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Staphylococcus aureus-Induced Septic Arthritis and Septic Death Is Decreased in IL-4-Deficient Mice: Role of IL-4 as Promoter for Bacterial Growth

Olof Hultgren,* Manfred Kopf,† and Andrzej Tarkowski*

Lack of IL-4 has been shown to be protective in some experimental models of infectious diseases in mice such as cutaneous leishmaniasis. At the same time IL-4, together with other Th2 cytokines, including IL-10 and IL-13, is known as an anti-inflammatory cytokine with the potential to down-regulate proinflammatory cytokine production. To investigate the role of IL-4 in experimental Staphylococcus aureus-induced and T lymphocyte-mediated arthritis, IL-4-deficient C57BL/6 mice (IL-4−/−) and their congenic controls (IL-4+/+) were inoculated with a toxic shock syndrome toxin-1-producing S. aureus strain. In IL-4+/+ mice, arthritis peaked 14 days after bacterial inoculation, whereas, at that time, IL-4−/− mice displayed significantly less frequent (p < 0.05) joint inflammation. Paralleling lower frequency of arthritis, IL-4-deficient mice showed a decreased bacterial burden in joints (p = 0.014) and kidneys (p = 0.029), as well as lower infection-triggered weight decrease and mortality. In vitro, IL-4 inhibited intracellular killing of S. aureus in infected macrophages, without affecting phagocytosis. This finding may explain the enhanced staphylococcal clearance observed in IL-4−/− mice in vivo. Our results suggest 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. The Journal of Immunology, 1998, 160: 5082–5087.

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We have recently described a murine model of hematogenously induced S. aureus infection (7), causing arthritis and mortality in animals injected i.v. with a toxic shock syndrome toxin-1 (TSST-1)-producing S. aureus strain originating from a spontaneously arthritic NZB/W mouse (8). Using this model, we have shown the disease to be T and B cell dependent and superantigen mediated (9–12). We have further used in situ hybridization technique to study cytokine mRNA expression after induction of septic arthritis. This study revealed early up-regulation of Th2 cytokines, including IL-4, locally in the joint (13). IL-4 is a central cytokine in T cell development. In the presence of IL-4, primed CD4+ T cells develop into Th2 cells (14, 15), which produce a polarized pattern of cytokines such as IL-4, IL-5, IL-10, and IL-13. IL-4-deficient mice display impaired Th2 development and related effector responses, including eosinophilia (16). In the early phase of infection, IL-4 can be produced by mast cells/basophils, eosinophils (17), and CD3+CD4+ NK1.1+ lymphocytes (18), and later by Th2 cells. Because of their anti-inflammatory properties, IL-4 and IL-10 have been suggested as possible treatment modality in Th1-dominated autoimmune disease (19–21). The aim of our study was to assess the role of IL-4 in the septic arthritis model, both regarding development of the arthritic process and host susceptibility to bacteria. For this purpose, we used C57BL/6 wild-type mice and C57BL/6 mice with disrupted IL-4 gene.

Materials and Methods

Mice, bacteria, and infection

Inbred male, 8- to 12-wk-old C57BL/6 mice, intact or defective with respect to IL-4 gene, were used throughout the study. Procedure of IL-4 gene disruption has been described in detail elsewhere (16). The mice were maintained in the animal facility of the Department of Rheumatology, University of Göteborg, Göteborg, Sweden. They were kept under standard conditions of temperature and light and fed standard laboratory chow and water ad libitum. S. aureus strain LS-1 was originally isolated from a swollen joint of a spontaneously arthritic NZB/W mouse. Before each experiment, bacteria were cultured on blood agar (5% human erythrocytes) for 24 h and then reincubated on blood agar for another 24 h. A bacterial
solution was prepared in PBS at a concentration of 5 × 10^7 (experiments 1, 2, and 4) or 1 × 10^6 bacteria/ml (the third experiment). Two hundred microliters of the solution were injected into one of the tail veins on day 0. Viable counts were used to check the concentration of bacteria injected.

**Clinical evaluation of arthritis and weight**

All mice were labeled and monitored individually. Limbs were inspected by two blinded observers (O.H. and A.T.) at regular intervals (3, 7, 14, and 21 days). Arthritis was defined as visible erythema and/or swelling of at least one joint. To evaluate the intensity of arthritis we used a clinical scoring in which macroscopic inspection yields a score of 0 to 3 for each paw (0 = normal; 1 = mild swelling and/or erythema; 2 = moderate swelling and erythema; 3 = marked swelling and occasionally ankylosis), resulting in an arthritis score ranging from 0 to 12 for each individual mouse. Arthritic index was constructed by a summation of scores from all four limbs in each mouse divided by the number of animals in each experimental group as previously reported (22). The weight was checked at days 0, 5, 7, 14, and 21. At day 21 all the mice were sacrificed.

**Experimental protocol**

In the first experiment we used 11 IL-4 knockout mice and 15 wild-type littermates. Twenty-one days after the bacterial inoculation all the surviving mice were bled and sacrificed. The left limbs were chosen for histopathologic examination and the right ones for evaluation of bacterial growth. The kidneys were also subjected to bacterial examination. In the second experiment, nine IL-4 knockout mice and nine wild-type littermates were used. In this experiment, the mice were bled 3 days after bacterial inoculation. At the time of sacrifice, at day 21, the right limbs were chosen for histopathologic and the left ones for immunohistochemical examination. The third experiment was performed with 15 IL-4−/− mice and 18 wild-type controls. Mice were bled and sacrificed 3 wk after bacterial inoculation. Kidneys were removed to study bacterial growth. A fourth experiment was performed to assess bacterial load in blood, spleen, liver, and kidneys. At days 1 (blood), 2, 6, and 12, mice (4–10 per group) were sacrificed. In addition, sera were collected at day 12 for analysis of NO3.

**Determination of bacterial load**

Bacterial samples from talocrural and radiocarpal joints were obtained using charcoal sticks. The bacterial presence was defined if 15 or more CFU per joint were found. Both kidneys were removed aseptically, placed on ice, homogenized, and diluted in 10 ml PBS. Viable counts were done to examine bacterial concentration. Colonies from every plate were then tested for catalase and coagulase, and with a Staphaurex kit (Murex Diagnostics, Dartford, U.K.).

**Histopathologic examination**

Limbs were fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin. Tissue sections were prepared and stained with hematoxylin and eosin. Sections were examined by a blinded observer with regard to synovial hypertrophy (membrane thickness of more than two cell layers), pan-nus formation (joint cartilage covered with synovial tissue), and destruction of cartilage and bone.

**Immunohistochemical analysis**

After the mice were killed the paws were removed and demineralized by a procedure detailed in an earlier report (23). The demineralized specimens were then embedded (Tissue-Tek, Miles, Elkhart, IN), frozen in isopentane prechilled by liquid nitrogen, and kept at −70°C until cryosectioned. Sections 6 μm thick were cut frontally to permit simultaneous inspection of most of the joints within the paw. All sections were fixed in cold acetone for 5 min, washed in PBS, and depleted of endogenous peroxidase by treatment with 0.3% H2O2 for 5 min. After additional washing in PBS, the sections were incubated overnight in a humid atmosphere at 4°C with unlabeled primary rat mAbs at appropriate dilutions. Biotin-labeled rabbit anti-rat Ig diluted in PBS-BSA were used as secondary Abs (Vector Laboratories, Burlingame, CA). Binding of biotin-labeled secondary Abs was detected by stepwise incubation with avidin-biotin-peroxidase complexes and 3-amin-9-ethylcarbazole containing H2O2. All sections were counterstained with Mayer's hematoxylin. The primary Abs used were: M1/70 (Mac-1, specificity; C3bi receptor; target cells: granulocytes, macrophages, NK-cells), H129.19 (specificity; CD4) and 53.6.7 (specificity; CD8). All the Abs were kindly provided by Dr. R. Holmdahl (Lund, Sweden). The stained cells were counted to evaluate the predominant cell type present in synovial tissue.

**Cytokine analysis**

For the measurement of IL-6, a murine hybridoma cell line B13.29, subclone B9, was selected for its IL-6 dependency. In our experimental model, we have recently shown that in response to septicemic doses of S. aureus IL-6 knockout mice express only background serum IL-6 levels (≤50 pg/ml) using the same assay system (O. Hultgren et al., unpublished data). For the assay, B9 cells were harvested by centrifugation, and after one wash in IL-6-free medium, they were seeded at 5 × 10^6 cells into microtitre plates (Nunc, Roskilde, Denmark) in the presence of serum samples and grown for 68 h before adding [3H]thymidine in order to study proliferative responses to IL-6. After 4 h, the cultures were harvested onto filters and counted in a beta counter. Mouse IL-6 (Genzyme, Cambridge, MA) was used as a standard. The serum samples and standards were all set up in triplicates.

IFN-γ levels were measured by an ELISA. Microtiter plates were coated overnight at 4°C with 2 μg/ml of rat anti-mouse IFN-γ mAb (PharMingen, San Diego, CA) dissolved in sodium bicarbonate, pH 9.6. Blocking was made with 1% BSA dissolved in 0.05 M Tris, pH 7.4, for 1 h. Mouse IFN-γ (Genzyme) was used to create a standard curve. Biotinylated rat anti-mouse IFN-γ, 2 μg/ml (PharMingen), was used as a catching Ab. The plates were incubated overnight at 4°C, washed, and further incubated with ExtrAvidin alkaline phosphatase 0.5 μg/ml and alkaline phosphatase substrate 1 mg/ml (Sigma). TNF-α levels were measured in the same way using 1.25 μg/ml mAb rat anti-mouse TNF-α, recombinant mouse TNF-α, and 0.5 μg/ml biotinylated rat anti-mouse TNF-α (PharMingen).

**Phagocytosis and intracellular killing**

Intrapertoneal macrophages from noninfected mice were extracted, adjusted to 2 × 10^6 cells/ml and incubated in a 24-well plate (Nunc) according to an earlier detailed procedure (25, 26). Adherent macrophages were incubated with 500 μl of S. aureus at a concentration of 5 × 10^6 bacteria/ml for 50 min at 37°C, and subsequently washed three times in Iscove’s medium. The macrophage content of bacteria was then measured after two incubation intervals, 0 and 4 h, to study phagocytosis and intracellular (IC)-killing capacity, respectively. To avoid extracellular bacterial growth in the 4-h plate, incubation medium contained 10 μg/ml of gentamicin. Antibiotics were washed away before lysing macrophages with distilled water. The impact of IL-4 on phagocytosis and IC killing was examined by preincubation of macrophages with varying concentrations of IL-4 (0, 0.2, 2, and 20 ng/ml) during 12 h, followed by wash and then exposure to S. aureus. IL-4 was obtained from a transfected cell line grown in antibiotics-free medium.

**Determination of nitrate**

Serum levels of nitrate, one of major metabolites of NO, was determined with a stable isotope (K15NO3) dialysis assay, utilizing positive ion-chemical ionization, gas chromatography-mass spectrometry, after conversion of endogenous and labeled nitrate in the samples to nitrobenzene (27). As an internal standard, a defined volume of serum was added with a known amount of K15NO3. The detection limit for endogenous nitrate was 0.1 nmol/ml.
involved, compared with the control animals, resulting in a higher clinical arthritis in affected joints and a higher number of joints that arthritic mice in the IL-4-deficient group had a more severe defective with respect to IL-4 gene. Mice (24 IL-4+/+; 20 IL-4−/−) were i.v. injected with 1 × 10⁷ TSST-1-producing S. aureus strain LS-1 at day 0. Limbs were inspected at regular intervals (at days 3, 7, 14, and 21).

Statistics
The t test, Mann-Whitney U test, χ² test with Yates' correction, and Fisher's exact test were used. Values are expressed as mean ± SEM.

Results
IL-4 deficiency decreases frequency of Staphylococcus aureus-induced arthritis
C57BL/6 mice defective (IL-4−/−) or intact (IL-4+/+) with respect to IL-4 gene were injected i.v. with 1 × 10⁷ S. aureus, and the clinical course of disease was followed for 3 wk. Two weeks after bacterial inoculation, at the peak of the inflammatory response (24), mice with defective IL-4 gene displayed significantly lower frequency of arthritis than wild-type controls (40% vs 76%, Fig. 1). However, comparing the two groups of animals, only minor differences were found with respect to 1) the severity of arthritis measured by arthritic index (1.0 ± 0.2 in IL-4−/− vs 1.2 ± 0.2 in IL-4+/+ mice; ns); 2) the cellular composition (expression of Mac-1⁺, CD4⁺, and CD8⁺ cells) of the synovium; and 3) the frequency of synovial hypertrophy and erosivity (60% in IL-4−/− vs 57% in IL-4+/+ mice, ns). These findings may be explained by the fact that arthritic mice in the IL-4-deficient group had a more severe clinical arthritis in affected joints and a higher number of joints involved, compared with the control animals, resulting in a higher index per arthritic animal, 2.4 ± 0.5 vs 1.6 ± 0.2; ns. The beneficial effect on the frequency of arthritis in IL-4−/− mice was overcome by exposure to a higher, septicemic dose of bacteria (2 × 10⁷ per mouse).

Weight loss and mortality rate is reduced in IL-4−/− mice
During the first week of infection, IL-4+/+ mice showed a substantial weight loss, which reached the maximum of 16% of body weight at day 7 (Fig. 2A). In parallel, a substantial number of IL-4−/− mice succumbed to infection during the first 2 wk (Fig. 2B). The mortality rate in this group was 17% (7/42). In contrast, 7 days after bacterial inoculation IL-4−/− mice showed both a reduced weight loss and mortality rate with a maximum of 5% and 3% (1/35), respectively. While weight loss was significantly decreased in IL-4-deficient mice throughout the experiment (p < 0.05 and p = 0.01), mortality rate was not (ns). Notably, the differences between groups become measurable immediately after inoculation indicating that early IL-4 response plays a detrimental role.

Enhanced bacterial clearance in IL-4−/− mice
Three weeks after inoculation, we determined the bacterial burden in joints and kidneys of infected mice. Figure 3A shows that 50% of IL-4−/− mice contained live bacteria in their joints, whereas joints of IL-4−/− mice were free of bacteria (p = 0.014). Bacterial growth in joints showed the same pattern in earlier stages of the infection. At 6 days after the inoculation IL-4-deficient mice did not show any bacterial growth in their joints. In contrast, 25% of wild strain mice displayed staphylococcal growth. Similar relationship was observed at day 12 (56 vs 70%). In addition, bacterial growth was less common in more than one joint in the IL-4 knockout mice as compared with wild-type mice (median CFU 30,000 vs 120,000; p = 0.029) (Fig. 3B). Staphylococcal growth in kidneys analyzed at days 2, 6, and 12 showed throughout lower number of bacteria in IL-4 knockout mice compared to wild-type controls (median: day 2, 12,000 vs 31,500; day 6, 110,000 vs 4,509,500; and day 12, 224,000 vs 702,500). Due to wide variation of the above results, no significant differences were found. Bacteria were not found in blood, spleen, or liver at day 6 postinoculation. No differences with respect to the number of bacteria were seen in blood at days 1 and 2 (results not shown). Spleen tissues displayed

![FIGURE 1. Frequency of S. aureus arthritis in C57BL/6 mice, intact or defective with respect to IL-4 gene. Mice (24 IL-4+/+; 20 IL-4−/−) were i.v. injected with 1 × 10⁷ TSST-1-producing S. aureus strain LS-1 at day 0. Limbs were inspected at regular intervals (at days 3, 7, 14, and 21).](https://example.com/figure1)

![FIGURE 2. A. Weight change in IL-4+/+ and IL-4−/− mice after inoculation of 1 × 10⁷ S. aureus. The weight was checked at days 0, 3, 7, 14, and 21. Each group consisted of 9 mice. B. Cumulative mortality rates in IL-4 knockout (n = 35) and wild-type (n = 42) mice.](https://example.com/figure2)

![FIGURE 3. Enhanced bacterial clearance in IL-4−/− mice. Three weeks after inoculation, we determined the bacterial burden in joints and kidneys of infected mice. Figure 3A shows that 50% of IL-4−/− mice contained live bacteria in their joints, whereas joints of IL-4−/− mice were free of bacteria (p = 0.014). Bacterial growth in joints showed the same pattern in earlier stages of the infection. At 6 days after the inoculation IL-4-deficient mice did not show any bacterial growth in their joints. In contrast, 25% of wild strain mice displayed staphylococcal growth. Similar relationship was observed at day 12 (56 vs 70%). In addition, bacterial growth was less common in more than one joint in the IL-4 knockout mice as compared with wild-type mice (median CFU 30,000 vs 120,000; p = 0.029) (Fig. 3B). Staphylococcal growth in kidneys analyzed at days 2, 6, and 12 showed throughout lower number of bacteria in IL-4 knockout mice compared to wild-type controls (median: day 2, 12,000 vs 31,500; day 6, 110,000 vs 4,509,500; and day 12, 224,000 vs 702,500). Due to wide variation of the above results, no significant differences were found. Bacteria were not found in blood, spleen, or liver at day 6 postinoculation. No differences with respect to the number of bacteria were seen in blood at days 1 and 2 (results not shown). Spleen tissues displayed](https://example.com/figure3)
Specific Ab responses against *S. aureus* are decreased in IL-4−/− mice

Specific Ig responses were measured to both TSST-1 and *S. aureus* cell wall Ags. As shown in Table I, in IL-4−/− the Ag-specific IgG responses were reduced by 35 to 55% (p < 0.05), whereas IgM responses were largely unaffected. Analysis of IgG isotypes revealed that IL-4−/− mice showed a five- and twofold reduction in IgG1 response against TSST-1 and *S. aureus* cell wall, respectively. In contrast, the IgG2a levels were increased threefold in response to TSST-1 and reduced slightly against *S. aureus* cell wall in IL-4−/− mice (data not shown). The switch to IgG1 can apparently be overcome to some extent in IL-4-deficient mice during *S. aureus* infection. This could be due to another cytokine, e.g., IL-13, or other cytokine-independent signals.

**IL-4 deficiency does not give rise to up-regulation of IFN-γ responses**

As previously demonstrated, IL-4−/− mice display typically impaired Th2 and up-regulated Th1 cytokine responses (16). Administration of IFN-γ, a Th1 cytokine, before or 3 days after bacterial inoculation, has been shown to ameliorate the outcome of *S. aureus* infection (28). Thus, it was of interest to assess the levels of IFN-γ in IL-4−/− mice during infection with *S. aureus*. At day 3 after bacterial inoculation, circulating IFN-γ was detectable in only 2 of 11 IL-4-deficient mice and in none of the controls. Three weeks later, at sacrifice, IL-4−/− mice had somewhat lower serum levels of IFN-γ compared with wild-type controls (1068 ± 104 vs 1558 ± 405 U/ml), which might simply indicate a milder course of infection. Serum levels of the proinflammatory cytokine IL-6 were similar in both groups, 1516 ± 590 vs 1558 ± 619 U/ml, which might simply indicate a milder course of infection. This could be due to another cytokine, e.g., IL-12, or other cytokine-independent signals.

**Preincubation of macrophages with IL-4 decreases bactericidal capacity**

Intrapitoneal macrophages from noninfected IL-4−/− and IL-4+/+ mice were extracted to study their efficiency with respect to phagocytosis and IC-killing of *S. aureus*. No differences could be seen either in phagocytosis (IL-4−/−: 3300 ± 199 bacteria/105 macrophages vs IL-4+/+: 2970 ± 172) or in IC-killing (viable bacteria, IL-4−/−: 186 ± 66 vs IL-4+/+: 154 ± 33). Since macrophages are not able to produce IL-4, we preincubated macrophages for 12 h with different concentrations of IL-4 (0.2, 2, and 20 ng/ml) prior to exposure to *S. aureus*. Phagocytosis was not influenced by preincubation with IL-4. In contrast, the IC killing of bacteria (Fig. 4) was significantly down-regulated by addition of extrinsic IL-4. This effect was most pronounced in relatively high concentrations of IL-4. However, even low concentrations of IL-4 (200 pg/ml) was enough to result in a twofold decrease of *S. aureus* killing (p < 0.05). This decreased IC-killing capacity was seen in both IL-4−/− and IL-4+/+ macrophages preincubated with IL-4.

**Discussion**

Utilizing IL-4-deficient mice, we studied the importance of IL-4 in *S. aureus*-induced sepsis and septic arthritis. The clinical benefit of
IL-4 absence on the course of infection by hematogenously spread *S. aureus* is shown with regard to both survival and development of septic arthritis. In addition to the clinical observations, an enhanced bacterial clearance from both joints and kidneys was found in IL-4−/− mice compared with wild-type mice. Surprisingly, we found somewhat higher serum levels of IFN-γ, a Th1 cytokine, in wild-type mice than in IL-4−/− mice. We believe that these somewhat unexpectedly high IFN-γ levels in IL-4−/+ mice mirror simply a considerably more severe infection/inflammation process in vivo.

What is the mediator of the harmful effects of IL-4 on the infection and inflammation with superantigen-producing staphylococci? One potential possibility could be that IL-4, a Th2 cytokine with potent B lymphocyte-differentiating properties, would increase autoantibody production and hence development of immune complex-mediated arthritis. Indeed, our results show significantly higher IgG levels in IL-4−/+ mice in comparison with IL-4−/− congenic strain. In addition, we have previously demonstrated that during the course of *S. aureus* arthritis there will be development of hypergammaglobulinemia and autoantibody production to, e.g., collagen type II and Fc fragment of IgG (i.e., rheumatoid factors) (24); both of these Abs are known to participate in the arthritic process (29). Further support for this hypothesis is provided by our recent study indicating that mice with X-linked immunodeficiency, which are unable to produce IL-4 on their own, have a similar capacity for phagocytosis and intracellular killing of staphylococci, exposure to even a low concentration of extrinsic IL-4 will result in a significant and dose-dependent inhibition of intracellular bactericidal capacity. The latter finding could have been due to the known property of IL-4 to decrease NO production, as shown previously in case of infection with *Candida albicans* (30). Indeed, NO plays an important role also in the control of *S. aureus* infections, since treatment of mice with NO synthase inhibitors resulted in more frequent and severe *S. aureus*-induced arthritis along with higher mortality rates (31). However, no difference could be detected between groups with regard to serum NO3 levels, indicating either 1) influence of IL-4 on NO-independent bactericidal macrophage activities or 2) local differences in NO production presumably not measurable in serum.

The early and profound weight decrease by IL-4+/* as compared to IL-4−/* mice following inoculation of staphylococci indicates that a rapid IL-4 response might be determining the severe outcome of infection. Indeed, in a recent study we reported the highest IL-4 transcript levels in synovia found day 2 after *S. aureus* inoculation and then declining (13). A possible source for this early IL-4 production in synovia are mast cells situated in peritendinous tissue (32). In contrast to synovium, virtually no IL-4 mRNA-expressing cells could be detected in the spleen after the onset of *S. aureus* infection, indicating that other tissues may be responsible for early systemic IL-4 production. Although virtually every organ contains mast cells, they are particularly abundant in lung, skin, and the gastrointestinal tract. The high content of mast cells in pulmonary tissue (33, 34) could definitely be of importance, since lung is one of the first organs exposed to bacteria after i.v. inoculation.

In noninfectious models of arthritides, such as collagen II-induced arthritis, IL-4 facilitates natural remission of disease (35). In analogy to collagen II-induced arthritis, administration of IL-4 suppresses the destructive phase of streptococcal cell wall arthritis, another model of septic joint disease (36). In our model of septic arthritis, IL-4, rather than preventing disease, gave rise to more frequent disease manifestations. However, despite the significantly increased frequency of arthritis, the severity of disease did not increase in IL-4+/+ mice in comparison with IL-4−/− mutants. Considering the fact that the joints of IL-4+/+ mice are exposed to a higher number of highly destructive staphylococci, without developing a more severe arthritis compared with IL-4−/− mice, one may argue that IL-4 per se exerts anti-inflammatory properties in the disease process, in agreement with observations from noninfectious arthritides.

In conclusion, we propose that IL-4 plays a detrimental role in the immune response to *S. aureus* infection causing a more severe systemic infection and, consequently, a higher mortality rate. IL-4 promotes the development of septic arthritis by enhancement of bacterial growth and/or decrease of clearance, which eventually results in an increased bacterial load in joint tissue.

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