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Septic Arthritis Following *Staphylococcus aureus* Infection in Mice Lacking Inducible Nitric Oxide Synthase

Iain B. McInnes, Bernard Leung, Xiao-Qing Wei, Curtis C. Gemmell, and Foo Y. Liew

Nitric oxide (NO), produced in large amounts by inducible NO synthase (iNOS), has emerged recently as an important microbicidal and immunomodulatory mediator. We have investigated its role in bacterial septic arthritis caused by *Staphylococcus aureus* infection using iNOS-deficient mice. The incidence, rate of development, and severity of arthritis were greater in iNOS-deficient than in heterozygous or wild-type control mice. Similarly, the incidence and severity of septicemia and mortality were significantly higher in iNOS-deficient mice compared with controls. Increased TNF-α synthesis in vivo and in vitro and enhanced IFN-γ compared with IL-4 production in vitro in iNOS-mutant mice demonstrated exaggerated Th1 polarization of the host response. These data indicate that high output NO production is not a prerequisite for severe articular destruction and imply that NO is of importance in synovial defense against staphylococcal infection. The Journal of Immunology, 1998, 160: 308–315.

Although NO clearly has been shown to mediate cytotoxicity against intracellular pathogens, including protozoa, viruses, bacteria, and fungi (14–17), its role in extracellular Gram-positive bacterial infections is less well understood. NO has been implicated in *S. aureus* killing by cytokineplasts from human neutrophils (18, 19), and in a cell-free system, NO donors are bactericidal for *S. aureus*, although the time course of bacterial killing is delayed compared with that mediated by reactive oxygen intermediates (20). In vivo, injection of certain staphylococcal exotoxins, such as toxic shock syndrome toxin (TSST)-1 or SEB, leads to a T lymphocyte-mediated shock syndrome in BALB/c mice, which can be exacerbated with exogenous NOS inhibitors (21). However, staphylococcal cell wall components, such as peptidoglycan and lipo-teichoic acid, synergistically induce multiple organ failure in rats by an NO-dependent mechanism (22). NO production may therefore have potential deleterious, as well as beneficial, effects during *S. aureus* infection in vivo.

Intravenous inoculation of Swiss mice with an exotoxin-producing strain of *S. aureus* leads to development of severe septic arthritis, but rarely induces septicemia (23, 24). The articular lesion exhibits synovial hyperplasia, containing neutrophils, macrophages, and a prominent T lymphocyte infiltrate, with associated erosion of underlying cartilage and bone. Anti-CD4 or anti-Vβ11 Ab administration to delete superantigen-responsive T cell subsets improves disease outcome following infection (25, 26), confirming a central role of T cell activation in pathogenesis. Moreover, amplification of this T cell response by infection of Vβ3 transgenic mice with a strain of TSST-1-producing *S. aureus* leads to significantly enhanced disease severity, with systemic involvement and increased mortality (27). This indicates that regulatory pathways normally operate to limit immunologically mediated systemic complications following *S. aureus* infection. Such pathways include the production of glucocorticoids, anti-inflammatory cytokines, principally IL-10, and perhaps, the generation of NO (28).

We previously generated mice lacking iNOS, which exhibit normal constitutive endothelial NOS and neuronal NOS expression (29). This provides an ideal model in which to address the role of high output NO synthesis on the clinical and immunologic sequelae to *S. aureus* infection. We now report that i.v. inoculation with *S. aureus* induced significantly increased clinical severity of septic arthritis, with attendant septicemia, in iNOS-deficient mice.
compared with similarly infected heterozygous or wild-type mice. These observations were associated with enhanced production of IFN-γ and TNF-α in vivo and in vitro, indicating Th1 polarization of the cell-mediated immune response. Our data define an important net protective role for NO in the host response to infections with *S. aureus*. They also strongly caution against the clinical use of selective NOS-inhibitor therapy in the management of septic arthritis.

**Materials and Methods**


inOS-deficient mice were constructed as previously described (29). Disruption of the murine inOS gene was achieved by homologous recombination in 129sv embryonic stem cells. The recombinant allele was passed through the germline following mating of embryonic stem cell chimeras with MF1 mice (Harlan Ltd, Oxon, UK). The homozygous, heterozygous, and wild-type mice thus generated were backcrossed to MF1 for three generations. All of the mice used were from littermate matings, and should therefore have similar MF1 genetic background. In later experiments, homozygous, heterozygous, and wild-type littermates of the 129x129sv strain were used which were derived from mating the chimeras with 129 mice (Harlan). Data from spleen cell cultures from these mice were identical to those obtained in 129x129xFMF1 mice. In some experiments, spleen cells from mice maintained in a specific pathogen-free (SPF) environment were also used. Extensive experiments using a variety of parameters demonstrated phenotypic similarity between the heterozygous and wild-type littermates. Peritoneal cells from the mutant mice did not express an iNOS protein following activation with IFN-γ and LPS in vitro, as judged by Western blot. They also did not produce detectable amounts of nitrate up to 48-h culture with IFN-γ and LPS. By 72 h, however, low levels of nitrite were detected in culture supernatants of cells from the mutant mice, which may have reflected the accumulation of nitrite produced by constitutive NOS2, or the induction of constitutive NOS (29).

**Induction and assessment of septic arthritis**

This was performed as previously described (23, 24). Briefly, LS-1, a TSST-1-producing *S. aureus* originally isolated from a spontaneous out-break of murine septic arthritis (gift of Dr. T. Bremell, University of Lund, Lund, Sweden), was stored in nutrient agar at 4°C, and before each experiment was cultured on 5% blood agar for 24 h at 37°C before resuspension in sterile PBS. The cell suspension was standardized spectrophotometrically to contain 5 x 10^8 CFU/ml. Male and female mice aged 3 to 4 wk received either 5 x 10^7 CFU *S. aureus* in 100 μl PBS injected i.v. via the tail vein, or 100 μl PBS alone. Wild-type or iNOS-deficient heterozygote mice served as controls for comparison with inOS-deficient homozygote mice (29). Individual mice were observed daily for up to 14 days, blind to genotype or infection status. Incidence of arthritis, number of limbs involved, and footpad/intermalleolar diameter (calliper measurement; Kroepelin, Metallp, Paris, France) were measured, and an arthritic index was derived, <=3 points/limb: 1, erythema alone; 2, swelling and erythema; and 3, erythema, swelling, and extension/loss of function. In one experiment, arthritic limbs (arthritic index = 3) from five iNOS-deficient and five heterozygote mice were fixed in 10% neutral buffered Formalin, and 5-μm sections were subsequently stained with hematoxylin and eosin (Sigma Chemical Co., Poole, U.K.) for assessment in a genotypically blinded manner. The severity of septicemia was judged clinically by characteristic changes in coat appearance (1), posture (1), presence of spontaneous movement (1), and mucocutaneous abscess formation (1), from which a septic index (maximum score 4/mouse) was derived for each animal by a blinded observer and also by daily weight measurement (g). Autopsy was performed on every animal to determine internal skeletal or large organ involvement. Viable *S. aureus* tissue distribution was estimated by whole organ culture, as described (27). Mice (n = 3/group) were killed before, or 3, 7, and 11 days after i.v. staphylococcal injection. Splenectomized kidneys and spleens were dissected, homogenized, then diluted in 10-fold steps in sterile PBS. Blood (200 μl) was similarly diluted in sterile PBS. Each dilution (100 μl) was plated on to prewarmed 5% blood agar and cultured for 24 h at 37°C to determine the number of CFU present, expressed per ml of blood, or per whole organ. Serum was also collected and stored at -70°C until assay either for cytokine concentration by ELISA, or for estimation of nitrate/nitrite concentration using a chemiluminescence method (11, 29). Briefly for the latter, individual sera were incubated with nitrate reductase (Sigma Chemical Co.) at 37°C for 2 h, then introduced through boiling glacial acetic acid, with 6% sodium iodide, into a chemiluminescence NO analyzer (Dasibi Environmental Corporation, Osaka Japan).

**Spleen cell culture**

Spleen single cell suspensions, obtained by gently mincing spleens, followed by filtration through Nitex membrane, were cultured at 2 x 10^6 cells/ml for up to 96 h in RPMI (Life Technologies, Paisley, U.K.) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (all Life Technologies) at 37°C. Cells were stimulated with either the staphylococcal exotoxins SEA and TSST-1 (both Sigma Chemical Co.), or heat-killed *S. aureus* at concentrations indicated in figure legends. Proliferation assays were performed in triplicate in U-bottom 96-well culture plates (Nuncnic, Roskilde, Denmark), with addition of 1 μCi of [3H]Thymidine (Amersham Life Sciences, Bucks, U.K.) in 25 μl during the final 6 h of culture before harvesting onto a glass fiber filter (Packard, Wallac, Milton Keynes, UK) using a Micromate 196 Harvester (Packard). [3H]Thymidine incorporation was measured using a Matrix 96, direct beta counter (Packard). In some experiments, duplicate 500-μl cultures were performed for up to 96 h in 48-well plates (Costar Corning Costar, Burks, U.K.), and supernatants were stored at -70°C until estimation of cytokine content by ELISA. Spleens from SPF mice aged 4 to 6 wk were obtained from Dr. Charles Lam (Sandoz, Vienna, Austria).

**Cytokine estimation**

Murine TNF-α, IFN-γ, IL-4, and IL-6 were detected in supernatants or serum by ELISA, with paired capture and biotinylated detection mAbs for each cytokine (PharMingen). The lower limit of detection in each assay was as follows: murine TNF-α, 10 pg/ml; murine IFN-γ, 30 pg/ml; murine IL-4, 40 pg/ml; and murine IL-6, 20 pg/ml.

**Statistical analysis**

This was performed using Minitab software for Macintosh. Analysis was by Log-Rank, χ², Mann-Whitney, or Student’s t tests, as indicated.

**Results**

**Incidence and severity of septic arthritis**

We first investigated the incidence and severity of septic arthritis in iNOS-deficient mice. Homozygous iNOS-deficient (n = 33), heterozygous (n = 28), or wild-type (n = 20) mice received 5 x 10^7 CFU/ml *S. aureus* i.v. in three independent experiments. Clinical signs of arthritis were observed within 2 days of infection. After 10 days, whereas arthritic inflammation was evident in only 50% of heterozygous and wild-type mice, up to 93% of iNOS-deficient mice developed arthritis (p < 0.005; Fig. 1a). Moreover, the mean arthritic index was significantly higher in arthritic homozygous mice than in arthritic control mice from day 5 (p < 0.05 to p < 0.01; Fig. 1b). The peripheral distribution of arthritis was similar between groups, but iNOS-deficient mice showed a higher incidence of paraspinal abscess formation, often with hind-limb paralysis and urinary incontinence (39% vs 10.7%; p < 0.05). Paraspinal abscess formation was usually a premorbid presentation, but in 9% of mutant mice was asymptomatic and found only at postmortem. Histologic examination of arthritic joints of equivalent severity from iNOS-deficient and control mice confirmed the presence of cartilage and bone erosion by synovial pannus, but revealed no difference in appearance between groups (data not shown). Thus, in contrast to murine autoimmune arthritis models,
in which inhibition of NO synthesis is protective (6–8), mice lacking the capability for high output NO synthesis display an increased incidence and severity of septic arthritis following S. aureus infection.

Incidence and severity of septicemia

Clinical evidence of septicemia was evident in 65% of iNOS-deficient mice within 48 h, compared with 14% of controls, rising to 83% compared with 20%, respectively, after 4 days (p < 0.005; Fig. 2a). Disease severity in animals developing sepsis was greater in homozygous than control mice during the acute phase up to day 6 (p < 0.001 to p < 0.04; Fig. 2b). These observations were reflected in significantly greater weight loss in iNOS-deficient mice following infection compared with controls (p < 0.01; Fig. 2c), or with uninfected iNOS-deficient littermates (data not shown). Increased mortality was observed in mutant mice, which reached 30% after 8 days (p < 0.005 to p < 0.03; Fig. 2d). No significant clinical differences between heterozygote and wild-type mice were observed.
24 h after inoculation. In contrast, basal levels of serum nitrite/nitrate were lower in iNOS-deficient mice and remained unaltered by staphylococcal infection (day 0, 19 ± 1.3 μM vs day 1, 16.4 ± 1.6 μM; n = 3). Thus, increased NO generation rapidly followed staphylococcal infection in vivo, and this response was absent in homozygous mice.

To determine whether these observations were reflected in altered bacterial distribution and survival in vivo, sequential cultures of blood and tissues were performed. Viable staphylococci were not detected in blood in any group at day 3, but were present in iNOS-deficient mice at days 7 and 11. In contrast, control mice had few viable blood-borne bacteria even at day 7 (Fig. 3a). High numbers of bacteria were present in the spleens of both groups after day 3, but persisted to days 7 and 11 only in mutant mice (Fig. 3b). These data indicated that although both groups similarly localized staphylococci to spleen, subsequent bacterial viability within, and dissemination from, the spleen were increased in iNOS-deficient mice. This was relevant to end-organ disease since greater numbers of viable staphylococcal CFU were also detected in kidneys of mutant mice (Fig. 3c).

**Immunoregulatory effects of iNOS deficiency**

We next investigated whether the altered clinical phenotype in iNOS-deficient mice was reflected in the immune response following staphylococcal infection. TNF-α has been implicated previously in the pathogenesis of Gram-positive bacterial shock (30). Serum TNF-α levels were significantly higher at day 3 (p < 0.05) and day 7 (p < 0.009) in mutant compared with control mice (Fig. 4a), corresponding to that period of maximal clinical sepsis and weight loss (Fig. 2). Moreover, spleen cells from iNOS-deficient donors cultured with heat-killed staphylococci in vitro generated significantly higher levels of TNF-α compared with controls (Fig. 4b). Spontaneous IFN-γ production in identical cultures was higher in spleen cells from iNOS-deficient than control mice (p < 0.005, **p < 0.001). Significant enhancement of IFN-γ production by killed S. aureus was observed in spleen cell cultures from heterozygote, but not iNOS-deficient mice (p < 0.01 vs baseline). Peak production was not significantly different between groups. (Fig. 4c, d).
Elevated levels of IL-6 were also present in serum, but in contrast to TNF-α, there was no significant difference observed between the groups at any time point (range 710–824 pg/ml, days 3–7 postinfection).

Neither IFN-γ nor IL-4 was detected in serum in any infected group at days 0, 3, 7, or 11. IFN-γ and IL-4 production were therefore compared in spleen cell suspensions, obtained from infected mutant or heterozygous mice, cultured in the presence or absence of heat-killed *S. aureus*. Spontaneous IFN-γ production was significantly higher in cultures from iNOS-deficient mice at each time point (p < 0.005 to p < 0.001; Fig. 4c). Addition of heat-killed *S. aureus* led to significant up-regulation of IFN-γ synthesis in cultures from control mice, to levels similar to those spontaneously produced by iNOS-deficient spleen cells (Fig. 4d), showing that diminished spontaneous IFN-γ production was not due to some inherent deficit in control mice. No further up-regulation of IFN-γ synthesis in cultures from mutant mice was observed, indicating that maximal induction of IFN-γ production had been achieved in vivo (Fig. 4d). No IL-4 was produced in vitro by cultures from any mice, even after *S. aureus* stimulation (data not shown). These observations are consistent with the presence of a dominant Th1 response during staphylococcal infection, which is enhanced in iNOS-deficient mice.

**Superantigen responses in mice lacking iNOS**

The diverse immunoregulatory properties of NO raised the possibility that iNOS-deficient mice generated altered immune responses a priori, thereby contributing to T lymphocyte-mediated pathology, irrespective of bacterial distribution (Fig. 3a–c). Spleen cells from uninfected mutant or control mice were cultured with TSST-1, the exotoxin secreted by the *S. aureus* LS-1. Significantly enhanced proliferation (p < 0.005; Fig. 5a) and IFN-γ production (p < 0.005; Fig. 5b) than did spleen cells from controls. Elevated levels of IL-6 were also present in serum, but in contrast to TNF-α, there was no significant difference observed between the groups at any time point (range 710–824 pg/ml, days 3–7 postinfection).
FIGURE 6. Spleen cells from naive SPF iNOS-deficient mice (○) showed enhanced proliferation, compared with SPF wild-type controls (□), in response to TSST-1 (a) or SEA (b) after 48 h. Data are mean ± SEM (**p < 0.01, *p < 0.001). IFN-γ synthesis was also enhanced in iNOS-deficient compared with control cultures in response to both TSST-1 (c) and SEA (d) after 72 h (*p < 0.005).

Discussion

Data presented in this work demonstrate directly that high output NO synthesis is an essential protective mechanism during Gram-positive bacterial infection. This was reflected in increased local and systemic pathology in iNOS-deficient mice, most strikingly in the development of severe articular destruction. NO is likely to function both as a direct effector and as an immunoregulatory molecule. iNOS-deficient mice were unable to limit bacterial growth despite the presence of high levels of Th1 cell activity and concomitant production of cytokines such as IFN-γ and TNF-α, which should have ensured maximal phagocytic activity within the reticuloendothelial system. This is in accordance with in vitro observations indicating that NO is an important cofactor in staphylococcal phagocytosis and subsequent killing by polymorphonuclear cells (18, 19, 31). Studies indicating that NO promotes multiple organ failure following injection of staphylococcal cell wall products (22) do not address either phagocytosis and killing of viable bacteria, nor the crucial regulatory role of NO in exotoxin-mediated pathogenesis. The potential for articular and systemic pathology resulting from superantigen-mediated T cell activation has been documented clearly in studies in which superantigen-specific T cell subsets have been targeted using mAbs (25, 26). Such responses mediate a predominantly Th1-lead process (27, 32, 33). Our data demonstrate that failure to regulate such Th1 activity in iNOS-deficient mice is likely to contribute significantly to pathology.

NO appears critical in down-regulating immune responses capable of potential damage to the host. Since uninfected iNOS-deficient mice exhibited enhanced Th1 polarization in responses to superantigens in vitro, it seems likely that the exaggerated Th1 response observed during staphylococcal infection in vivo reflected an inherent failure to properly regulate T cell responses. That this was due to altered susceptibility to infection...
with other pathogens during development was unlikely since similar observations were made in SPF animals. These data are consistent with our earlier finding that iNOS-deficient mice infected with Leishmania major develop elevated Th1 responses (29), and with observations in vitro that proliferation and cytokine production by Th1 clones are inhibited by high concentrations of NO donors (34). Moreover, macrophage-mediated suppression of SEA- or SEB-induced T cell activation in vitro has been attributed to NO production (35). The mechanism whereby NO influences such immunoregulation is currently unknown. It is possible that NO preferentially regulates the production of cytokines implicated in Th1/Th2 differentiation through modification of cytokine gene transcription (36). As NO has also been implicated in apoptotic pathways (37), activation-induced cell death, which normally follows superantigen stimulation, could be impaired in iNOS-deficient mice. Endothelial expression of P-selectin, which selectively mediates Th1 recirculation to inflammatory sites, is down-regulated by peroxynitrite (38), providing a further potential regulatory role for NO during Th1-mediated staphylococcal arthritis. Other immunoregulatory circuits operate following SEB injection in mice, including production of IL-10 and granulocyte-macrophage CSF and of endogenous glucocorticoids (28). The interaction of NO with such pathways is currently unclear.

Previous studies of autoimmune inflammatory arthritis in rodents indicated that lack of NO is protective since NOS inhibitors retard or abrogate collagen-induced, streptococcal cell wall or adjuvant-induced arthritis (6–8). Our data clearly indicate that the opposite is the case in infectious arthritis. NO up-regulates metalloproteinase and cyclooxygenase function, impairs chondrocyte chemotaxis and biosynthetic activity, and promotes bone resorption (39–42). Such proinflammatory effects for NO mediated locally, as demonstrated in other arthritis models (6–8), cannot be ruled out in septic arthritis. However, it appears that enhanced systemic Th1 cytokine production, leading to increased TNF-α synthesis, dominates the host response to S. aureus with resultant articular destruction, and in this context NO is a necessary protective regulator. Thus, there appears to be a clear distinction between arthritis caused by live organisms compared with that induced by either nonliving organisms or their secreted/cell wall components. It will be important to establish whether other cytokines, such as IL-15 (43), which are thought to be important in articular inflammation, are also up-regulated in iNOS-deficient mice.

We have demonstrated that NO is an important component of the host response to extracellular Gram-positive bacteria. In the absence of high output NO synthesis, mice developed an exaggerated Th1 response and exhibited increased frequency and severity of septic arthritis, with associated septicemia. This increased susceptibility to sepsis may reflect a role in staphylococcal killing for NO, either directly, or through the synthesis of peroxynitrite together with superoxide. Other pathogenetic mechanisms, including the altered immunoregulation described in this work, which led to enhanced TNF-α production, may also influence systemic disease manifestations. These findings suggest that selective inhibitors of iNOS, keenly sought for the treatment of septic shock, are unlikely to be of benefit in septic arthritis. Moreover, the potential for reduced synovial defense against staphylococcal infection, and the removal of an important Th1 regulatory element, suggests that iNOS inhibitors may perhaps be of reduced value in the management even of noninfectious inflammatory arthropathies, which are believed to be Th1 mediated.

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