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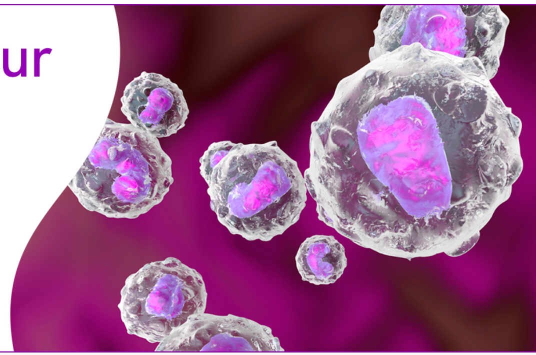

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Production of Granulocyte Colony-Stimulating Factor in the Nonspecific Acute Phase Response Enhances Host Resistance to Bacterial Infection[1]

Mahdad Noursadeghi,*† Maria C. M. Bickerstaff,* Jeff Herbert,* David Moyes,† Jonathan Cohen,[2]† and Mark B. Pepys[2,3]*

Mice mounting an acute phase response, induced by sterile inflammation after a single s.c. injection of casein 24 h beforehand, were remarkably protected against lethal infection with Gram-positive or Gram-negative bacteria. This was associated with enhanced early clearance of bacteremia, greater phagocytosis and oxidative burst responses by neutrophils, and enhanced recruitment of neutrophils into tissues compared with control, nonacute phase mice. Casein-induced inflammation was also associated with increased concentrations of G-CSF in serum, and administration of neutralizing Ab to this cytokine completely abrogated protection against *Escherichia coli* infection after casein pretreatment. Injection of recombinant murine G-CSF between 3 and 24 h before infection conferred the same protection as casein injection. In contrast, the casein-induced acute phase response affected neither serum values of TNF-α, IL-1β, or IL-6 after *E. coli* infection nor susceptibility to LPS toxicity. Furthermore, protection against infection was unaffected in IL-1R knockout mice, which have deficient acute phase plasma protein responses, or after nonspecific inhibition of acute phase protein synthesis by D-galactosamine or specific depletion of complement C3 by cobra venom factor. Increased production of G-CSF in the acute phase response is thus a key physiological component of host defense, and pretreatment with G-CSF to prevent bacterial infection in at-risk patients now merits further study, especially in view of increasing bacterial resistance to antibiotics. *The Journal of Immunology,* **2002, 169: 913–919.**

Increasing microbial resistance to antibiotics, resulting in therapeutic failure and consequently significant morbidity, mortality, and escalating healthcare costs, is an issue of immediate worldwide concern (1). Augmentation of nonspecific host defenses may provide an alternative or adjunctive approach to the prevention and management of bacterial infections, and elucidation of underlying mechanisms is therefore both of interest and of potential practical importance.

After most forms of tissue injury, infection, and inflammation, a complex cascade of cytokine production occurs in all endothermic species and mediates the nonspecific acute phase response (2). This comprises alterations in expression of genes encoding a wide range of plasma proteins, including proteinase inhibitors, clotting, complement, and transport proteins (3). The responses of serum amyloid A protein and of the pentraxins, C-reactive protein, and, in the mouse, serum amyloid P component are exquisitely sensitive, even to subclinical processes. This phenomenon and its stable evolutionary conservation strongly suggest that the acute phase response and its components have survival value. Indeed many of the cytokines and acute phase plasma proteins are postulated to have functions that may contribute to host defense and repair (4). Here, we show that the acute phase response induced by local sterile inflammation dramatically enhances resistance to acute pyogenic bacterial infections and that a single cytokine, G-CSF, is a key mediator.

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Materials and Methods

Mice and acute phase proteins

BALB/c, C57BL/6, and CD1 mice were purchased from B & K Universal (Hull, U.K.). IL-1R$^{-/-}$ (BALB/c genetic background) mice were kindly provided by M. Kopf (Basel, Switzerland). A single s.c. injection of 0.5 ml of 10% (w/v) casein (ICN Pharmaceuticals, Basingstoke, U.K.) in 0.05 M $NaHCO_3$ buffer was used to stimulate the acute phase response in mice as described previously (5). All control animals received s.c. injections of 0.5 ml of 0.05 M $NaHCO_3$ buffer alone. D-galactosamine (10 mg; Sigma-Aldrich, Poole, U.K.) in saline was given i.p. at the same time as casein or control buffer injection to inhibit de novo hepatic protein synthesis (6). Cobra venom factor, 10 U, was injected i.p. at the same time as casein or control buffer injection to deplete complement component C3 (7). Recombinant murine G-CSF and rabbit polyclonal IgG Ab to mouse G-CSF were kindly provided by G. Senaldi (Amgen, Thousand Oaks, CA). Ab to G-CSF, 0.5 mg, was injected i.v. at the same time as casein or control buffer to neutralize endogenous circulating G-CSF. Normal rabbit IgG was used as a control for the anti-G-CSF Ab.

Bacterial infections and LPS toxicity

Groups of 10–20 weight- and sex-matched 8- to 10-wk-old mice received either casein or control buffer 24 h before infection. *Escherichia coli* O111:B4 (from B. J. Applemelk, Vrije Universiteit, Amsterdam, The Netherlands) or an M1T1 clinical isolate of *Streptococcus pyogenes* (8) was grown to log phase. The bacteria were then washed and resuspended in saline. Mice were infected with 5–10 × 10^7 *E. coli* by i.v. injection or with 1–5 × 10^7 *S. pyogenes* by i.m. or i.p. injection. Morbidity and mortality were then recorded until no further deaths occurred. In some experiments,

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CD1 mice infected with *E. coli* O111:B4 were partially treated with gentamicin as previously described (9). For investigation of LPS-induced toxicity, mice were injected with 10 mg/kg LPS (from *E. coli* O111:B4; Sigma-Aldrich) i.p. 24 h after casein or control buffer injection.

Quantitative bacteriology, cytokine assays, and acute phase protein assays

Mice were sacrificed at sequential time points after infection, and viable bacteria were quantified by colony counts from serial dilutions of heparinized blood. Immunofluorescence staining was used to visualize bacteria within tissues. Sections, 30 μm, of liver and spleen from mice 12 h after infection with *E. coli* were incubated with a rabbit polyclonal Ab to the *o*-polysaccharide side chain of LPS from *E. coli* O111:B4 (kindly provided by D. Heumann, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland) and subsequently with a fluorescein-conjugated goat anti-rabbit Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) before conventional fluorescence microscopy using a Nikon Eclipse E600 (Nikon, Kingston, U.K.). ELISA immunoassays (R&D Systems Europe, Rennes, France) were used for quantification of cytokines, and electroimmunoassays were used to measure serum amyloid P component (SAP)[4] and C3 in mouse serum, as previously described (5).

Neutrophil function assays

Neutrophil phagocytosis and respiratory burst responses were measured by a modified flow cytometric assay (10) using *E. coli* O111:B4, which was transformed with the plasmid pFPV25.1 containing *gfp-mut 3a* under the constitutive S13 ribosomal protein promoter (from D. Holden, Imperial College School of Medicine, London, U.K.) to express green fluorescent protein (GFP) (3). Dihydroethidine (Sigma-Aldrich), 0.1 μg/ml, was added to whole heparinized mouse blood, followed by 10^7 GFP-expressing bacteria in early log phase (~10 bacteria per neutrophil). Phagocytosis was allowed to take place for 15 min, rotating at 37°C, and then was stopped by FACS Lysing Solution (BD Biosciences, San Jose, CA). Ingestion of GFP-expressing bacteria and respiratory burst responses, indicated by oxidation of dihydroethidine to red fluorescent ethidium bromide, were quantified by flow cytometry (FACSCalibur flow cytometer with CellQuest software, BD Biosciences). Granulocytes, 10^4, identified by light scatter parameters were analyzed in each sample. Relative mean cellular green and red fluorescence in cells that were positive for both fluorochromes quantified phagocytosis and respiratory burst responses. Neutrophil recruitment to tissues was measured by a myeloperoxidase assay (11). Livers from mice 3 h after infection were washed with PBS, homogenized mechanically (Ultramax T25; IKA Labortechnik, Staufen, Germany) in 0.5% (w/v) hexadecyltrimethylammonium bromide in 50 mM K_2PO_4, pH 6.0, and then sonicated on ice for 20 s, and cell suspensions were further lysed by three freeze-thaw cycles. Tissue debris was removed by centrifugation and samples were standardized for protein content using a detergent-compatible protein assay (Bio-Rad Laboratories, Hemel Hempstead, U.K.). Relative myeloperoxidase activity in samples was compared in microtiter plates by incubating equal volumes of sample and substrate (0.1% (w/v) *o*-dianisidine dihydrochloride in homogenization buffer with 0.001% (v/v) H_2O_2), stopping the enzymatic reaction with 1 M H_2SO_4 and reading the OD at 460 nm using a microplate reader. Samples were diluted to give ODs in the linear range.

Results

Prestimulation of the acute phase response in murine models of bacterial infection

In mice, s.c. injection of casein, a classical sterile inflammatory stimulus (5), induces a rise in serum levels of the acute phase proteins that is maximal at 24 h (Fig. 1). Mice that received an s.c. injection of casein 24 h before i.v. infection with *E. coli* or i.p. or i.m. infection with *S. pyogenes* had markedly reduced morbidity and improved survival compared with control animals injected with solvent alone (Fig. 2). The same results were obtained in outbred CD1 as well as inbred C57BL/6 and BALB/c mice, indicating that this is a general phenomenon. Casein-pretreated mice had significantly fewer viable bacteria in the blood within 1 h of infection and at least 1000-fold fewer live bacteria by 12 h (Fig. 3*A*). Estimation of bacterial load by immunofluorescence showed accelerated clearance of bacteria from the tissues of "acute phase" mice with strikingly greater numbers of organisms in the livers and spleens of control animals (Fig. 3*B*). In contrast, pretreatment with

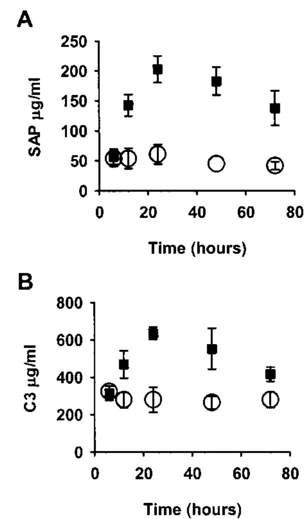

FIGURE 1. Acute phase response in BALB/c mice after s.c. injection of casein. Serum concentrations of the acute phase proteins SAP (*A*) and C3 (*B*) after injection of casein (■) or control buffer (○) in groups of five mice sacrificed at times shown; each point represents the mean ± SD.

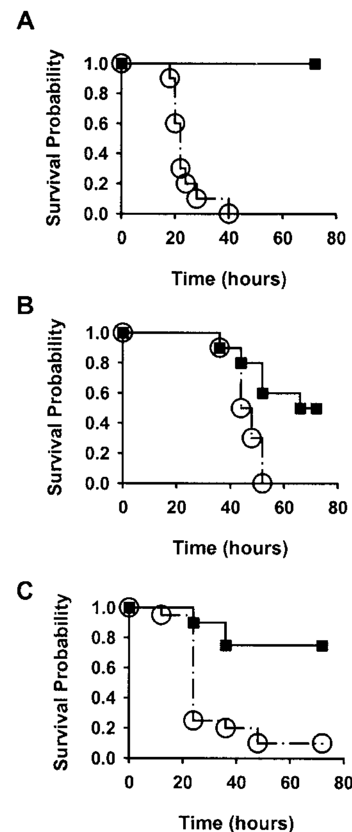

FIGURE 2. Survival of casein-pretreated and control mice in models of Gram-negative and Gram-positive bacterial infection. Stimulation of the acute phase response by s.c. injection of casein (■) 24 h before infection of BALB/c mice with *E. coli* O111:B4 (*A*), of BALB/c mice with i.m. *S. pyogenes* (*B*), or of C57BL/6 mice with i.p. *S. pyogenes* (*C*) significantly ($p < 0.001$, log rank test) improved survival compared with control groups (○). Each result shown is typical of at least two identical experiments.

[4] Abbreviations used in this paper: SAP, serum amyloid P component; GFP, green fluorescent protein.

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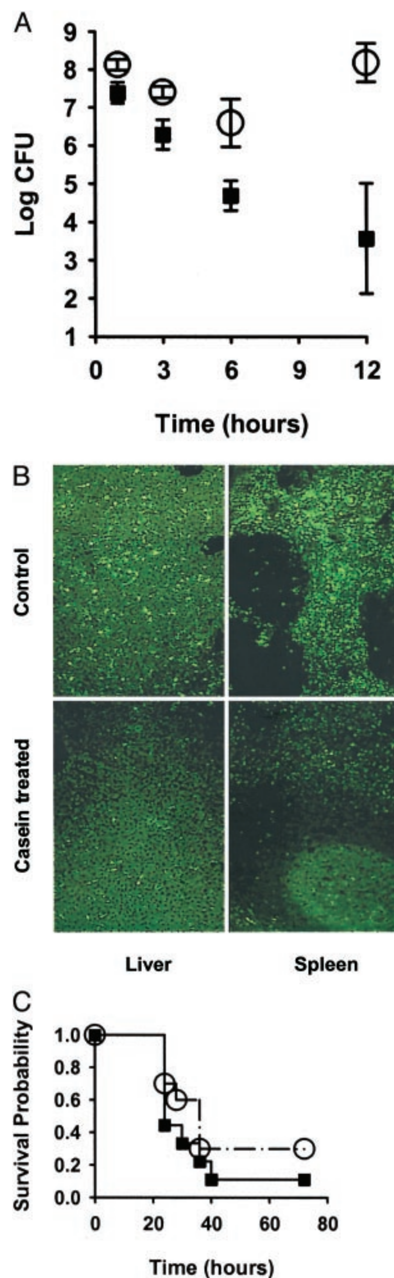

FIGURE 3. Bacterial clearance and response to LPS in casein-pretreated and control mice. *A*, Clearance of bacteremia in casein-pretreated (■) BALB/c mice infected with *E. coli* was significantly lower than in controls (○) ($p < 0.00001$ by one-way ANOVA). Each point represents the mean (±SD) of groups of five mice. *B*, Typical staining patterns of liver and spleen sections from mice killed 12 h after infection and stained by immunofluorescence with an Ab to the *O*-polysaccharide of *E. coli* O111:B4 show a strikingly greater number of bacteria in the controls than in the casein-pretreated mice. *C*, The preexisting acute phase response in casein-pretreated mice (■) did not protect against LPS-induced toxicity compared with controls (○).

casein did not improve the survival of mice receiving a lethal parenteral dose of Gram-negative bacterial LPS (Fig. 3*C*). The improved survival of casein-pretreated mice after infection with *E. coli* thus does not result from prior exposure to LPS, which may contaminate the casein preparation and could potentially increase tolerance to LPS toxicity (12). This observation also suggests that pretreatment with casein does not protect against the proinflammatory host response, which has been widely implicated in the pathogenesis of sepsis (13). Indeed, we found that after i.v. infection of mice with *E. coli* there were no significant differences between casein-pretreated and control groups in the serum concentrations of the major proinflammatory cytokines TNF-α, IL-1, and IL-6, (Fig. 4).

Role of acute phase proteins

A number of acute phase proteins have well-described roles in host defense against bacterial infections (4). Indeed, in previous studies pretreatment of mice with IL-1, which was found to enhance survival from subsequent *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* infection, was attributed to the synthesis of acute phase proteins, because inhibition of hepatic protein synthesis by D-galactosamine completely abrogated the protective effect of IL-1 (6). Therefore, we investigated the possible role of IL-1 in our casein model by using mice with targeted deletion of the IL-1 receptor (IL-1R$^{-/-}$) (14). As expected, the serum rise in acute phase proteins in response to casein was blunted compared with that of wild-type controls (Fig. 5*A*). Nonetheless, pretreatment of IL-1R$^{-/-}$ mice with casein still led to significant protection against lethal i.v. infection with *E. coli* (Fig. 5*B*). Clearly, therefore, neither IL-1 nor acute phase protein synthesis induced by IL-1 make physiologically significant contributions to host defense against bacterial infection in this model. To further explore the contribution of major acute phase plasma proteins in our model, we investigated the role of global de novo hepatic acute phase protein synthesis in wild-type mice and the role of the complement system in particular. The complement system plays an important role in host defense against bacteria, and many complement proteins, including the pivotal C3 component, are acute phase reactants. However, specific depletion of circulating C3 by prior administration of cobra venom factor (7) (Fig. 5*C*) did not abrogate the protective effect against infection conferred by prior induction of the acute phase response (Fig. 5*D*). Furthermore, the protective effect remained intact even when mice received D-galactosamine (Fig. 5*F*), a specific inhibitor of de novo hepatic protein synthesis (6), at a dose that did not cause significant hepatic necrosis but still globally reduced the acute phase plasma protein response by 50% (Fig. 5*E*). Higher doses of D-galactosamine, which completely block the acute phase response, cause severe liver damage that compromises the infection models and therefore could not be used (data not shown). Nevertheless, these results indicate that the enhanced resistance to acute lethal systemic bacterial disease, displayed by animals mounting an acute phase response at the time of infection, is not dependent on increased production of the classical acute phase plasma proteins.

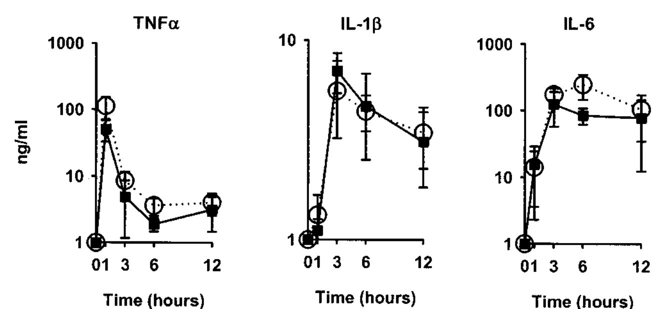

FIGURE 4. Comparison of proinflammatory cytokine responses in casein-pretreated and control mice. Stimulation of the acute phase response in mice (■) 24 h before infection with *E. coli* had no effect on serum concentrations of TNF-α, IL-1β, or IL-6 (at the time points shown) after infection, compared with controls (○). Each point represents the mean (±SD) of groups of five mice.

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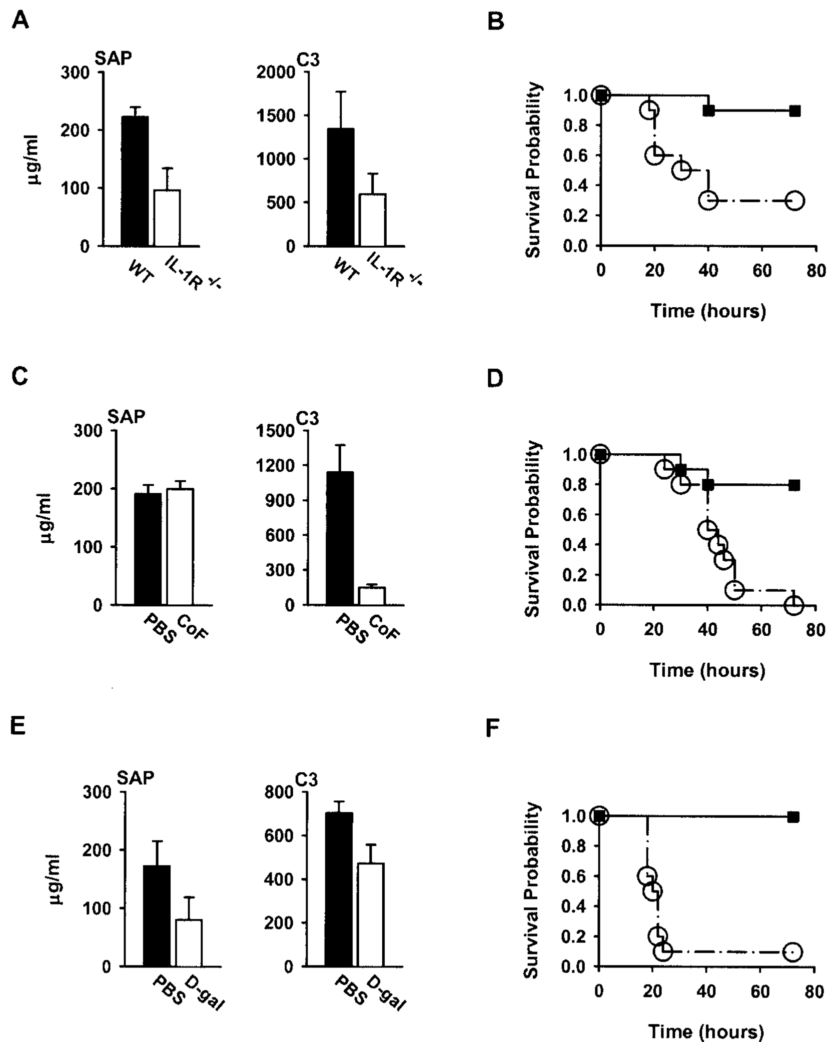

FIGURE 5. The effect of reduction in acute phase protein concentrations on protection against infection conferred by the acute phase response. *A*, Compared with wild type (filled bar), IL-1R$^{-/-}$ mice (open bar) had significantly reduced serum SAP and C3 levels at 24 h after s.c. injection of casein. *B*, Survival of IL-1R$^{-/-}$ mice given s.c. casein (■) 24 h before infection with *E. coli* was significantly greater than that of mice receiving control buffer (○). *C*, In wild-type BALB/c mice, 10 U of cobra venom factor (CoF, open bar) at the same time as receiving an s.c. casein injection significantly depleted serum C3 levels measured 24 h later, but did not affect serum SAP levels. Controls (filled bar) received casein alone. *D*, Among mice treated with cobra venom factor, those given s.c. casein (■) 24 h before i.v. infection with *E. coli* showed significantly greater survival than mice receiving control buffer (○). *E*, 10 mg of D-galactosamine (D-gal) given to wild-type BALB/c mice at the same time as an s.c. casein injection significantly reduced serum levels of SAP and C3 measured 24 h later (open bar), compared with those of controls given casein alone (filled bar). *F*, Among mice treated with D-galactosamine, those given s.c. casein (■) 24 h before i.v. infection with *E. coli* showed significantly greater survival than mice receiving control buffer (○). Bars represent the mean (±SD) of groups of five mice. Significant differences in *A*, *C*, and *E* were $p < 0.05$ (Mann-Whitney test), and in *B*, *D*, and *E* they were $p < 0.01$ (log rank test). Each survival curve is typical of results from at least two identical experiments.

Neutrophil function in the acute phase response

The major effect of an existing acute phase response was clearly to enhance host capacity to kill and clear virulent bacteria. Neutrophils are the major cell type primarily involved in defense against pyogenic bacteria, but there was no difference in circulating neutrophil counts between control mice and those receiving casein 30 min, 12 h, or 24 h previously, nor was there a difference between these two groups at 1, 3, 6, or 12 h after i.v. infection with *E. coli* (data not shown). However, the neutrophils from casein-pretreated mice showed significantly enhanced phagocytosis of bacteria in vitro and greater respiratory burst responses than neutrophils from controls, suggesting that they may have increased capacity for killing bacteria (Fig. 6, *A* and *B*). Furthermore, there was significantly enhanced neutrophil recruitment and accumulation within the tissues of the casein-pretreated animals in vivo (Fig. 6*C*), suggesting that whole body neutrophil numbers may have been higher.

Role of G-CSF in resistance to infection associated with the acute phase response

This critical priming of neutrophil function was most likely to be mediated by cytokines. Therefore, we measured serum levels of candidate cytokines after casein injection. As expected, there was a rise in IL-6, which is the major mediator of increased production of acute phase proteins by the liver (15). Serum IL-6 values peaked at 6 h and normalized by 24 h (Fig. 7*A*). The circulating concentration of G-CSF also rose and, in contrast to IL-6, detectable levels were sustained at 24 h. However, no TNF-α, IL-1β, IFN-γ, or GM-CSF were detected in the serum at the sensitivities of the assays used, nor were any cytokines detected in the serum of control mice receiving buffer alone. The G-CSF response after casein injection was intact in IL-1R$^{-/-}$ mice, indicating that IL-1 activity is not required.

G-CSF is a variably glycosylated, 20- to 23.5-kDa monocyte/macrophage-derived cytokine with well-documented effects on neutrophils. It increases circulating neutrophil counts by mobilizing cells from bone marrow storage pools and by stimulating differentiation of committed stem cells. It also has wide-ranging effects on neutrophil phenotype, increasing cell surface expression of complement and FcR and enhancing phagocytic activity, respiratory burst responses, chemotaxis, and recruitment (16). The increase in serum G-CSF after injection of casein therefore suggests a mechanism for priming of neutrophil function in our model. Interestingly, casein injection did not affect circulating neutrophil counts within 24 h, suggesting that higher or more sustained levels of G-CSF may be necessary for this effect.

Mice with targeted deletion of the G-CSF gene are severely neutropenic (17) and cannot be used in the present models of infection to analyze underlying mechanisms. Therefore, we used specific neutralizing Ab to G-CSF to block its effects in vivo after injection of casein. This had no effect on neutrophil counts or the rise in serum acute phase proteins (data not shown), but it completely abrogated the protective effect against i.v. infection with *E.*

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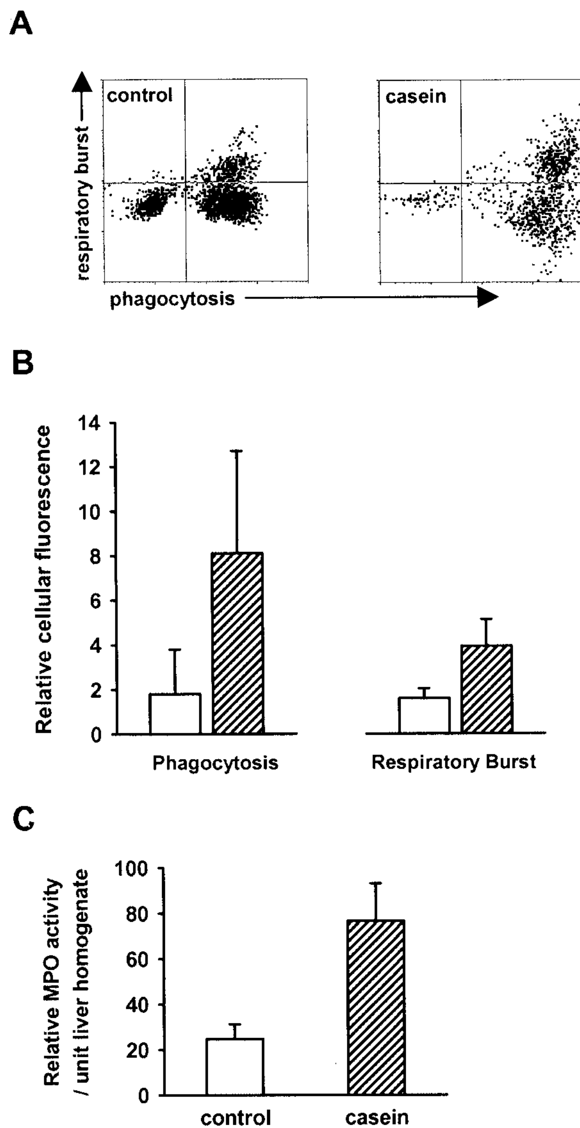

FIGURE 6. Comparison of neutrophil function in casein-pretreated and control mice. *A*, Flow cytometric analysis of neutrophil phagocytosis and respiratory burst responses in blood from casein-pretreated or control BALB/c mice. Cells with increased green fluorescence (*x*-axis on dot plots), by virtue of association with GFP-expressing *E. coli*, and increased red fluorescence (*y*-axis on dot plots), demonstrating a respiratory burst response to phagocytosis, were included in the analysis (outlined by the rectangular boundaries), thereby excluding clumps of bacteria or cells with passively adhering bacteria. *B*, Relative phagocytosis and respiratory burst responses by neutrophils quantified by comparison of mean cellular green and red fluorescence from casein-pretreated mice (▨) were significantly greater than those from controls (□). *C*, Neutrophil accumulation into liver tissue 3 h after infection of BALB/c mice with *E. coli* demonstrated significantly greater neutrophil recruitment in casein-pretreated mice (▨) compared with controls (□). Bars represent the mean (±SD) of groups of five mice; $p < 0.05$ (Mann-Whitney test).

coli (Fig. 7*B*). The key role of G-CSF itself in mediating protection was then directly confirmed by pretreating mice with a single s.c. injection of 1 μg of recombinant murine G-CSF or control buffer 24 h before infection. Pretreatment with G-CSF alone, which produced no stimulation of acute phase plasma protein production, provided the same significant survival benefit, as the casein induced acute phase response (Fig. 7*C*). G-CSF was similarly effective when given at 3 or 6 h before infection, but not 48–72 h before, immediately before, or at 2 h after infection (Fig. 8).

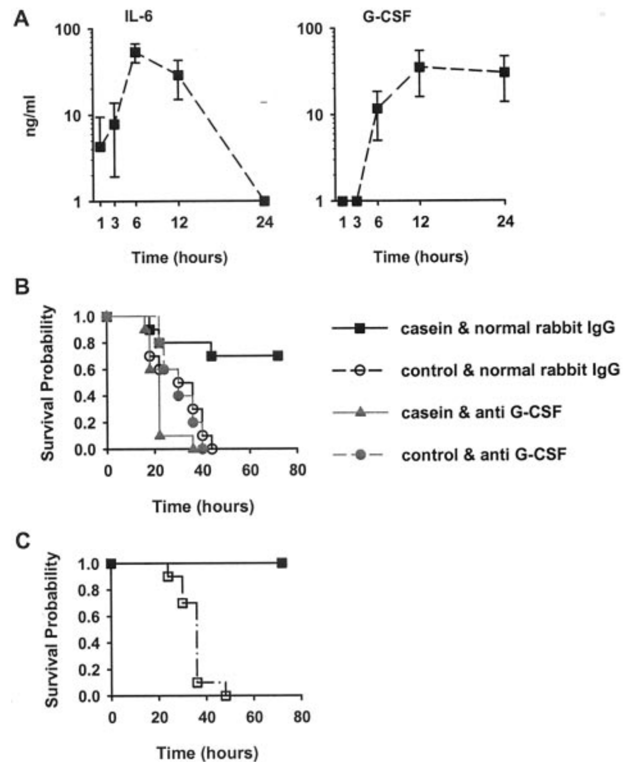

FIGURE 7. The role of G-CSF in casein-induced protection against infection. *A*, After s.c. injection of casein in BALB/c mice, serum levels of IL-6 were detectable by 1 h and peaked by 6 h. Serum levels of G-CSF were raised by 12 h and maintained at 24 h. TNF-α, IL-1β, IFN-γ, and GM-CSF were not detected. *B*, BALB/c mice were given rabbit IgG anti-G-CSF Abs or normal rabbit IgG at the same time as receiving casein or control buffer, 24 h before i.v. infection with *E. coli*. The neutralizing Ab to G-CSF totally abrogated the protective effect of pretreatment with casein. *C*, BALB/c mice receiving a single s.c. injection of recombinant murine G-CSF (■) 24 h before infection with *E. coli* were completely protected in contrast to mice receiving control buffer (□), in which there was 100% mortality ($p < 0.001$, log rank test). Results are representative of at least two experiments with 10–20 mice per group.

Discussion

The present finding that local inflammation, such as that produced by a local infection or by an injury likely to breach barrier defenses against bacterial invasion, evokes a potent response nonspecifically protective against systemic infection is of considerable evolutionary significance and is potentially of clinical value. A number of previous studies have similarly demonstrated in murine models that pretreatment with a proinflammatory stimulus, such as muramyl dipeptides derived from Gram-positive cell walls, glucans derived from yeast cell walls, or IL-1, can enhance host resistance to subsequent bacterial infection (4). We have presented a new model in which IL-1 is clearly not a major mediator. In most studies using nonspecific stimuli, no other specific host factor has been identified as the critical mediator of host defense against bacterial infection. A recent study demonstrating increased resistance to *K. pneumoniae* infection after pretreatment with turpentine, another classical acute phase stimulant in murine models, suggested a major role for the acute phase protein α_1-acid glycoprotein (18). However, in our model, nonspecific inhibition of acute phase protein synthesis did not diminish the protective effect of pretreatment with casein.

Use of nonspecific proinflammatory stimuli in previous reports has not led to the development of a therapeutic strategy to increase

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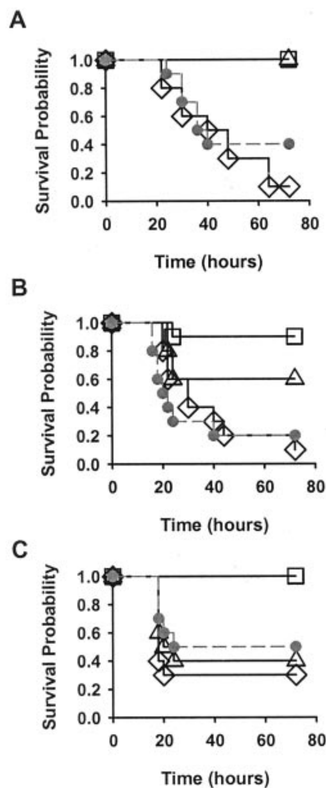

FIGURE 8. The effect of timing of G-CSF administration on protection against infection. *A*, BALB/c mice receiving a single s.c. injection of recombinant murine G-CSF (□) 24 h or (△) 6 h before infection with *E. coli* had significantly greater survival than those receiving G-CSF 2 h after infection (◇) or control mice (◍). *B*, BALB/c mice receiving a single s.c. injection of recombinant murine G-CSF (□) 6 h or (△) 3 h before infection with *E. coli* had significantly greater survival than those receiving G-CSF at the time of infection (◇) or control mice (◍). *C*, BALB/c mice receiving a single s.c. injection of recombinant murine G-CSF (□) 24 h before infection with *E. coli* had significantly greater survival than control mice (◍) and those receiving G-CSF 48 (△) h or 72 (◇) h before infection. In all experiments there were 10 mice per group and control groups were not given G-CSF. The significance of all differences cited was $p < 0.01$ by the log rank test.

the resistance of patients at risk of bacterial infections. Our study demonstrates clearly that G-CSF is a cytokine component of the acute phase response and is the critical mediator therein responsible for enhancing nonspecific host resistance to bacterial infection. Our results confirm and extend previous studies in which pretreatment with supraphysiological doses of exogenous G-CSF provided nonspecific protection against bacterial infection. These include rodent models of *P. aeruginosa* (19, 20), *K. pneumoniae* (21), and *S. pneumoniae* (22) infection and polymicrobial sepsis after cecal ligation and puncture (23). However, the present model significantly demonstrates that endogenous production of G-CSF can induce this protective effect, and it highlights the physiological importance of this cytokine as part of the acute phase response.

Our results are of particular importance because they suggest an immediately applicable strategy for increasing host resistance to infection in clinical practice. Although pretreatment here with G-CSF effectively improved survival in subsequent bacterial infection, administration of G-CSF at the time of infection or afterward provided no benefit. This is consistent with clinical studies in a number of different contexts where concurrent treatment of infection with G-CSF has been of little benefit. For example, G-CSF is widely used after cytotoxic chemotherapy (24), but although it is effective at reducing the duration of neutropenia, it has had no impact on morbidity or mortality (25). G-CSF has also recently been evaluated as an adjunctive agent for treatment of infection in nonneutropenic patients, but with mixed results. The addition of G-CSF to standard therapy in severe community-acquired pneumonia had no beneficial effect (26), although in diabetic patients with foot infections, administration of G-CSF accelerated resolution of cellulitis, reducing antibiotic use and hospital length of stay (27). Our findings suggest that pretreatment with G-CSF before infection becomes manifest, particularly in patients at risk such as those undergoing surgical procedures, may be an effective adjunct or alternative to antibiotic prophylaxis in preventing bacterial infection.

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