). Kidneys and paws were removed and homogenized before dilution in PBS. Blood (100 \mu l) was also diluted in PBS. Each dilution (50 \mu l) was plated onto prewarmed blood agar and cultured for 18 h at 37°C to determine the number of CFU present, expressed per milliliter of blood, or per whole organ. Serum was also collected and stored at \(-70^\circ\)C until cytokine assay by ELISA. The spleens were removed for determination of proliferation and cytokine production in response to killed S. aureus, TSST-1, or Staphylococcal enterotoxin A (SEA).

Spleen cell proliferation and cytokine production

Splenic single cell suspensions, from L. major-infected or S. aureus-infected mice were cultured at 2 \times 10^6 cells/ml for up to 96 h in RPMI (Life Technologies/BRL, Gaithersburg, MD). The medium was supplemented with 5% FCS and 10 mM HEPES (pH 7.4). After 18 h of culture, spleen cell proliferation was determined by [3H]thymidine uptake and cytokine production by ELISA.
with 10% FCS, 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies/BRL), and 50 μM 2-mercaptoethanol (Sigma). Cells were stimulated with killed S. aureus, SEA, or TSST-1 (last two obtained from Sigma) for S. aureus-infected mice (24). Proliferation assays were performed in triplicate in 96-well culture plates (Nunc, Roskilde, Denmark), with addition of 1 μCi of [3H]thymidine (Amersham, Aylesbury, U.K.) in 25 μl during the final 6 h of culture before harvesting onto a glass-fiber filter (Packard, Wallac, Milton Keynes, U.K.) using a Micromate 196 Harvester (Packard), [3H]Thymidine incorporation was measured using a Matrix 96, direct beta counter (Packard). [3 H]Thymidine incorporation was measured using a Matrix 96, direct beta counter (Packard). 

FACS phenotyping
Splenoc single cell suspensions were washed in serum-free RPMI, and then 10⁵ cells were incubated with 5 μl of conjugated specific anti-CD3(PE), anti-CD4(FITC), anti-CD8(FITC), anti-ThB (FITC), and anti-Pan NK (FITC) Abs (all from Sigma) or normal IgG control (PharMingen, San Diego, CA) for 20 min at room temperature in the dark and then processed as previously described (24).

Statistical analysis
Statistical analysis was performed using Minitab software (Minitab, State College, PA). Analysis was performed using log-rank, χ², Mann-Whitney, Student’s t, or two-tailed log-rank test.

Results
Generation of IL-18⁻/⁻ mice
The IL-18 gene was disrupted by homologous recombination in E14 ES cells with a replacement targeting construct (Fig. 1A). Seven targeted clones were identified among 278 G418- and gancyclovir-resistant clones by Southern blot using 3' external probe, and confirmed by 5' external probe. Southern blot using the neo-
log_{10} higher than those in the heterozygous mice. Experiments were repeated twice by comparing the phenotypes of the mutant and wild-type mice. Again, the IL-18^{+/−} mice were highly susceptible to *L. major* infection compared with the IL-18^{+/+} mice (Fig. 3A). These results show directly that IL-18 plays a critical role in host resistance against the intracellular protozoa parasite, *L. major*.

**Immunological analysis of *L. major*-infected mice**

Serum IL-12 and IFN-γ were measured on day 40 after infection. Serum from the mutant mice contained markedly higher IL-12 (p<0.01 plus p<0.05), but significantly less IFN-γ, than serum from the heterozygous littermates. IL-4 concentrations for both groups were significantly less IFN-γ from the IL-18^{+/−} mice than serum from the *IL-18^{+/+}* mice (data not shown). However, IL-18^{+/−} mice produced significantly more IL-4 than cells from the IL-18^{+/+} mice. In contrast, cytokine levels were generally below detection levels, except for IL-12 which was comparable in the two groups of mice on day 7 after infection (+/+ vs 138 ± 16 vs 83 ± 7 mg; n = 10; p < 0.05; cumulative data for mice 4–14 days after infection). Their spleen cells also produced stronger proliferative responses than those of the mutant mice (Fig. 4A). Spleen cells from the IL-18^{+/−} mice produced significantly less IFN-γ and TNF-α, but more IL-4, IL-5, and IL-10, compared with those from the +/- mice when cultured with killed parasite (Fig. 4). IL-12 was not detectable in the culture supernatants. Serum cytokine levels were generally below detection levels, except for IL-12 which was comparable in the two groups of mice on day 7 after infection (+/+ vs 95.22 ± 48.37; −/−, 104.88 ± 48.87 pg/ml). Thus, the absence of IL-18 predisposes the animals to develop a Th2 phenotype following immunological activation, as illustrated in vivo by the prototypic Th1/Th2 polarization model, murine cutaneous leishmaniasis.

**IL-18^{+/−} mice developed less septicemia but more severe arthritis than intact mice after *S. aureus* infection**

Within 3 days after i.v. *S. aureus* infection, all the wild-type mice showed clinical evidence of septicaemia, which progressed up to 7 days. In contrast, only 28% of the IL-18^{+/−} mice developed sepsicaemia by day 5 after infection (Fig. 5A). Interestingly, despite the lower levels of septicemia, the IL-18^{+/−} mice developed markedly more severe septic arthritis than the control wild-type mice. Clinical signs of arthritis were observed within 2 days of infection. After 7 days, whereas articular inflammation was evident in only 50% of wild-type mice, up to 75% of IL-18^{+/−} mice developed arthritis (Fig. 5B). Moreover, the mean arthritic score was significantly higher in arthritic homozygous mice than in arthritic control mice from day 2 (Fig. 5C).

To determine whether these observations were reflected in altered bacterial distribution and survival in vivo, cultures of blood cultured with killed parasites, and culture supernatants were analyzed for a panel of cytokines. The wild-type mice had significantly larger spleens than the infected mutant mice (138 ± 16 vs 83 ± 7 mg; n = 10; p < 0.05; cumulative data for mice 4–14 days after infection). Their spleen cells also produced stronger proliferative responses than those of the mutant mice (Fig. 4A). Spleen cells from the IL-18^{+/−} mice produced significantly less IFN-γ and TNF-α, but more IL-4, IL-5, and IL-10, compared with those from the +/- mice when cultured with killed parasite (Fig. 4). IL-12 was not detectable in the culture supernatants. Serum cytokine levels were generally below detection levels, except for IL-12 which was comparable in the two groups of mice on day 7 after infection (+/+ vs 95.22 ± 48.37; −/−, 104.88 ± 48.87 pg/ml). Thus, the absence of IL-18 predisposes the animals to develop a Th2 phenotype following immunological activation, as illustrated in vivo by the prototypic Th1/Th2 polarization model, murine cutaneous leishmaniasis.
and tissues were performed. Viable staphylococci were detected in blood on day 3 after infection, but not in paws or kidneys of the wild-type mice. In contrast, bacteria were present in blood and, more strikingly, in paws and kidneys of the mutant mice on day 3 (Fig. 6A). By day 7 the levels of bacteria in the paws and kidneys were comparable in the two strains of mice. The mutant mice, however, showed lower bacterial count in blood than the control mice (Fig. 6B).

**Immunoregulatory effect of IL-18 deficiency**

We next investigated whether the altered clinical phenotype in the IL-18−/− mice was reflected in the immune response following staphylococcal infection. Seven days after infection, spleen cells from the IL-18−/− mice displayed significantly lower proliferative responses to TSST-1 (an exotoxin secreted by the *S. aureus* LS-1) and SEA than cells from the control wild-type mice (Fig. 7A). Culture supernatant of cells from the IL-18−/− mice also produced markedly lower concentrations of IFN-γ, but larger amounts of IL-4, compared with cells from wild-type mice (Fig. 7, B and C). Interestingly, spleen cells from the infected mutant mice also produced significantly less TNF-α compared with cells from the control mice when stimulated with the superantigens TSST-1 and SEA (Fig. 7D).

We then compared the kinetics of cytokine production in the mutant and wild-type mice following *S. aureus* infection. Groups of mice were sacrificed on days 1, 2, 3, and 7 after infection, and their production of cytokines was analyzed by ELISA. Sera from the −/− mice contained significantly lower concentrations of IFN-γ and TNF-α than those from the +/+ mice. IL-12 concentrations were comparable in the two groups of mice (Fig. 8). IL-4 and IL-10 were below detectable levels. Spleen cells were cultured with graded numbers of heat-killed bacteria. Cells from the mutant mice consistently produced less IFN-γ, TNF-α, and IL-6 than those from the +/+ mice (Fig. 9). IL-4, IL-10, and IL-12 were not detected in the culture supernatants of both groups of mice.

**Discussion**

The results presented here provide direct evidence that IL-18 plays a critical role in the host resistance against an intracellular infection. In contrast to intact mice, IL-18-deficient mice were highly susceptible to *L. major* infection, with uncontrolled disease progression. This was accompanied by depressed Th1, but enhanced Th2, cell response. This is consistent with recent reports that IL-18 is a costimulator of Th1 and NK cell activities (1–3, 26). Furthermore, we have recently demonstrated that IL-18R is selectively expressed on murine Th1, but not Th2, cells (27). It is intriguing that despite their inability to control the infection, infected IL-18−/− mice produced markedly more IL-12 than similarly infected control mice 40 days after infection. These results, therefore, demonstrate that IL-18 has a direct and profound effect on the activation and development of Th1 cells in vivo. This is consistent with a recent report that IL-18 may be involved in the negative regulation of IL-12 production (12). Importantly, our results also demonstrate that IL-12 alone, in the absence of IL-18, is insufficient for the induction of Th1 cell expansion in vivo. We have recently demonstrated that IL-18 could up-regulate the expression of the
IL-12Rβ2 gene (27). The enhanced levels of IL-12 produced by the infected IL-18−/− mice could be the consequence of stronger antigenic stimulation of macrophages from increased parasite load. This is in line with our observation that the levels of serum IL-12 are comparable in IL-18−/− and IL-18+/+ mice up to 14 days of infection.

Northern blot analysis also showed that spleen cells from the two groups of mice expressed similar levels of IL-12Rβ2 mRNA up to 40 days after infection with L. major (data not shown). This is consistent with an earlier report that IL-18-deficient mice could respond to IL-12 (26). IL-18 was thought to play a secondary role to IL-12 in the induction of Th1 cell development because, unlike IL-12, it was unable to drive Th1 cells expansion in vitro (2, 3). However, our results indicate that in the absence of IL-18, IL-12 alone is insufficient for the full development of Th1 cells in vivo. It should, however, be noted that the lymphoid cell population from naive IL-18−/− mice appeared to have the same normal Th1/Th2 ratio as the intact mice. Spleen cells from the two strains of uninfected mice proliferated and produced comparable levels of IFN-γ and IL-4 in response to a T cell mitogen (Con A) or polyclonal activation by anti-CD3 Ab. IL-18, therefore, enhances Th1 cell differentiation only in the presence of antigenic stimulation.

It is also of considerable interest that the IL-18-deficient mice developed significantly less septicemia but more severe septic arthritis than similarly infected intact mice. This was surprising because, compared with wild-type mice, the mutant mice developed a markedly reduced Th1 response, which has been frequently associated with septic arthritis (20, 28, 29). The reduced septicemia could well be due to the decreased TNF-α synthesis in the IL-18−/− mice, because this cytokine has been implicated to have a direct effector role in the pathogenesis of Gram-positive bacterial shock (30, 31). Our results are consistent with a recent report showing that IL-18 could up-regulate TNF-α synthesis by human PBMC (32). The mechanism by which IL-18 influences TNF-α synthesis, however, remains to be addressed. The more severe septic arthritis observed in the IL-18−/− mice could be due to the higher initial bacterial loads in the paws compared with those in wild-type mice. The higher bacterial load in the mutant mice, in turn, could be the consequence of higher concentration of IL-4 produced by these mice compared with the intact mice. It has been recently reported that IL-4 and IL-4-dependent Th2 responses promote septic arthritis and sepsis-related mortality by inhibition of bacterial clearance during S. aureus infection (33).

In conclusion, we have demonstrated directly the crucial role of IL-18 in host resistance against intracellular L. major infection and in the septicemia and septic arthritis of extracellular Gram-positive S. aureus infection. Furthermore, IL-18 plays a critical role in the in vivo regulation of Th1 and Th2 balance, which frequently determines the outcome of many important infectious and autoimmune diseases. The role of IL-18 in other infections and immunopathology can now be addressed by direct experimentation.

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References


