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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.

Bacterial arthritis is a rapidly progressive and highly destructive joint disease in humans with an incidence rate ranging from 0.034 to 0.13% (1). The typical case of this disease is characterized by high fever and a red, warm, and swollen joint, in 80 to 90% of cases displaying a monoarticular pattern. Rheumatoid arthritis (RA)3 is the most common predisposing factor for septic arthritis, present in 10 to 40% of septic arthritis cases (2–4). The reasons for that include previous joint destruction and immunosuppressive drug therapy prevalently used in RA patients. All patients with primary or secondary immunodeficiencies as well as those who have passed through prosthetic joint surgery display signs of RA arthritis (1, 4, 5). The major human pathogen in septic arthritis is hematogenously spread Staphylococcus aureus, causing 75% of cases in RA patients (6). Despite medication with antibiotics and, hence, eradication of bacteria, complete recovery is seen only in a minority of patients (4). This might be due to continuous course of joint inflammation due to autoreactive phenomena. Therefore, it is of interest to better understand the host pattern of immune responsiveness to the infectious agent.

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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 × 10^8 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 arthritic 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, 3, 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 immunohistochimica 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 synovial tissue.

**Phagocytosis and intracellular killing**

Intaperitoneal macrophages from noninfected mice were extracted, adjusted to 2 × 10^7 cells/ml and incubated in a 24-well plate (Nunc) according to an earlier detailed procedure (25, 26). Adherent macrophages were incubated 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 iIFN-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).

**Determination of bacterial load**

Bacterial samples from talocural 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-

**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 detected 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-amino-9-ethylcarbazole containing H2O2. All sections were counterstained with Mayer’s hematoxylin. The primary Abs used were: M170 (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.

**Determination of nitrate**

Serum levels of Abs to S. aureus cells were measured by an ELISA using 10 μg/ml methylated BSA (Sigma, St. Louis, MO) to coat wells and 50 μl of whole, formalin-treated (1%, 20 min) S. aureus LS-1 cells (10^8/ml) to coat the wells. All the sera were serially diluted in PBS-BSA (0.5%) and incubated in wells. To measure the level and class specificity of Abs bound to the solid phase, affinity-purified and biotinylated F(ab')2 fragments of goat anti-mouse IgG and IgM (The Jackson Laboratory, Bar Harbor, ME) diluted 1:3000 in PBS-TWEEN-20 were added, followed by stepwise incubation with 0.5 μg/ml of avidin-biotin-peroxidase (Sigma) and 2.5 mg/ml of the enzyme substrate 2,2-azino-bis(3-ethylbenzothiazoline sulfonylic acid) (Sigma) in citrate buffer (pH 4.2) containing 0.0075% H2O2. The absorbance was measured in a spectrophotometer (Labsystems Multiskan, Chicago, IL). All OD values were converted to Ag-specific arbitrary units with calibration curves based on the OD values obtained from serial dilutions of a reference pool. IgG1 and IgG2a levels of Abs to S. aureus cells and TSST-1 were measured by an ELISA technique. Coating and blocking procedure are described in detail elsewhere (12, 24). Goat anti-mouse IgG1 and IgG2a were purchased from Sigma and biotin-conjugated rabbit anti-goat from Cooper Biomedical (Malvern, PA).
Index per arthritic animal, 2.4 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 1 defective with respect to IL-4 gene. Mice (24 IL-4−/−; 20 IL-4+/+) were i.v. injected with 1 × 10^7 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 S. 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 × 10^7 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 × 10^7 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 became measureable 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 than in the controls (11 vs 40%). Similarly, 3 wk after inoculation staphylococcal growth in kidneys of IL-4−/− mice was reduced 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
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 ± 274 pg/ml in IL-4−/− and 1435 ± 219 in wild-type littermates, whereas serum TNF-α levels remained undetectable.

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

Intraperitoneal 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.

Twelve days postinoculation, serum levels of nitrate, a metabolite of NO, did not appear to differ between IL-4-deficient mice and wild-type controls (48.0 ± 6.7 vs 49.6 ± 4.9 nmol/ml; ns).

**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, another model of aseptic 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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