

Are B Lymphocytes of Importance in Severe *Staphylococcus aureus* Infections?

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To investigate the role of B cells in experimental, superantigen-mediated *Staphylococcus aureus* arthritis and sepsis, we used gene-targeted B-cell-deficient mice. The mice were inoculated intravenously with a toxic shock syndrome toxin 1 (TSST-1)-producing *S. aureus* strain. The B-cell-deficient and thus agamma-globulinemic mice showed striking similarities to the wild-type control animals with respect to the development of arthritis, the mortality rate, and the rate of bacterial clearance. Surprisingly, we found that the levels of gamma interferon in serum were significantly lower ($P < 0.0001$) in B-cell-deficient mice than in the controls, possibly due to impaired superantigen presentation and a diminished expression of costimulatory molecules. In contrast, the levels of interleukin-4 (IL-4), IL-6, and IL-10 in serum were equal in both groups. Our findings demonstrate that neither mature B cells nor their products significantly contribute to the course of *S. aureus*-induced septic arthritis.

We have previously described a murine model of hematogenously induced *Staphylococcus aureus* arthritis and sepsis (7, 8). Using this model, approximately 80 to 90% of mice inoculated with *S. aureus* LS-1 develop clinical arthritis. Immunohistochemical analysis of arthritic joints demonstrated the presence of phagocytes and T cells, predominantly of the CD4 phenotype (4). The infected mice displayed increased levels of inflammatory cytokines, such as tumor necrosis factor and interleukin-6 (IL-6) in serum (7). We have also shown that toxic shock syndrome toxin 1 (TSST-1), a superantigen produced by *S. aureus* LS-1, contributes to the arthritogenicity of *S. aureus* (3, 5). A series of studies using this model suggested that *S. aureus* arthritis is a T-cell-dependent and superantigen mediated disease.

As to the role of B cells in *S. aureus* arthritis, we have found that a striking feature in this model is the occurrence of polyclonal B-cell activation with highly increased levels of immunoglobulins and autoantibodies in serum (7). Using X-linked immunodeficiency (xid) mice to investigate the contribution of the B1 subset of B cells to the development of septic arthritis, it was found that this defect provided resistance (21). Since the B1 subset is considered to be of importance in the production of autoantibodies, it was hypothesized that the outcome of the experiment might have been due to this fact.

The aim of this study was to investigate if mature B cells, irrespective of their B1 or B2 phenotype, and their products including cytokines, autoantibodies, and antibodies to bacterial constituents would affect the outcome of *S. aureus*-induced arthritis and sepsis. We report here that a complete absence of mature B cells has no impact on the outcome of these very severe and life-threatening conditions.

MATERIALS AND METHODS

Mice. Gene-targeted B-cell-deficient μ MT mice (C57BL/6 \times 129) (11) were backcrossed to B10.Q (*H-2^d*) mice for eight generations and then further inter-

crossed for two generations to provide homozygous B10.Q mice lacking functional B cells (μ MT-BQ) (19). All the offspring were investigated for the presence of serum immunoglobulins (IgM and IgG). The mice were maintained in the animal facility of the Department of Rheumatology, University of Göteborg. Up to 11 mice were kept in each cage, and they were fed standard laboratory chow and water ad libitum. Three independent experiments were performed when the mice were 6, 20, and 24 weeks old.

Bacteria and inoculation. *S. aureus* LS-1 used in the experiments has been previously described (8). One of the characteristics of this strain is that it produces large amounts of TSST-1, an exotoxin with superantigenic properties (7). The bacteria were cultured on blood agar for 24 h and then reincubated on blood agar for another 24 h. They were kept frozen at -20°C in phosphate-buffered saline (PBS) (0.13 M sodium chloride, 10 mM sodium phosphate [pH 7.4]) containing 5% bovine serum albumin and 10% dimethyl sulfoxide ($\text{C}_2\text{H}_6\text{OS}$) until use. Before the experiments were started, the bacterial solution was thawed, washed in PBS once, and diluted in PBS to achieve the desired concentration of bacteria. Mice were inoculated in one of the tail veins with 0.2 ml of bacterial solution. One group received a low (suboptimal) arthritogenic dose (1×10^7 mouse), the second group received a moderate (optimal) arthritogenic dose (4×10^7 CFU/mouse), and the third group was injected with an high, septic dose (1×10^9 CFU/mouse) of the bacteria. Viable counts in the leftover solution were determined to ascertain the number of bacteria injected.

Clinical evaluation of arthritis. All the mice were followed up individually, and arthritis was evaluated. The limbs were evaluated by a blinded observer on days 0, 2 to 4, 7, and 10 to 11 after bacterial inoculation. The joints inspected included finger/toe and ankle/wrist joints. Arthritis was defined as visible erythema and/or swelling of a joint. To evaluate the intensity of arthritis, a clinical scoring (arthritis index) was carried out using a system where macroscopic inspection yielded a score of 0 to 3 points for each limb (0, neither swelling nor erythema; 1, mild swelling and/or erythema; 2, moderate swelling and erythema; 3, marked swelling and erythema) (2). The total score was calculated by adding all the scores for each animal tested. The overall condition was evaluated by assessment of weight and general appearance.

Histopathologic examination. Histopathologic examination of the joints was performed after routine fixation, decalcification, and paraffin embedding. Tissue sections from fore- and hindpaws from the experiment with the lowest (1×10^7) bacterial dose were cut and stained with hematoxylin-eosin. All the slides were coded and evaluated by two blinded observers. The specimens were evaluated with regard to synovial hypertrophy, pannus formation, and cartilage/subchondral-bone destruction. The degree of synovitis and destruction yielded each a score from 0 to 3 in every joint, i.e., finger/toes, wrists/ankles, elbows, and knees.

Bacteriological examination. Bacterial growth in blood was examined after 18 h and 3 days in the "septic-dose" experiment. The bacterial content in both kidneys and the liver was examined at the time of sacrifice. The different organs were aseptically removed, ground, and diluted with 10 ml of PBS. Appropriate dilutions were made, and 0.1-ml samples of tissue suspension or blood were plated on agar dishes containing 5% horse blood. Samples for bacteriological examination of joints were obtained using sterilized cotton sticks, after dissection of talocrural and radiocarpal joints, and transferred to 5% horse blood agar.

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TABLE 1. Absence of B cells does not affect histopathological progression of *S. aureus* arthritis in wild-type or μ MT mice^a

Mice	Frequency of synovitis (%)	Severity of synovitis ^b	Severity of bone or cartilage destruction	Frequency of pannus (%)
μ MT ($n = 11$)	91	4.5 \pm 1.6	4.4 \pm 2.3	36
Wild type ($n = 11$)	91	3.4 \pm 0.7	2.7 \pm 1.1	27

^a In all cases, the differences between μ MT and wild-type mice are not statistically significant.

^b For a definition of the numbers used to indicate severity, see the text.

After incubation for 48 h the colonies were counted and the results were expressed as the number of CFU per milliliter blood or per whole organ.

Serological analyses. (i) Immunoglobulins. Levels of IgG and IgM in serum were measured by radial immunodiffusion (12). Antiserum and Ig were purchased from Sigma Chemical Co. (St. Louis, Mo.).

(ii) IL-6 assay. Cell line B13.29, subclone B9, which is dependent on IL-6 for its growth, was used for IL-6 determinations (1, 10). B9 cells were harvested from tissue culture flasks, seeded into microtiter plates (Nunc, Roskilde, Denmark) at 5,000 cells/well, and cultured in Iscove's medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 5% fetal calf serum (Integro B.V., Leuvenheim, The Netherlands), and 50 μ g of gentamicin per ml and serum samples were added. [³H]thymidine was added after 68 h of culturing, and the cells were harvested 4 h later. The samples were tested in twofold dilutions and compared with a recombinant mouse IL-6 standard (Genzyme, Cambridge, Mass.) (6). B9 cells were previously shown not to react with several recombinant cytokines, including IL-1 α , IL-1 β , IL-2, IL-3, IL-5, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor, and gamma interferon (IFN- γ). There was only a weak reactivity with IL-4 (10).

(iii) IFN- γ assay. Levels of IFN- γ were measured by an enzyme-linked immunosorbent assay using 2 μ g of purified rat anti-mouse IFN- γ monoclonal antibody (PharMingen, San Diego, Calif.) per ml in sodium bicarbonate (pH 9.6) for coating. All sera were serially diluted in Tris-NaCl and incubated in wells. Biotinylated rat anti-mouse IFN- γ monoclonal antibody (2 μ g/ml) (PharMingen) was added to measure the level of IFN- γ bound to the solid phase. This procedure was followed by stepwise addition of streptavidin alkaline phosphatase (Dako, Glostrup, Denmark). The enzyme substrate was then added, and the absorbance was measured in a SpectraMax PLUS photometer (Molecular Devices) at 405 nm. The samples were tested in twofold dilutions and compared with a recombinant mouse IFN- γ standard (Genzyme).

(iv) IL-4 and IL-10 assay. Kits for detection of these cytokines were purchased from R&D Systems. Detection limits in our assays were 47 pg/ml and 2 pg/ml, respectively.

Statistical analysis. The mortality rate and the frequency of arthritis were analyzed using the χ^2 test with Yates' correction. All the remaining parameters were analyzed by the Mann-Whitney U test. All data are expressed as means \pm standard errors of the means unless otherwise indicated.

RESULTS

B lymphocytes do not affect the course of *S. aureus* arthritis or sepsis. The clinical outcome of arthritis varied among the three experiments due to inoculation of different numbers of bacteria. However, no statistically significant differences with respect to arthritis between the μ MT and the wild-type mice were observed in any of the experiments. In the experiment where the mice were inoculated with an optimal arthritogenic dose of bacteria (4×10^7 /mouse), 70% of the μ MT mice ($n = 10$) developed arthritis by day 7 whereas the corresponding rate for their littermate controls ($n = 11$) was 81%. The mice in the low-dose experiment (1×10^7 /mouse) had a lower frequency of arthritis: on day 7, 36% of the μ MT mice ($n = 11$) had developed arthritis and 27% of the wild-type controls had done so. In the sepsis experiment, the mice were inoculated with a high dose of bacteria (1×10^9 CFU/mouse); 86% of the μ MT mice ($n = 11$) and 60% of the controls ($n = 7$) developed arthritis by day 4. Also, the severity of arthritis was similar between μ MT and wild-type controls in all three experiments (data not shown). The clinical observations were confirmed by histopathological analysis of joints (Table 1).

No deaths were recorded as a result of inoculation with the

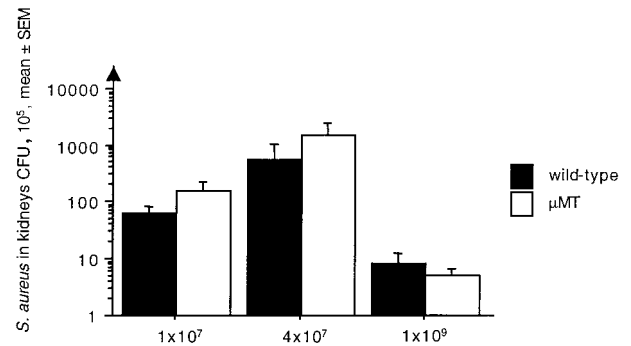


FIG. 1. B-cell deficiency does not influence bacterial clearance in the kidneys. In the low-dose (1×10^7 bacteria/mouse) experiment, μ MT ($n = 11$) and wild-type ($n = 11$) mice were sacrificed on day 14; in the moderate-dose (4×10^7 bacteria/mouse) experiment, μ MT ($n = 11$) and wild-type ($n = 10$) mice were sacrificed on day 11; and, finally, in the septic-dose experiment (1×10^9 bacteria/mouse), the μ MT ($n = 6$) and wild-type ($n = 6$) mice were sacrificed on day 7.

arthritogenic and subarthritogenic doses of bacteria (1×10^7 and 4×10^7 , respectively). Inoculation of B-cell-deficient mice with a septic dose (1×10^9) of *S. aureus* resulted in a somewhat increased mortality compared to that of wild-type controls (45% [5 of 11] and 13% [1 of 7], respectively). However, these data did not reach statistical significance. Furthermore, the general condition of the mice as measured by weight gain or loss showed no differences between the groups (data not shown).

B cells do not influence the elimination of *S. aureus* in vivo. To assess the elimination of *S. aureus* during infection, bacterial counts in blood were measured after 18 h and 3 days. In addition, bacterial counts in the kidneys, liver, and joints were measured at the time of sacrifice. There were no significant differences between the mice, irrespective of their B-cell status, in any of the experiments (Fig. 1). The shorter life span of the mice in the septic-dose experiment explains the lower bacterial burden.

Decreased production of IFN- γ in response to *S. aureus* infection in B-cell-deficient mice. To further study the cellular basis of responses to *S. aureus*, levels of cytokines in serum were determined. As shown in Fig. 2, there was a striking reduction of IFN- γ levels in the μ MT mice following infection with *S. aureus*. No differences in the levels of IL-4, IL-6, and IL-10 were found (Table 2).

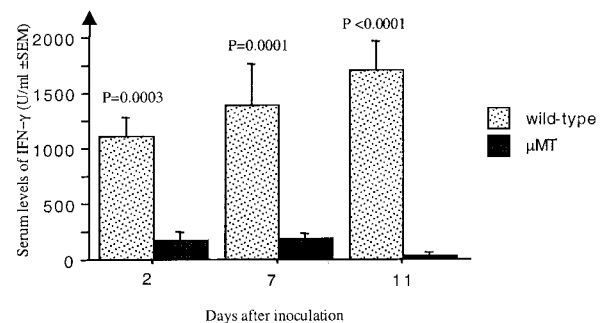


FIG. 2. Levels of IFN- γ in serum are significantly reduced in the B10.Q ($H-2^d$) μ MT mice compared to the wild-type controls. The levels were measured in the moderate-dose (4×10^7 bacteria/mouse) experiment on days 2, 7, and 11.

TABLE 2. Levels of cytokines in serum following *S. aureus* infection in B-cell-deficient (μ MT) and wild-type congenic mice

Cytokine	Level of cytokine ^a (mean \pm SEM) at days after inoculation:							
	2		3		7		11	
	μ MT	Wild type	μ MT	Wild type	μ MT	Wild type	μ MT	Wild type
IL-4	62 \pm 15	47 \pm 0 ^b	49 \pm 2	47 \pm 0 ^b	ND	ND	119 \pm 50	55 \pm 8 ^b
IL-6	2,962 \pm 908	1,544 \pm 437 ^b	ND ^f	ND	4,631 \pm 1,859	6,967 \pm 349 ^b	1,761 \pm 406	1,449 \pm 448 ^b
IL-10	5 \pm 1	5 \pm 1 ^b	4.0 \pm 0	58 \pm 52 ^b	ND	ND	5 \pm 1	5 \pm 1 ^b
IFN- γ	166 \pm 79	1,107 \pm 172 ^c	ND	ND	185 \pm 51	1,396 \pm 364 ^d	34 \pm 34	1,704 \pm 267 ^e

^a Levels of IL-4, IL-6, and IL-10 are expressed in picograms per milliliter. Levels of IFN- γ are expressed in units per milliliter.

^b Not statistically significant.

^c $P = 0.0003$.

^d $P = 0.0001$.

^e $P < 0.0001$.

^f ND, not done.

DISCUSSION

This study demonstrates that a complete absence of functional B cells in the μ MT mice affects neither susceptibility to nor outcome of *S. aureus*-induced septic arthritis and sepsis-related mortality. In addition, B cells do not influence the rate of in vivo elimination of staphylococci.

We have previously shown that mice with X-linked immunodeficiency (xid mice) are less susceptible to septic arthritis than are their congenic controls, probably because of a changed cytokine profile, characterized by decreased production of IL-1 β and IL-6 and increased synthesis of IFN- γ combined with poor antibody responses (21). The xid mice have relatively few functional B cells of the B2 subset and have no B1 cells. Since the B1 subset of B cells is an important source of autoantibody production, it may be hypothesized that deletion of this population might have had a beneficial impact on the outcome of *S. aureus* arthritis, a disease characterized by the production of high levels of rheumatoid factors, collagen II antibodies, and anti-DNA antibodies (7). In contrast, the B2 subset of B cells has the capacity to produce antibodies to extrinsic molecules, e.g., bacterial antigens. These antibodies might, at least under certain circumstances, be important in the defense against *S. aureus* sepsis and arthritis. Indeed, we have recently shown that antibodies specific to staphylococcal collagen adhesin (15) and enterotoxins (16) have a protective capacity. Thus, we hypothesize that the opposing properties of B1 versus B2 cell products might have neutralized each other's effect.

It has recently been shown that B cells are crucial to the progression of collagen type II-induced arthritis (19). It was suggested that B cells were important antigen-presenting cells and that some of them (i.e., those specific for collagen type II) were important for antigen uptake and thereby enhanced macrophage activity. The type of challenge used in different studies of B-cell-deficient mice is vital to the experimental outcome. In the present study, rather than a single antigen, live bacteria encompassing a multitude of antigens, mitogens, and superantigen were employed.

Is the ability of the B cells to present antigens important to the acquisition of an efficient immune response to the infectious agent? In this study, this does not seem to be the case. Previously it has been shown that B cells are not mandatory as antigen-presenting cells for T-cell priming, at least with respect to soluble protein antigens (9, 17, 20). Interestingly, a recent study indicated that intraperitoneal inoculation of μ MT mice with *Listeria monocytogenes* did not affect the clinical outcome of the disease but led to significantly lower production of IFN- γ (13). This outcome is similar to our findings with *S.*

aureus, an extracellularly growing bacterium. The mechanism responsible for the decrease of IFN- γ production is unclear but may be dependent on the expression of costimulatory molecules, such as B7 and CD40, on antigen-presenting cells. There are several studies which show the importance of CD40/CD40L costimulation for regulation of Th1 responses. Thus CD40L-deficient ($-/-$) mice fail to produce IFN- γ during the induction phase of 2,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis (18). In addition, stimulation with a CD40L agonist enhanced IFN- γ production by human peripheral blood mononuclear cells (14).

Our findings showing deficient production of IFN- γ are confirmed by the results of Matsuzaki et al. (13) and may be explained by a diminished number of major histocompatibility complex class II molecules, CD40, B7-1, and B7-2 molecules, and thereby an impaired T-cell priming. In addition, the decreased number of class II-expressing cells due to lack of the B-cell population might have affected the superantigenic responses, since the IFN- γ is a cytokine whose secretion is readily triggered by TSST-1 (22). Finally, it has recently been shown that clonal expansion of superantigen-reactive T cells is diminished in μ MT mice compared to intact controls (20).

This study demonstrates that the clinical and histopathological outcome of septic arthritis and sepsis-related mortality in response to intravenously inoculated *S. aureus* is identical in B-cell-deficient mice and their congenic controls. Interestingly, significantly decreased IFN- γ levels and absence of Ig production in the μ MT mice did not affect the in vivo clearance of bacteria.

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