Role of Neutrophils in Experimental Septicemia and Septic Arthritis Induced by *Staphylococcus aureus*

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We have previously described a murine model of hematogenously induced *Staphylococcus aureus* sepsis and arthritis. In this model, large numbers of granulocytes can be observed both in the circulation and locally in the inflamed synovium within 24 h after bacterial inoculation. To assess the role of neutrophils in this severe infection, mice were given granulocyte-depleting monoclonal antibody RB6-8C5 before being inoculated with *S. aureus*. All the control mice survived their intravenous injection with 3×10^7 CFU of *S. aureus*, whereas all the mice given RB6-8C5 antibody died of sepsis within 2 to 3 days. Even when the inoculum size was reduced sixfold (i.e., 6×10^6 CFU/mouse), 50% of the RB6-8C5-treated animals died within 6 days. The RB6-8C5-treated mice had a significantly higher burden of bacteria in their blood and kidneys 24 and 48 h after bacterial inoculation. In addition, when a suboptimal dose of bacteria was administered, the neutrophil-depleted animals displayed a higher frequency of arthritis than did the controls. The granulocyte-depleted animals exhibited increased levels of the proinflammatory cytokines tumor necrosis factor alpha, interleukin-6, and gamma interferon, reflecting the severity of their disease. This is the first direct demonstration of neutrophils playing a crucial protective role in the early phase of *S. aureus* infection.

Neutrophils are host immune defense cells that are the first to migrate into tissues in response to invading pathogens. These cells are capable of chemotaxis toward mediators present at the infection site. One of their principal roles in inflammatory and immune responses is thought to be the phagocytosis and killing of bacteria via the generation of reactive oxygen intermediates and the release of lytic enzymes stored in granules (13, 18). *Staphylococcus aureus*, an extracellularly growing bacterium and one of the most important pathogens in human sepsis and septic arthritis, is still associated with severe sequelae and mortality despite the use of antibiotics. We have recently described a murine model of hematogenously induced *S. aureus* sepsis and arthritis (5, 9). Mice given an injection of a single dose of the toxic shock syndrome toxin 1-secreting *S. aureus* LS-1 develop clinical signs of arthritis within 48 h. Depending on the injected dose of bacteria, the mice may develop sepsis, characterized by weight loss, diminished physical activity, chills, skin abnormalities, and eventually death. T lymphocytes contribute to the development of the disease since depletion of CD4$^+$ T cells or αβ T-cell receptor-expressing cells improves the outcome of the disease (1, 2, 7). Astonishingly, B cells and B-cell-derived cytokines also contribute to the pathogenesis of *S. aureus* infection (24). In contrast, our previous study suggested that monoclonal antibody (MAb)-mediated depletion of CD43$^+$ cells increased *S. aureus*-mediated morbidity and mortality, indicating that the cells expressing this molecule (present at its highest density on granulocytes) might be important in protection against staphylococci (6). The aim of the present study was to evaluate the role of neutrophils in the induction, progression, and outcome of *S. aureus*-induced septicemia and arthritis. Our results indicate that neutrophils play a protective role in the early phase of the disease.

MATERIALS AND METHODS

Mice. Male BALB/c mice, 5 to 6 weeks old, were obtained from B&K Universal AB (Stockholm, Sweden). They were housed in the animal facility of the Department of Rheumatology, University of Göteborg, Göteborg, Sweden, under standard conditions of light and temperature and fed standard laboratory chow and water ad libitum.

Bacteria and infection. *S. aureus* LS-1 was originally isolated from a swollen joint of a spontaneously arthritic NZB/W mouse (8). This bacterial strain has been shown to produce large amounts of toxic shock syndrome toxin 1 and is coagulase and catalase positive. The bacteria were kept frozen at −20°C in phosphate-buffered saline (PBS) (0.13 M NaCl, 10 mM sodium phosphate [pH 7.4]) plus 5% bovine serum albumin and 10% dimethyl sulfoxide (C_2H_6OS) until used. Before use, the bacterial solution was thawed and washed in PBS. Viable counting was used to check the number of bacteria in each bacterial solution. Mice were given intravenous (i.v.) injections (in the tail vein) of 0.2 ml of bacterial solution. Mice that were in septic shock, i.e., dehydrated and unable to move, eat, or drink, were sacrificed.

Production and purification of MAbs. MAb RB6-8C5 is a rat immunoglobulin G2b (IgG2b) antibody that selectively binds to and depletes mature mouse neutrophils and eosinophils. The hybridoma cells secreting RB6-8C5 were a kind gift from R. Coffman, DNAX Research Institute, Palo Alto, Calif. The hybridoma cells were expanded in Iscove's medium (Gibco, Paisley, United Kingdom) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Seralab, Crawley Down, United Kingdom), 50 µg of gentamicin per ml, 2 mM L-glutamine, and 5×10^{-5} M β-mercaptoethanol. The cells were grown to a maximum density, and the Igs were precipitated with 50% of saturated ammonium sulfate, dialyzed against PBS, and filter sterilized. The concentration of Igs was determined by the radial immunodiffusion method (16). As a control, monoclonal Ig-class-matched anti-ovalbumin antibodies were used (kindly provided by E. Telemo, Department of Clinical Immunology, University of Göteborg, Göteborg, Sweden).

Clinical evaluation. All the mice were monitored individually. Their limbs were inspected visually at regular intervals. Arthritis was defined as visible joint erythema and/or swelling of at least one joint. To evaluate the intensity of arthritis, a clinical scoring system of 0 to 3 points for each limb was used (1 point, mild swelling and/or erythema; 2 points, moderate swelling and erythema; 3 points, marked swelling and erythema). The arthritic index was constructed by adding the scores from all four limbs for each animal. Previous study has shown that there is a good correlation between the clinical and histopathological appearance of arthritis (5). The overall condition of each mouse was evaluated by assessing its weight, general appearance, alertness, and skin abnormalities.

Hematological analyses. Mice were bled from the tail into heparinized tubes. Total leukocyte counts were determined in a hemacytometer (Toa Medical Electronics, Kobe, Japan). Blood smears were prepared and stained by the May-Grunewald-Giemsa method for differential counts.

Determination of bacterial growth. At 24 and 48 h after bacterial inoculation, the mice were sacrificed. Bacterial samples from the joints were obtained with charcoal-treated sticks and transferred directly to plates containing 5% horse blood. The kidneys were aseptically removed, passed through a nylon mesh, and

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TABLE 1. Percentage of circulating granulocytes in BALB/c mice after an i.v. inoculation of *S. aureus* and a single pretreatment with either MAb RB6-8C5 or rat IgG

Treatment	% of granulocytes after[a]:			Total no. of granulocytes (10^6/ml) after 6 days (n = 9–10)[a,c]
	24 h[b] (n = 4)	48 h[b] (n = 4)	6 days[c] (n = 9–10)	
RB6-8C5	4.0 ± 1.5	13.0 ± 4.1	65.0 ± 10.5	11.7 ± 2.0
Rat IgG	39.0 ± 4.4	60.0 ± 3.5	9.2 ± 1.7	0.9 ± 0.2
P	<0.05	<0.05	<0.001	<0.001

[a] Mean ± SEM.
[b] Injection of 3×10^7 CFU of *S. aureus*.
[c] Injection of 1.5×10^6 CFU of *S. aureus*.

suspended in PBS. Blood and organ suspensions were plated in appropriate dilutions on agar plates and incubated at 37°C for 48 h. The bacterial colonies were tested for catalase and coagulase activity.

Assessment of cytokine levels in sera. (i) IL-6 assay. The cell line B13.29, subclone B9, which is dependent on interleukin-6 (IL-6) for its growth, was used for IL-6 determinations (5). The cells were seeded into microtiter plates at 5,000 cells/well, and samples at different dilutions were added. [^{3}H]thymidine was added after 68 h of culture, and the cells were harvested 4 h later. The results were compared with those obtained with a recombinant IL-6 standard (Genzyme, Cambridge, Mass.). The B9 cells were previously shown not to react with several recombinant cytokines including IL-1α, IL-1β, IL-2, IL-3, IL-5, granulocyte/macrophage colony-stimulating factor, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ). There was only a weak reactivity with IL-4 (14).

(ii) IL-1β and TNF-α assays. The levels of IL-1β and TNF-α were determined with enzyme-linked immunosorbent assay kits (Genzyme).

(iii) IFN-γ assay. The IFN-γ levels in sera were quantified with a previously described ELISA system (27). Briefly, 96-well microplates (Dynatech, Chantilly, Va.) were coated overnight at 4°C with 2 μg of rat anti-mouse IFN-γ MAb (Pharmingen, San Diego, Calif.) per ml, dissolved in sodium bicarbonate (pH 9.6). Blocking was performed for 1 h with 1% bovine serum albumin (Sigma) dissolved in 0.05 M Tris (pH 7.4). Sera, recombinant IFN-γ (Genzyme), and biotinylated antibodies were all diluted in 0.05 M Tris (pH 7.4)–0.015 M NaCl. A biotinylated anti-mouse IFN-γ antibody (2 μg/ml; Pharmingen) was used as a catching antibody. 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). Absorbance was measured at 405 nm in a Titertek Multiscan photometer (Flow Laboratories, McLean, Va.).

In vivo cell-mediated inflammatory responses. (i) Immunization procedure and registration of DTH. To assess the effect of granulocyte depletion on T-cell-mediated in vivo responses, the mice were sensitized by epicutaneous application, on the shaved abdomen and thorax skin, of 50 μl of a mixture of absolute ethanol and acetone (3:1) containing 3% 4-ethoxymethylene-2-phenyloxazolone (Sigma). Seven days after sensitization, all the mice were challenged by application, on both sides of the right ear, of 15 μl of 1% oxazolone dissolved in olive oil. At 2 h before the challenge, the mice were pretreated with 1 mg of MAb RB6-8C5 or 1 mg of control rat IgG. The thickness of the ear before and 24 h after challenge was measured with an Oditest spring caliper (Kröplin,

Schluchtern, Germany) as previously described (12). All challenges and measurements were performed under light pentobarbital anesthesia. The intensity of the delayed-type hypersensitivity (DTH) reaction was expressed as (ear thickness at 24 h minus ear thickness at 0 h) $\times 10^{-3}$ cm.

(ii) Induction and registration of olive oil-mediated inflammation. Olive oil induces in vivo a strong granulocyte-mediated but T-cell-independent inflammatory response (15). Inflammation was induced by injection of 30 μl of olive oil (Apoteksbolaget AB, Göteborg, Sweden) intradermally into the hind footpad. The mice were pretreated with 1 mg of MAb RB6-8C5 or 1 mg of control rat IgG 2 h before injection of olive oil. Footpad swelling was registered with an Oditest spring caliper 24 h after injection. The intensity of the olive oil-induced inflammation was expressed as (footpad thickness at 24 h minus footpad thickness at 0 h) $\times 10^{-3}$ cm.

Statistics. Statistical comparisons were made by the Mann-Whitney test and chi-square test with Yates correction. All values are reported as the mean ± standard error of the mean (SEM).

RESULTS

Efficiency of the granulocyte depletion procedure. The mice were given intraperitonal injections of 1 mg of MAb RB6-8C5 or an IgG rat anti-ovalbumin MAb as a control 2 h before intravenous injection of bacteria. Analysis of blood smears showed that the MAb RB6-8C5 depletes the granulocyte population to a significant degree. Thus, 24 and 48 h after bacterial inoculation with 3×10^7 CFU of *S. aureus*, the blood smears from the anti-granulocyte-pretreated animals exhibited significantly lower levels of granulocytes than did those from the control mice (Table 1). However, when blood smears and the total blood cell count were analyzed 6 days after pretreatment with MAb RB6-8C5 and bacterial inoculation with 1.5×10^6 CFU, the levels of circulating granulocytes in the RB6-8C5-treated group were significantly higher than in the control group (Table 1).

Effect of neutrophil depletion on *S. aureus*-induced mortality and arthritis. The mice treated with MAb RB6-8C5 2 h before being inoculated with 3×10^7 CFU of *S. aureus* LS-1 suffered from severe illness, which resulted in a high level of mortality (Fig. 1A). Thus, 2 days after the bacterial inoculation, 10 of 13 mice in the experimental group but 0 of 14 in the control group died of sepsis ($P < 0.001$). On day 3 after the bacterial inoculation, the remaining three animals in the granulocyte-depleted group had died but all the control mice were still alive ($P < 0.001$). To minimize mortality, the dose of injected bacteria, after pretreatment with granulocyte-depleting MAb and control MAb, was lowered fourfold (8×10^6 CFU per mouse). This resulted in a lower rate of mortality in the RB6-8C5-treated group (Fig. 1B). However, 6 days after the bacterial inoculation, 7 of 12 mice in the granulocyte-depleted group but only 1 of 12 in the control group had died ($P < 0.05$). When, after pretreatment with MAb RB6-8C5, a nonlethal dose of
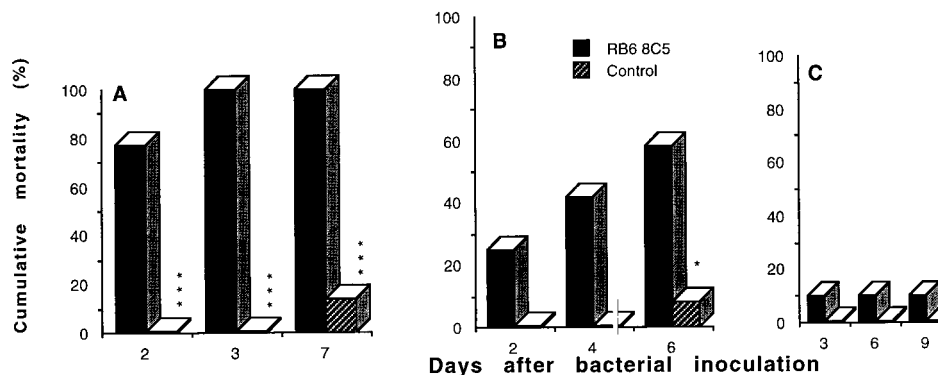

FIG. 1. Mortality in BALB/c mice treated with 1 mg of RB6-8C5 or an equivalent amount of control rat IgG 2 h before an i.v. injection of 3×10^7 CFU (A), 8×10^6 CFU (B), or 1.5×10^6 CFU (C) of *S. aureus* LS-1. n = 10 to 14 per group. *, $P < 0.05$; ***, $P < 0.001$.
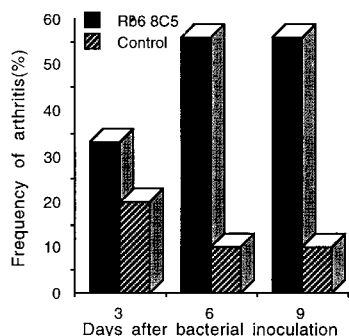
FIG. 2. Prevalence of arthritis in BALB/c mice treated with 1 mg of RB6-8C5 ($n = 9$) or rat IgG ($n = 10$) 2 h before an i.v. injection of 1.5×10^6 CFU of *S. aureus*.

TABLE 3. Growth of *S. aureus* in kidneys and blood of mice pretreated with MAb RB6-8C5 or control rat IgG 2 h before injection of 3×10^7 CFU of *S. aureus* LS-1[a]

Treatment	Bacterial growth in kidney (10^3 CFU) at:		Bacterial growth in blood (CFU/ml) at:	
	24 h	48 h	24 h	48 h
RB6-8C5	109 ± 38	$15,575 \pm 4,946$	$2,375 \pm 2,056$	15 ± 12
Controls	<1	19 ± 12	0	0
P	<0.05	<0.05	<0.05	<0.05

[a] The number of CFU in homogenates of two kidneys and blood from each animal were determined on blood agar plates. The results are expressed as mean $\pm$ SEM for a group of four animals.

bacteria (1.5×10^6 CFU per mouse) was administered, only 1 of 10 mice in the RB6-8C5-treated group and none in the controls had died 9 days after bacterial inoculation (Fig. 1C).

The frequency of arthritis could be properly evaluated when the mice did not die of sepsis. Six days after antibody treatment and inoculation with 1.5×10^6 CFU of bacteria, 5 of 9 mice in the neutrophil-depleted group displayed clinical signs of arthritis compared to 1 of 10 animals in the control group (Fig. 2).

The weight loss by the RB6-8C5-treated mice compared to the controls, reflecting the overall severity of the infection, was striking. Six days after antibody treatment and injection with 8×10^6 CFU of *S. aureus*, the granulocyte-depleted animals had lost 35% of their original weight whereas those in the control group had lost only 3%. When a suboptimal dose of bacteria (1.5×10^6 CFU) was used, the controls gained weight from the very beginning of the experiment whereas the neutrophil-depleted animals never achieved their original weight (Table 2).

Decreased elimination of bacteria in granulocyte-depleted animals. To examine the elimination of *S. aureus* during the infection, the magnitude of bacterial growth was evaluated in the blood and kidneys of mice pretreated with anti-granulocyte MAb ($n = 8$) or control MAb ($n = 8$) before administration of 3×10^7 CFU of *S. aureus*. At 24 and 48 h after bacterial administration, the blood and kidneys from the RB6-8C5-treated mice displayed significantly larger numbers of live bacteria than did those from the control group (Table 3). At 48 h after bacterial inoculation, staphylococci were recovered from the joints in three of four animals in the granulocyte-depleted group and in two of four in the control group.

Increased proinflammatory cytokine levels in MAb RB6-8C5-treated animals. As previously described, mice inoculated with *S. aureus* will produce proinflammatory cytokines (5, 25).

Levels of IL-6 in the serum of granulocyte-depleted animals were significantly higher at 24 and 48 h after bacterial inoculation than were these of the controls in the group inoculated with 3×10^7 CFU and at 48 h and 6 days after bacterial inoculation in the group inoculated with 8×10^6 CFU of *S. aureus* (Fig. 3).

At 48 h after inoculation with 8×10^6 CFU of *S. aureus*, 2 of 3 mice in the granulocyte-depleted group but 0 of the 11 controls displayed circulating levels of TNF-α. At 6 days after infection, all 5 mice in the RB6-8C5-treated group but only 1 of 11 controls had detectable levels (>60 pg/ml) of TNF-α in serum.

The group of mice treated with RB6-8C5 displayed higher levels of IFN-γ than did the controls 48 h after inoculation with 3×10^7 CFU of *S. aureus* (875 ± 226 versus 275 ± 164 U/ml [not statistically significant]).

There were no detectable levels of IL-1β in the sera of animals inoculated with 3×10^7 or 6×10^6 CFU of *S. aureus*.

Granulocyte-dependent and -independent inflammatory responses. The reaction to olive oil is a granulocyte-dependent inflammatory response. The swelling of the footpad in the RB6-8C5-treated, noninfected animals ($n = 5$) was significantly less pronounced than in the controls ($n = 5$) ([25 ± 11] $\times 10^{-3}$ versus [71 ± 18] $\times 10^{-3}$ cm; [$P = 0.01$]). There were no differences in the DTH responses to oxazolone, a T-cell-dependent but granulocyte-independent inflammatory skin reaction, between the two noninfected groups ([32 ± 5] $\times 10^{-3}$ versus [28 ± 4] $\times 10^{-3}$ cm [not statistically significant]).

DISCUSSION

Neutrophils, the main phagocytizing cell type, are important effector cells in the defense against bacteria. They are rapidly recruited to infected sites of the body both by chemotaxis to the bacteria themselves and by inflammatory mediators, e.g., IL-8, that are generated at the initial site of infectious injury.

TABLE 2. Changes of body weight in BALB/c mice at different times after injection of *S. aureus* LS-1[a]

Treatment	Wt loss[b] (% of original wt) after initial dose of:						
	8×10^6 CFU				1.5×10^6 CFU		
	n	Day 2	n	Day 6	n	Day 3	Day 5	Day 7
RB6-8C5	9	-19.7 ± 1.0	5	-35.3 ± 2.0	9	-17.5 ± 3.5	-18.0 ± 4.3	-17.3 ± 3.5
Controls	12	-0.3 ± 0.6	11	-3.1 ± 2.6	10	$+1.6 \pm 0.8$	$+3.1 \pm 0.8$	$+4.7 \pm 0.8$
P		<0.001		<0.001		0.001	<0.001	<0.001

[a] The mice were treated with MAb RB6-8C5 or control rat IgG 2 h before bacterial inoculation.
[b] Mean $\pm$ SEM.
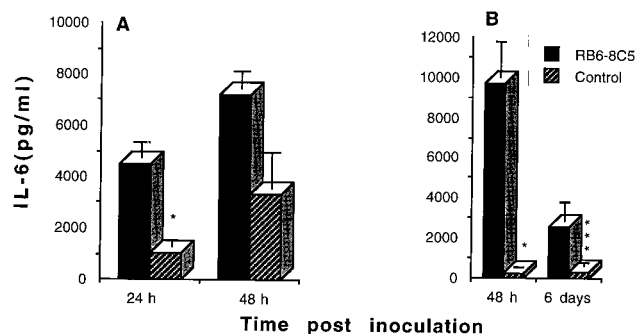
FIG. 3. Levels of IL-6 at different time intervals in sera of mice pretreated with RB6-8C5 or control rat IgG 2 h before inoculation with 3×10^7 CFU ($n = 4$ per group) (A) or 8×10^6 CFU ($n = 3$ to 11 per group) (B) of *S. aureus*. *, $P < 0.05$; ***, $P < 0.001$.

Once there, they phagocytize and kill the invaders (11, 18). We have previously demonstrated that the acquired immune system, i.e., T and B lymphocytes, is enhancing the *S. aureus*-induced septicemia and septic arthritis (1–3, 7, 24). In contrast, intact expression of CD43 was protective from the point of view of both survival and joint inflammation during *S. aureus* infection (6). Although the CD43 molecule is expressed on practically all hematopoietic cells, its highest density can be found on phagocytes (19). Bearing this in mind, as well as the occurrence of large numbers of granulocytes in the circulation and locally in the joints during septicemia and septic arthritis, we decided to evaluate the role of neutrophils in the pathogenesis of *S. aureus* infection.

In the present study, we depleted the neutrophils prior to inoculation with *S. aureus* by giving the mice a single injection of MAb RB6-8C5. The efficiency and selectivity of this procedure were ascertained by in vivo experiments showing that (i) the T-cell-dependent but granulocyte-independent DTH reaction to oxazolone was not affected by this procedure whereas (ii) the granulocyte-dependent but T-cell-independent olive oil-induced inflammation was greatly inhibited. In addition, up to 90% of peripheral blood granulocytes were depleted early during the course of infection after treatment with MAb RB6-8C5. This treatment had a detrimental effect on the outcome of the disease, since the granulocyte-depleted animals displayed severe signs of septicemia and high mortality rates. In addition, the frequency of arthritis was clearly increased.

Typically, the peak bacterial burden after i.v. staphylococcal inoculation occurs in blood within the first 1 to 3 days and in kidneys somewhat later (7 days) (23). The clearance of bacteria in the RB6-8C5-treated animals was defective due to the lack of phagocytizing neutrophils. These mice had a significantly higher burden of bacteria in the blood and kidneys compared to the controls 24 and 48 h after bacterial inoculation. Also, 6 days after the start of infection, 2 of the 6 spleens of the granulocyte-depleted animals but 0 of 11 spleens of control animals had abscess formations (data not shown).

In normal mice, polymorphonuclear leukocytes can be seen in the synovial tissue of the joint within 24 h after i.v. injection of *S. aureus*, and they are dominating in the early joint lesions as well as in extra-articular sites of infection (5, 7). On one hand, neutrophils are an effective defense against bacterial pathogens, but on the other, they also act as mediators of tissue-destructive events in many inflammatory diseases. In this respect, a massive infiltration of granulocytes into the joint may mediate cartilage and bone destruction by a series of well-defined mechanisms. Thus, neutrophils adherent to the artic-

ular cartilage surface may discharge latent and active proteinases and products of the NADPH oxidase into the joint space, leading to latent proteinase activation and destruction of cartilage by proteinases specific for collagen and/or proteoglycan (18).

The nonphagocytized, tissue-bound bacteria and, more importantly, their exotoxins activate macrophages and monocytes to release the proinflammatory cytokines TNF-α, IL-1, IL-6, and IFN-γ, which enter the circulation and trigger a general inflammatory response (3, 5, 17, 21, 22). The levels of these cytokines in serum are associated with the severity of the infectious process (20). The presence of inflammatory cytokines prolongs the survival of neutrophils engaged in phagocytosis (4), but it also mediates the symptoms of disease, including sepsis-induced death and septic arthritis.

The granulocyte-depleted mice exhibited raised levels of the proinflammatory cytokines TNF-α and IL-6. IL-6 is triggered early and produced in large amounts throughout the course of arthritis and septicemia with *S. aureus* (5). It is one of the most pleiotropic of the ILs released at sites of injury or infection. The production of IL-6 is highly correlated with death, both in patients and in animal models of sepsis (10). The circulating levels of IL-6 were significantly higher in the granulocyte-depleted animals than in the controls, reflecting the poor condition of the former group.

IFN-γ is a cytokine that exerts many biological activities. It can activate macrophages and neutrophils to enhance microbiocidal activity. We have found that IFN-γ has a beneficial effect in the early stage of the disease by increasing phagocytosis and increasing bacterial clearance (26). The sera tested for IFN-γ were obtained at 24 and 48 h postinfection, when the RB6-8C5-treated animals were still neutropenic. Despite somewhat raised levels of IFN-γ in the RB6-8C5-treated group, efficient bacterial clearance could not occur due to the lack of neutrophils. This finding shows that mononuclear phagocytes (i.e., monocytes and macrophages) alone are not sufficiently effective to clear invading bacteria despite intrinsic activation with IFN-γ.

Altogether, our results show that phagocytosis by recruited neutrophils in the initial stage of *S. aureus* infection is critical for the outcome of staphylococcal infection. Further studies with recombinant granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, and IL-8 will enable potentially more efficient treatments of patients at risk of gram-positive sepsis.

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