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

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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)\(^{1}\) 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 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 also enhanced susceptibility to septic 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.

1 Abbreviations used in this paper: RA, rheumatoid arthritis; TSST-1, toxic shock syndrome toxin-1; IC, intracellular; ns, not significant; NO, nitric oxide.
solution was prepared in PBS at a concentration of 5 × 10³ (experiments 1, 2, and 4) or 1 × 10⁶ 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 (25). 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 immunohistochemical examination. The third experiment was performed with 15 IL-4−/− mice and 18 wild-type controls. Mice were bled and sacrificed 2 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 harvested 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. All tissue 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 precooled 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 monitored using a 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-horseradish peroxidase (Sigma) and 2.5 mg/ml of the enzyme substrate 2,2'-azino-bis(3-ethylbenzothiazoline sulfonic 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 IgG and IgG2a were purchased from Sigma and biotin-conjugated rabbit anti-goat from Cooper Biomedical (Malvern, PA).

Cytokine analysis

For the measurement of IL-6, a murine hybridoma cell line B13.29, subclone 21, 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 × 10⁵ cells into microtiter plates (Nunc, Roskilde, Denmark) in the presence of serum samples and grown for 68 h before adding [H]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 rIFN-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

Intrapertioneal macrophages from noninfected mice were extracted, adjusted to 2 × 10⁶ 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 × 10⁶ 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) dilution 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 (Sigma). The detection limit for endogenous nitrate was 0.1 nM/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 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 × 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 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 mice compared to 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
In contrast, the IgG2a levels were increased threefold in IL-4−/− mice against TSST-1 and S. aureus cell wall in IL-4−/− 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−/− 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−/− 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 ± 6.7 vs 49.6 ± 4.9 nmol/ml; ns).

**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.

**Table 1. Serum levels of Abs to S. aureus cell walls, and to TSST-1 in C57BL/6 mice congeneric for IL-4, 3 wk after inoculation with TSST-1-producing S. aureus LS-1 strain**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Anti-TSST-1</th>
<th>Anti-S. aureus Cell Wall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>IL-4+/+</td>
<td>11,927 ± 3,073</td>
<td>11,216 ± 1,642</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>ns</td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>5,514 ± 1,955</td>
<td>10,580 ± 1,824</td>
</tr>
</tbody>
</table>

* Mice, intact or defective with respect to IL-4 gene, were inoculated i.v. with 1 × 10⁷ S. aureus. Surviving mice (IL-4+/+, n = 12; IL-4−/−, n = 11) were sacrificed after 3 wk. Blood samples were obtained, and serum levels of anti-S. aureus cell wall and anti-TSST-1 Abs were determined. Values are expressed as arbitrary units per milliliter (mean ± SEM).
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 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, giving rise to a profound B lymphocyte defect, display less frequent and less severe *S. aureus* arthritis in comparison with their congenic controls.

However, the explanation above does not explain the bacterial findings. The decreased frequency of septic arthritis in IL-4−/− mice is seen together with fewer live *S. aureus* in examined organs. Low frequency of arthritis in IL-4−/− mice might therefore be due to either a decreased number of bacteria reaching the joints as a consequence of increased systemic bacterial clearance, improved clearance of bacteria in situ (i.e., within the joint cavity), or a combination of these phenomena. The size of the bacterial inoculate is an important parameter for the development of septic arthritis (8). In this respect, reduction of the inoculum size by two-thirds in C57BL/6 wild-type mice would diminish arthritis down to 40%, a frequency similar to that found in IL-4−/− mice given a standard number of staphylococci. We investigated both the staphylococcal uptake and intracellular killing capacity mediated by phagocytic cells. Our results clearly show that IL-4 dose dependently augmented bacterial growth in macrophages in vitro (Fig. 4), without affecting phagocytosis. Whereas macrophages (being 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 bacterial 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 perisynovial 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 arthropathies, 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 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 arthropathies.

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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References


