The Journal of Immunology

RESEARCH ARTICLE | OCTOBER 15 2000

Increased Resistance Against Acute Polymicrobial Sepsis in Mice Challenged with Immunostimulatory CpG Oligodeoxynucleotides Is Related to an Enhanced Innate Effector Cell Response[1] FREE

Heike Weighardt; ... et. al

J Immunol (2000) 165 (8): 4537–4543.
https://doi.org/10.4049/jimmunol.165.8.4537

Increased Resistance Against Acute Polymicrobial Sepsis in Mice Challenged with Immunostimulatory CpG Oligodeoxynucleotides Is Related to an Enhanced Innate Effector Cell Response[1]

Heike Weighardt,* Carolin Feterowski,* Martin Veit,* Martina Rump,* Hermann Wagner,† and Bernhard Holzmann[2]*

Recent reports support the concept that the major defect in polymicrobial sepsis is an impaired immunologic response to infection. Oligodeoxynucleotides containing CpG sequence motifs (CpG-ODN) were previously shown to induce immune protection in models of chronic infection with intracellular bacteria, parasites, and viruses due to their ability to augment IFN-γ-dependent Th1 responses. Here, we demonstrate that challenging mice with CpG-ODN substantially increases the resistance against acute polymicrobial sepsis. Systemic levels of IL-12, IL-18, and IL-10 were not altered in CpG-ODN-treated mice as compared with controls. In contrast, administration of CpG-ODN resulted in a strongly enhanced accumulation of neutrophils at the primary site of infection. Neutrophils of CpG-ODN-treated mice exhibited an up-regulation of phagocytic receptors, an increased phagocytic activity, and an elevated production of reactive oxygen metabolites. These results suggest that the protective effects of CpG-ODNs in acute polymicrobial sepsis are related to an enhanced effector cell response of innate immunity. CpG-ODN may therefore represent potent agents for the treatment of sepsis-associated immunoparalysis. ***The Journal of Immunology,*** **2000, 165: 4537–4543.**

The current paradigm for the pathogenesis of sepsis, which is based on the development of organ injury as a result of an uncontrolled inflammatory response, has been called into question by the failure of multiple clinical trials based on antiinflammatory treatment strategies (1–5). Rather, numerous recent studies support the alternative concept that the major defect in sepsis is an impaired immunologic response to infection. Analysis of peripheral blood T cells and monocytes during sepsis revealed that an impaired cytokine production correlated with mortality and that defective cellular functions may develop as a primary response to sepsis without preceding immune hyperactivity (6–8). Additional clinical investigations, which examined cellular functions before infection, have demonstrated that a reduced monocyte IL-12 production may predispose patients for the development of severe septic complications (9, 10). Based on the concept of sepsis-associated immunoparalysis a clinical pilot trial was performed recently, in which IFN-γ was applied to sepsis patients with severely reduced monocyte expression of MHC class II proteins (11).

Bacterial DNA is discriminated from host-derived self DNA by pattern recognition systems of the innate immune system (12–14). Unmethylated CpG dinucleotides in the context of particular flanking sequences (CpG motifs) are regarded as the structural basis for this specific recognition. In contrast to prokaryotic DNA, vertebrate DNA exhibits a low abundance of CpG dinucleotides and a high degree of cytosine methylation. Bacterial DNA and oligodeoxynucleotides (ODN)[3] containing CpG motifs (CpG-ODN) trigger macrophages and dendritic cells to up-regulate MHC class II and costimulatory molecules and to secrete inflammatory cytokines such as TNF, IL-1, IL-6, and IL-12 (15–20). CpG DNA also activates humoral immunity by inducing B cell proliferation and secretion of IL-6 and IgM (21, 22). Cell activation by CpG DNA requires the endosomal uptake of DNA and the rapid stimulation of the stress kinase pathways and NF-κB (16, 23). Moreover, CpG DNA augments the IL-12-stimulated production of IFN-γ by NK cells (24). Recent reports also show that CpG DNA acts as strong adjuvant to promote Ag-specific responses and to enhance protective Th1 immune reactions in models of chronic infection with intracellular bacteria and parasites as well as viruses (25–30). However, excessive or inappropriate exposure to CpG DNA may also result in adverse effects such as septic shock and pulmonary inflammation (15, 16, 31).

The present study investigated the effects of CpG-ODN administration on the immune response against acute polymicrobial sepsis. We demonstrate that pretreatment of mice with CpG-ODN substantially improves host defense and that the protective effects of CpG-ODNs in this model are associated with an enhanced effector cell response of innate immunity. Thus, DNA sequences containing CpG motifs may represent valuable and potent agents for the treatment of sepsis-associated immunosuppression.

Materials and Methods

Materials

Phosphothioate modified ODN were custom synthesized by MWG Biotec (Munich, Germany). The sequence of the CpG-ODN equals ODN 1668 and was 5′-TTCATGACGTTCCTGATGCT-3′ (21). The sequence of the control ODN (5′-GCTTGATGACTCAGCCGGAA-3′) does not contain

*Department of Surgery, Klinikum rechts der Isar, and †Institute of Medical Microbiology, Immunology and Hygiene, Technische Universität, Munich, Germany

Received for publication May 4, 2000. Accepted for publication July 24, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant Si 208/5-4 from the Deutsche Forschungsgemeinschaft.

[2] Address correspondence and reprint requests to Dr. Bernhard Holzmann, Department of Surgery, Klinikum rechts der Isar, Technische Universität, Ismaninger Strasse 22, 81675 München, Germany. E-mail address: holzmann@nt1.chir.med.tu-muenchen.de

[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide; CASP, colon ascendens stent peritonitis.

0022-1767/00/$02.00

CpG motifs and equals ODN AP-1 (32). The control ODN was chosen, because it did not show any biological activity in various experimental systems tested (32, 33). Specific Abs and isotype-matched Ig controls were purchased from PharMingen (Hamburg, Germany) or Serotec (Darmstadt, Germany). In this study, rat Abs against murine Ly6G/Gr-1 (RB6-8C5), Mac-1 (M1/70), CD16/CD32 (2.4G2), and CD13 (R3-242) were used. LPS from *Escherichia coli* (serotype O127:B8) and PMA were obtained from Sigma (St. Louis, MO).

Animal model of acute polymicrobial peritonitis

Female BALB/c mice were purchased from Harlan Winkelmann GmbH (Borchen, Germany). Mice were bred and maintained under specific pathogen-free conditions. Experimental animals were used at 8–10 wk of age. To induce polymicrobial sepsis, the colon ascendens stent peritonitis (CASP) procedure was performed as described previously (34). Briefly, a 16-gauge venous catheter was prepared by creating a notch at a distance of 2 mm from the orifice. One millimeter beyond, the catheter was cut with a scalpel. Following laparotomy, the colon ascendens was exteriorized and a 7/0 ethilon thread (Ethicon, Nordersted, Germany) was stitched through the antimesenteric portion of the colon ascendens ~10 mm distal of the ileocecal valve. The prepared venous catheter was punctured antimesenterically through the colonic wall, proximal of a pretied knot, and fixed. A second dication was performed proximal of the stent. The inner needle was removed and the stent was cut at the prepared site. To ensure proper intraluminal position of the stent, stool was milked from the cecum into the colon ascendens, until a drop of about 1 mm in diameter appeared. Fluid resuscitation of animals was performed by flushing 0.5 ml of sterile saline solution into the peritoneal cavity before closure of the abdominal wall.

The CASP procedure was developed as a model for postoperative septic peritonitis. It was shown that survival of CASP is dependent on IFN-γ and IL-12 (9, 34). Importantly, investigation of surgical patients revealed a direct correlation between the development and mortality of postoperative intraabdominal infection and the monocyte production of IL-12 (7, 9). These observations therefore suggest that the CASP model reflects important aspects of host defense during postoperative intraabdominal infection.

Treatment protocol

ODNs were dissolved in PBS and 10 nmol (64 μg) were injected i.p. 6 days before ex vivo analysis or CASP surgery. In some control experiments, mice received PBS 6 days before analysis.

Previous work has shown that the protective effects of CpG-ODN pretreatment develop within 2–3 days and persist for at least 2 wk (25–28). Therefore, we argued that alterations of the resistance against septic peritonitis may have developed by day 6 and would persist throughout the experiments.

Bacterial load of peripheral organs

Mice were sacrificed 20 h after CASP surgery, and peritoneal lavage fluid was collected. Lungs, livers, and spleens were removed and homogenized in 10 ml sterile PBS. Serial dilutions of organ homogenates in PBS were plated on blood agar plates (Becton Dickinson, Heidelberg, Germany). CFU were counted after incubation at 37°C for 24 h and calculated as CFU per whole organ.

Cytokine analysis

Serum samples were collected either 6 days after ODN treatment or 20 h after CASP surgery. Spleen cell suspensions were plated on plastic dishes, and adherent cells were collected after incubation at 37°C for 1 h. Adherent spleen cells (1×10^6) were stimulated either with 50 ng/ml LPS, 1 μM (6.4 μg/ml) CpG-ODN, or 1 μM (6.4 μg/ml) control ODN for 16 h, and supernatants were collected. Cytokine levels in serum or supernatants were determined by specific ELISA according to the manufacturer's protocols. Except for IL-10 (Endogen, Woburn, MA), all ELISA kits were purchased from R&D Systems (Minneapolis, MN). The levels of sensitivity were 5 pg/ml for TNF, 4 pg/ml for IL-12, 8 pg/ml for IL-18, and 12 pg/ml for IL-10.

Flow cytometry

For analysis of peripheral blood leukocytes, heparinized blood was collected, erythrocytes were lysed for 5 min in lysis buffer (155 mM ammonium chloride, 15 mM sodium bicarbonate, 1 mM EDTA), and cells were used for Ab staining. In addition, peritoneal exudate cells were analyzed. Cells were incubated with fluorochrome-conjugated mAbs for 30 min at 4°C in PBS containing 1% BSA. In each experiment, appropriate isotype-matched controls were included. After washing with PBS fluorescence of unfixed cells was analyzed on an EPICS XL flow cytometer (Coulter, Hialeah, FL).

Oxidative burst and phagocytosis

Production of oxygen metabolites was assessed by a flow cytometric method as described (35). Briefly, heparinized blood from CpG and control ODN-treated mice was collected, and erythrocytes were lysed as described above. Leukocytes were washed in HBSS and loaded for 15 min at 37°C with 60 μM dihydrorhodamine 123. After addition of 2.5 μM sodium azide and 0.05 mM cytochalasin B, the cells were incubated with 500 ng/ml PMA for 20 min at 37°C and flow cytometry analysis was performed immediately thereafter. Alternatively, leukocytes were incubated with FITC-labeled and opsonized *E. coli* (Orpegen Pharma, Heidelberg, Germany) at 37°C for 10 min. Thereafter, fluorescence of bacteria adhering to the cells was quenched, and cells were washed and fixed with 0.1% paraformaldehyde. Phagocytosis rate was measured by flow cytometry using a Epics XL cytometer (Coulter, Hialeah, FL).

Statistical analysis

Statistical analysis of the data was performed using the Student's t test except for survival data, which were analyzed using the log rank test. The level of significance was set at $p < 0.05$.

Results

Improved survival of acute polymicrobial sepsis in CpG-ODN-treated mice

To determine whether treatment of mice with CpG-ODN modulates the host defense against acute polymicrobial peritonitis, mice were injected with 10 nmol CpG-ODN or control ODN, and, 6 days later, the CASP procedure (34) was performed. The results in Fig. 1 show that survival of CpG-ODN-treated mice was significantly improved compared with mice treated with control ODN ($n = 18$ for CpG-ODN treatment and $n = 17$ for controls). Survival of control ODN-treated mice, however, was not significantly different from mice receiving PBS ($n = 12$) as determined by log rank test ($p = 0.626$). To further corroborate the protective effects of CpG-ODN, the bacterial clearance in the peritoneal cavity and in peripheral organs (spleen, lungs, and liver) of CpG and control ODN-treated mice was determined. We observed that, 20 h after CASP, significantly lower numbers of viable bacteria were present in peritoneal cavity, spleen, and lungs of mice injected with CpG-ODN as compared with controls (Fig. 2). Although the bacterial numbers in the livers of CpG-ODN-treated mice were about 2-fold lower that in control mice, this difference did not reach statistical significance (Fig. 2). Collectively, these results demonstrate that

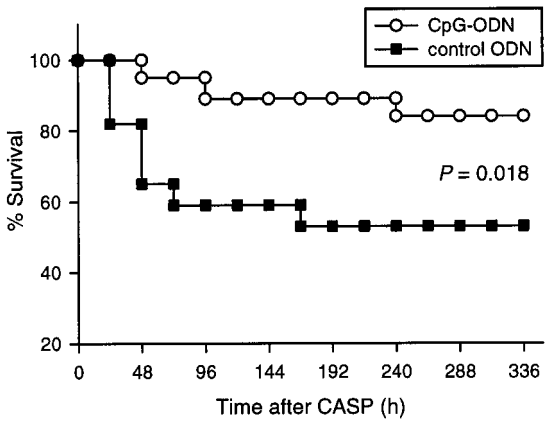

FIGURE 1. Improved survival of acute polymicrobial peritonitis following CpG-ODN treatment of mice. Mice were injected i.p. with a single dose of 10 nmol of CpG-ODN (○, $n = 18$) or control ODN (■, $n = 17$) 6 days before CASP. Survival was monitored over a period of 14 days. Three independent experiments were performed, and the data were pooled. Each experiment demonstrated an improved survival of CpG-ODN-treated mice as compared with controls. Statistical significance was determined using the log rank test.

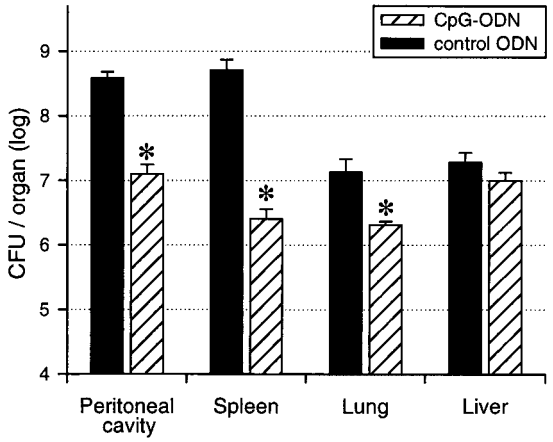

FIGURE 2. Increased bacterial clearance in CpG-ODN-treated mice. Mice were injected i.p. with 10 nmol of CpG-ODN (▨) or control ODN (■), and, 6 days later, the CASP procedure was performed. Peritoneal lavage fluid, lungs, spleens, and livers were obtained 20 h after CASP and organ homogenates were prepared. Bacterial numbers were determined by plating serial dilutions of each homogenate on blood agar. The results are represented as mean ± SEM and are derived from six to eight mice per treatment group that were analyzed in two independent experiments. *, $p < 0.05$ (CpG-ODN vs control ODN).

treatment of mice with CpG-ODN induces a state of increased resistance to acute polymicrobial peritonitis.

Effect of CpG-ODN treatment on systemic cytokine levels

CpG-ODN exhibit protective effects in models of chronic infection with intracellular pathogens that are related to increased Th1 responses (25–30). In addition, survival of acute polymicrobial sepsis in the CASP model was shown to be dependent on IFN-γ (34). Therefore, we analyzed the effects of CpG-ODN treatment on the systemic levels of the IFN-γ-regulating cytokines IL-12 and IL-18 as well as on IL-10 production 20 h after CASP. As depicted in Table I, baseline serum levels of IL-12, IL-18, and IL-10 were not significantly different between CpG and control ODN-treated mice. CASP surgery up-regulates production of IL-10 and IL-18, but not of IL-12 in both treatment groups (Table I). Again, no significant difference was observed between CpG-ODN-treated mice and controls (Table I). Serum levels of IFN-γ were below the limits of detection in both groups (data not shown). These results show that CpG-ODN treatment does not lead to alterations of systemic cytokine levels in response to acute polymicrobial sepsis.

Table I. *Effects of CpG-ODN treatment on serum cytokine levels during CASP* [a]

Treatment	Cytokine Levels (pg/ml)		*p* Values
	Control ODN	CpG-ODN	CpG vs control ODN
CASP 0 h			
IL-12	470 ± 118	390 ± 100	0.63
IL-18	335 ± 41	268 ± 18	0.19
IL-10	<12	45 ± 18	0.06
CASP 20 h			
IL-12	298 ± 85*	309 ± 59*	0.92
IL-18	740 ± 199†	669 ± 140†	0.72
IL-10	1814 ± 199‡	1321 ± 376†	0.25

[a] Mice were injected i.p. with CpG-ODN or control ODN. After 6 days, serum samples were obtained either from mice prior to CASP (CASP 0 h) or from mice 20 h after CASP. The contents of IL-12, IL-18, and IL-10 were determined by specific ELISA. Values represent the mean ± SD of four to eight individual mice in each group that were analyzed in three independent experiments.

*, NS (CASP 0 h vs CASP 20 h); †, $p < 0.05$ (CASP 0 h vs CASP 20 h); ‡, $p < 0.01$ (CASP 0 h vs CASP 20 h).

Local increase of neutrophil accumulation during polymicrobial sepsis in CpG-ODN-treated mice

To further elucidate potential mechanisms of immune protection by CpG-ODN treatment, the local inflammatory response was examined. CpG-ODN administration without additional infectious stimulus caused a moderate increase in total leukocyte numbers or Gr-1$^+$ cells (Fig. 3). Leukocyte counts in peripheral blood were not altered by CpG-ODN or control ODN treatment (data not shown). When the CASP procedure was performed, peritoneal leukocyte accumulation was observed in both treatment groups 20 h later. It should be noted, however, that cell numbers were strongly elevated in CpG-ODN-treated mice as compared with controls (Fig. 3*A*). Fig. 3*B* shows that in both groups most of the accumulating cells were neutrophils as identified by the expression of both Gr-1 and Mac-1 (36). Thus, 20 h after CASP, there were about 4-fold more neutrophils in the peritoneal cavities of CpG-ODN than of control ODN-treated mice.

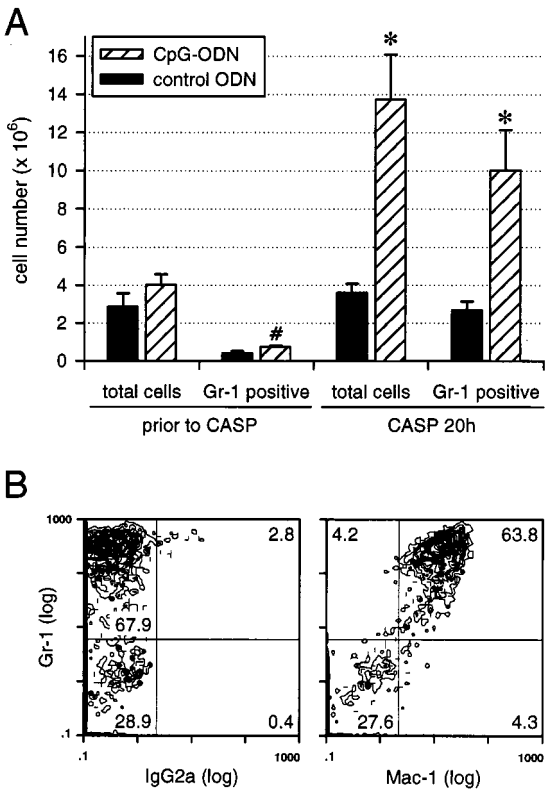

FIGURE 3. Enhanced peritoneal neutrophil accumulation in CpG-ODN-treated mice. Mice were injected i.p. with 10 nmol of CpG-ODN (▨) or control ODN (■), and, 6 days later, peritoneal lavage fluid was harvested either from untreated mice (prior to CASP) or 20 h after CASP. *A*, Total cell numbers were counted and the numbers of cells that stained positive for Gr-1 out of all peritoneal cells were determined by flow cytometry. Results are presented as mean ± SEM and are derived from five to eight mice per group that were analyzed in three independent experiments. *B*, Representative histograms show staining with control IgG2a (*left*) and expression of Mac-1 (*right*) on Gr-1$^+$ peritoneal cells that were isolated from CpG-ODN-treated mice 20 h after CASP. Numbers indicate the percentage of cells in each quadrant. *, $p < 0.005$ and #, $p < 0.05$ (CpG-ODN vs control ODN).

As shown in Fig. 4, administration of CpG-ODN also affected the expression of phagocyte receptors on peritoneal neutrophils. Expression of CD16/CD32, Mac-1, and CD13 was elevated on Gr-1$^+$ peritoneal cells of CpG-ODN-treated mice when compared with controls. Expression of CD16/CD32 and Mac-1, but not of CD13 was further increased 20 h after CASP surgery (Fig. 4, *A* and *B*). Importantly, the increase in CD16/CD32 and Mac-1 expression following CASP was significantly greater in CpG-ODN than in control ODN-treated mice (Fig. 4, *A* and *B*). Fig. 4*C* depicts representative histograms for Mac-1 expression on peritoneal Gr-1$^+$ cells following CASP, showing increased surface density of Mac-1 in CpG-ODN-treated mice as compared with controls. These results indicate that treatment of mice with CpG-ODN leads to an up-regulation of neutrophil phagocytic receptors at the primary site of infection.

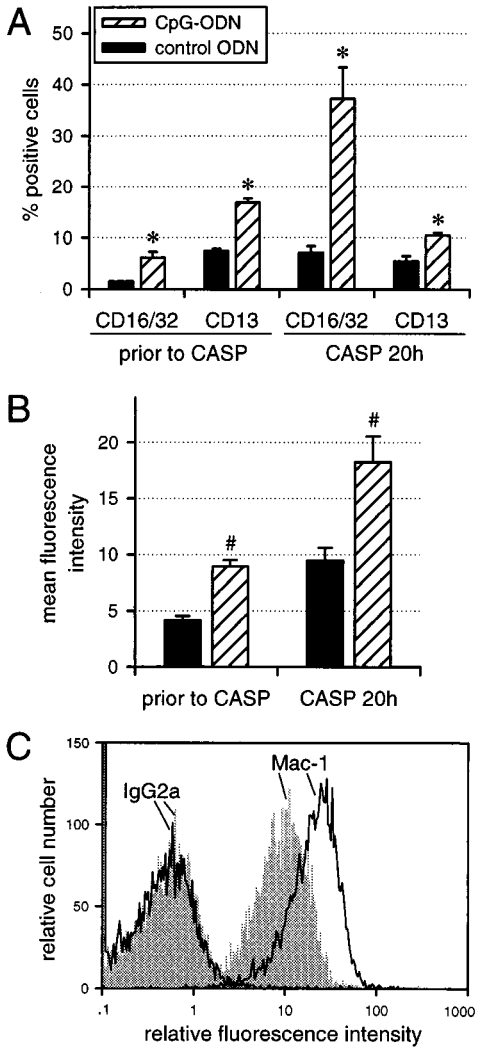

FIGURE 4. Increased expression of Fc receptors and Mac-1 on peritoneal neutrophils after CpG-ODN treatment. Mice were injected i.p. with 10 nmol of CpG-ODN or control ODN and peritoneal cells were obtained 6 days later, either from untreated mice (prior to CASP) or from mice 20 h after CASP. Expression of CD16/CD32 and CD13 (*A*) as well as Mac-1 (*B*) was determined on all Gr-1$^+$ peritoneal cells. Values are presented as percentage of positive cells (CD16/32 and CD13) or mean fluorescecence intensities (Mac-1) after subtraction of values for isotype-matched control IgG. Results are given as mean ± SEM and are derived from four to six mice per group that were analyzed in three independent experiments. *C*, Representative histograms show Mac-1 expression or staining with control IgG2a on Gr-1$^+$ peritoneal cells that were isolated from mice treated with CpG-ODN (solid lines) of control ODN (shaded graphs) 20 h after CASP. *, $p < 0.05$; #, $p < 0.005$ (CpG-ODN vs control ODN).

Improved oxidative burst and phagocytosis of peripheral blood neutrophils following CpG-ODN treatment

To determine the effects of CpG-ODN treatment on the antimicrobial functions of neutrophils, mice were injected with CpG-ODN or control ODN and, 6 days later, peripheral blood neutrophils were examined for their phagocytic capacity and their production of reactive oxygen metabolites. In these experiments, neutrophils were identified by their forward and side scatter profiles (Fig. 5*A*) to avoid a possible interference of Gr-1 and Mac-1 Abs with the functional assays. The results in Fig. 5*B* demonstrate that peripheral blood neutrophils of CpG-ODN-treated mice exhibited a significantly increased phagocytic capacity for opsonized *E. coli* when compared with cells from control mice. Moreover, the PMA-stimulated production of reactive oxygen metabolites by neutrophils was significantly greater following CpG-ODN than control ODN treatment of mice (Fig. 5*C*). Thus, priming of mice with CpG-ODN results in an improved antimicrobial activity of circulating neutrophils. Together with the results depicted in Figs. 3 and 4, these findings indicate that CpG-ODN treatment of mice leads to an amplification of the innate effector cell response against acute polymicrobial sepsis.

Effects of CpG-ODN treatment on cytokine production by mononuclear phagocytes

Pretreatment with LPS renders mice resistant against the toxic effects of a challenge with high doses of LPS (37). In addition, endotoxin tolerant mice are partially protected from the lethality induced by septic peritonitis (38, 39). Because the biological responses triggered by CpG-ODN are similar to those induced by LPS, we investigated whether treatment with CpG-ODN would alter cytokine production by mononuclear phagocytes. Mice were injected i.p. with PBS, LPS, CpG-ODN, or control ODN, and, 4–6 days later, spleens were removed. The plastic adherent fraction of splenocytes was restimulated in vitro with LPS, CpG-ODN, or control ODN, and the secretion of TNF and IL-12 was determined. The results in Fig. 6*A* indicate that TNF production of cells from mice challenged with endotoxin is diminished both after LPS and CpG-ODN restimulation. In contrast, adherence-purified spleen cells from CpG-ODN-treated mice showed an increased TNF release after CpG-ODN stimulation (Fig. 6*A*). Similar results were obtained for IL-12 secretion (Fig. 6*B*). These results show that endotoxin desensitizes adherent splenocytes against CpG-ODN-induced signals. However, treatment of mice with CpG-ODN does not induce tolerance against CpG-ODN or cross-tolerance against LPS stimulation.

Discussion

Treatment of mice with CpG-ODN was previously shown to increase the resistance against chronic infections with intracellular bacteria and parasites as well as viruses even when applied for up to 2 wk before pathogen exposure (25–30). In these models, immune protection by CpG-ODN was found to involve an elevated production of IL-12 and IFN-γ by cells of the innate immune system, which in turn may promote protective Th1 immune reactions in response to infection. The present report extends these studies and demonstrates that challenge of mice with CpG-ODN induces immune protection against acute polymicrobial sepsis. The protective effects were found to be associated with an augmented accumulation of neutrophils at the primary site of infection and an enhanced antimicrobial activity of these cells. The systemic levels

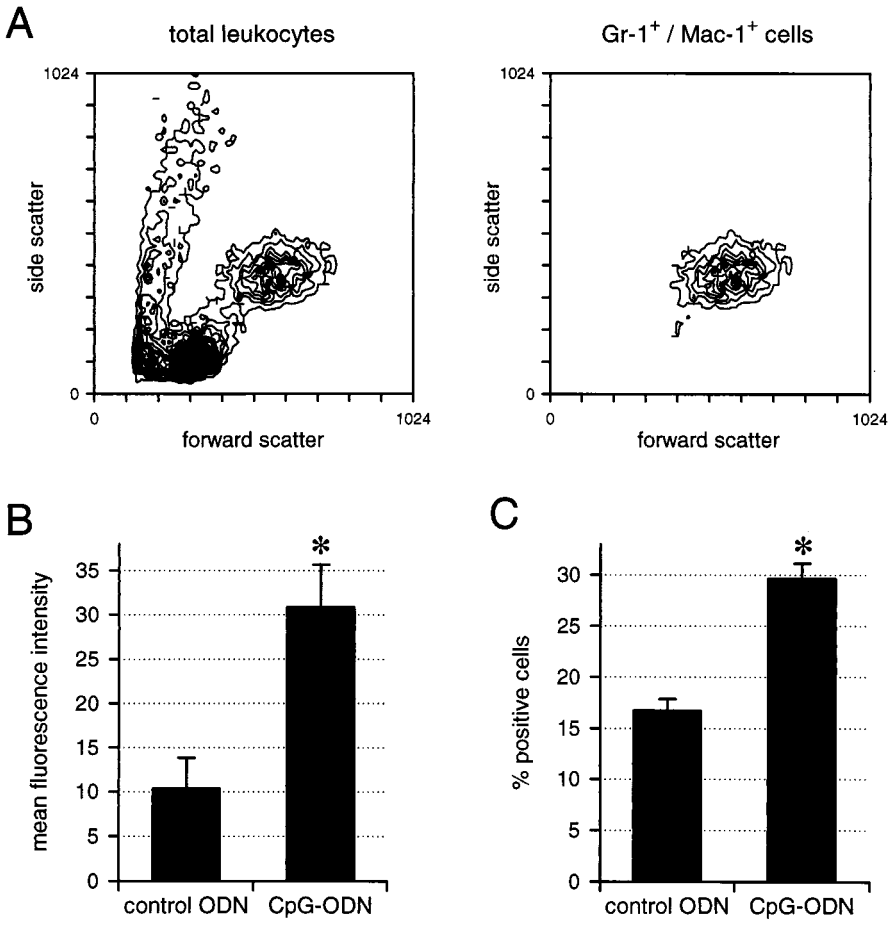

FIGURE 5. Increased antimicrobial activity of neutrophils in CpG-ODN-treated mice. Mice were injected i.p. with 10 nmol of CpG-ODN or control ODN and peripheral blood was collected 6 days later. *A*, Representative histograms show that Gr-1$^+$/Mac-1$^+$ cells (*right*) exhibit a distinct forward and side scatter profile as compared with total peripheral blood leukocytes (*left*). For analysis of oxidative burst and phagocytosis, neutrophils were identified by their light scatter profile. Staining of cells with Gr-1 and Mac-1 Abs was avoided, because the Abs may interfere with cellular functions either by triggering the cognate Ag or by Fc receptor interactions. *B*, The phagocytic activity of neutrophils was determined by incubation with fluorochrome-labeled and opsonized *E. coli*. *C*, The production of reactive oxygen intermediates was examined after PMA stimulation of cells. Phagocytosis and oxidative burst were analyzed by flow cytometry. Results are presented as mean ± SEM and are derived from three to four mice in each group that were analyzed in two independent experiments. *, $p < 0.005$ (CpG-ODN vs control ODN).

of IL-12 and IL-18, however, were not affected by CpG-ODN administration. Although locally restricted alterations of IFN-γ production cannot be excluded, these results suggest that the protective effects of CpG-ODN are not mediated by an enhanced Th1 response. Moreover, the rapid progression of infection and early mortality in the CASP model (34, 35) also indicate that host defense is mainly dependent on effector mechanisms of the innate rather than the adaptive immune system. Collectively, our observations provide strong evidence that the immune modulating activities of CpG-ODN are not restricted to the instruction and reprogramming of adaptive immunity but, in addition, may involve the amplification of the innate effector cell response during acute polymicrobial infection.

Polymicrobial sepsis induced by CASP leads to a rapid recruitment of neutrophils into the peritoneal cavity (34). CpG-ODN treatment was shown to augment this neutrophil accumulation by about 4-fold. In addition, administration of CpG-ODN increased the surface expression of Mac-1 and Fcγ receptors on resident peritoneal neutrophils and strongly enhanced the up-regulation of these receptors following CASP. Therefore, it is conceivable that the increase in neutrophil numbers and the elevated surface density of phagocytic receptors contribute to the improved bacterial clearance, which was observed in mice challenged with CpG-ODN. Thus, up-regulation of Mac-1 and Fcγ-receptors may improve phagocytosis of opsonized bacteria by neutrophils in CpG-ODN-treated mice and increased cell surface Mac-1 may facilitate cell recruitment from the circulation to the inflamed peritoneal cavity (40, 41).

In addition to phagocytosis of microorganisms, the production of antimicrobial substances, such as reactive oxygen metabolites, is a prerequisite for clearing bacterial infections (42). The importance of reactive oxygen intermediates for host defense is clearly demonstrated in patients suffering from chronic granulomatous disease, which is characterized by severe and life-threatening infections with bacteria and fungi (43, 44). Chronic granulomatous disease is caused by diverse mutations of components of the NAPDH oxidase, thereby abolishing the oxidative burst reaction of phagocytes (45). The mechanisms of immune pathology that underlie this genetic disorder were confirmed in mice deficient for p47^{phox} (46). It was therefore interesting to note that circulating neutrophils of mice treated with CpG-ODN showed an enhanced production of reactive oxygen metabolites after stimulation with PMA. Considered together, these studies support the view that an increased oxidative burst activity of neutrophils contributes to the protective effects of CpG-ODN in acute polymicrobial sepsis.

Challenging mice with CpG-ODN results in extramedullary hematopoesis and transient splenomegaly peaking at day 6 after injection (33). Spleen enlargement was associated with increased frequencies of B220/CD3 double-negative cells that contained myeloid and erythroid progenitor cells (33). In addition, CpG-ODN administration was found to accelerate the recovery of cytotoxic T cell responses and the resistance against *Listeria monocytogenes* infection after sublethal irradiation (33). In the present report, we show that treatment of mice with CpG-ODN alters the phenotype and function of neutrophils with an up-regulation of phagocytic receptors and an increased capacity to phagocytose bacteria and to produce reactive oxygen metabolites. Thus, the hematopoietic activities of CpG-ODNs might also cause alterations of the differentiation or activation state of neutrophils leading to these phenotypic changes. It is therefore conceivable that CpG-ODN may

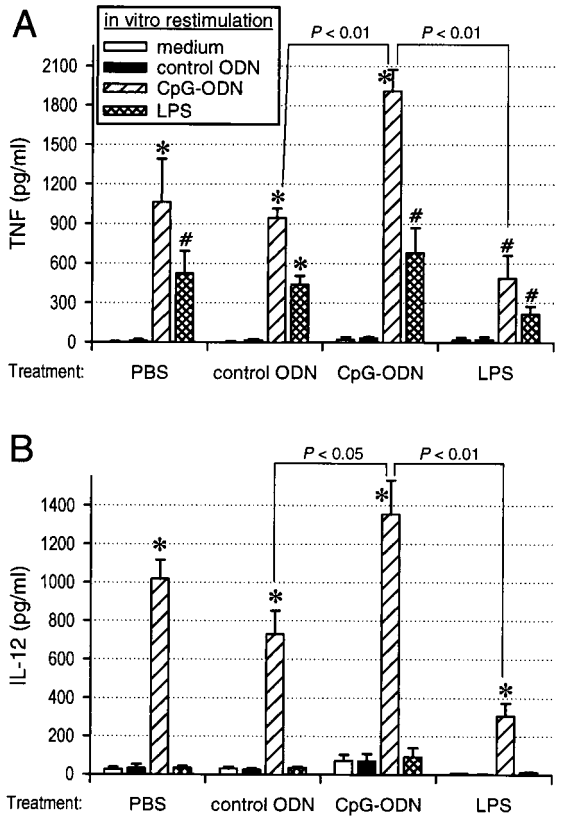

FIGURE 6. CpG-ODN treatment does not induce cross-tolerance with LPS. Mice were injected i.p. with PBS, 10 nmol of CpG-ODN, 10 nmol of control ODN, or 80 μg of LPS, and spleens were removed either 4 days (PBS and LPS treatment) or 6 days later (ODN treatment). Adherence purified splenocytes were either unstimulated (□), or stimulated in vitro with control ODN (■), CpG-ODN (▨), or LPS (▩) for 16 h. Production of TNF (*A*) or IL-12 (*B*) was quantitated by ELISA. Results are presented as mean ± SEM and are derived from four independent experiments each using cells pooled from three individual mice per group. *, $p < 0.01$ and #, $p < 0.05$ (vs medium control and control ODN of the same treatment group).

enhance immune defense against polymicrobial sepsis as a consequence of their effects on hematopoesis.

The biological responses triggered by CpG-ODNs are similar to those induced by LPS. For example, macrophage activation by CpG-ODNs and LPS involves similar signaling pathways (23, 47, 48) and results in the production of multiple inflammatory cytokines, which may cause septic shock in sensitized mice (15, 16). Because treatment of mice with LPS tolerizes against the pathologic effects of a subsequent challenge with high dose LPS (37) and protects from lethality of septic peritonitis (38, 39), we asked whether treatment of mice with CpG-ODN would lead to a similar state of reduced macrophage responsiveness. The results of the present report demonstrate, however, that challenging mice with CpG-ODN does not induce tolerance to a subsequent stimulation with CpG-ODN nor does it generate cross-tolerance with LPS. In contrast, LPS was shown to tolerize macrophages against CpG-ODN-mediated signals. These findings therefore suggest that despite certain similarities the in vivo response of macrophages to LPS and CpG-ODN also exhibits substantial differences and that the protective effects of CpG-ODN are not related to the induction of a functional status resembling LPS tolerance.

Previous work has shown that bacterial DNA and ODN containing CpG motifs may cause a TNF-dependent septic shock in mice that were sensitized with D-galactosamine (15, 16). These observations are reminiscent of studies demonstrating that lethal TNF-mediated shock is triggered by the injection of bacterial toxins such as LPS or staphylococcal enterotoxin B into sensitized mice (49, 50). However, several lines of evidence suggest that these toxin models do not correctly reflect the major pathogenic events in human sepsis. Several clinical trials failed to demonstrate beneficial effects of antiinflammatory treatment strategies or reported even adverse effects of mediator neutralization (1–5). Patient investigations have further indicated that an impaired cytokine production of monocytes and T cells is associated with an increased incidence or mortality of sepsis (6–10, 51). Moreover, mouse models of peritoneal sepsis have directly demonstrated essential protective functions of endogenously produced or exogenously administered proinflammatory cytokines (10, 34, 52–56). Collectively, these observations support the notion that immunosuppression may represent the major pathogenic process in sepsis and that therefore beneficial effects may be obtained by stimulating the proinflammatory response. The results of the present report indicating that pretreatment of mice with CpG-ODN enhances the neutrophil response and improves outcome of septic peritonitis are consistent with this concept. Nevertheless, it should be noted that an increased inflammatory response has the potential to result in adverse effects such as organ injury and shock. It is therefore conceivable that outcome is affected by a critical balance between antimicrobial and autoaggressive effects of CpG-ODN treatment. Although this balance may vary depending on the type of infection or the type of immune response triggered, our data argue that the protective effects predominate in sepsis.

In summary, the present report demonstrates that administration of CpG-ODN increases the resistance against acute polymicrobial sepsis and that immune protection is associated with an enhanced effector cell response of innate immunity. Thus, our study suggests that CpG-ODNs may represent inexpensive and potent agents for the treatment of sepsis-associated immunoparalysis.

Acknowledgments

We thank Felicitas Altmayr for expert technical assistance and Dr. Grayson Lipford for helpful discussions.

References

1. Abraham, E., A. Anzueto, G. Gutierrez, S. Tessler, G. S. Pedro, R. Wunderink, A. D. Nogare, S. Nasraway, S. Berman, R. Cooney, et al. 1998. Double-blind randomised controlled trial of monoclonal antibody to human tumour necrosis factor in treatment of septic shock. *Lancet 351:929.*
2. Fisher, C. J., J.-F. A. Dhainaut, S. M. Opal, J. P. Pribble, R. A. Balk, G. J. Slotman, T. J. Iberti, E. C. Rackow, M. J. Shapiro, R. L. Greenman, et al. 1994. Recombinant human interleukin 1 receptor antagonist in the treatment of patients with sepsis syndrome: results from a randomized, double-blind, placebo-controlled trial. *J. Am. Med. Assoc. 271:1836.*
3. Fisher, C. J., J. M. Agost, S. M. Opal, S. F. Lowry, R. A. Balk, J. C. Sadoff, E. Abraham, R. M. Schein, and E. Benjamin. 1996. Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. *N. Engl. J. Med. 334:1697.*
4. Greenman, R. L., R. M. H. Schein, M. A. Martin, R. P. Wenzel, N. R. MacIntyre, G. Emmanuel, H. Chmel, R. B. Kohler, M. McCarthy, J. Plouffe, J. A. Russell, and XOMA Sepsis Study Group. 1991. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of Gram-negative sepsis. *J. Am. Med. Assoc. 266:1097.*
5. Ziegler, E. J., C. J. Fisher, C. L. Sprung, R. C. Straube, J. C. Sadoff, G. E. Foulke, C. H. Wortel, M. P. Fink, R. P. Dellinger, N. N. H. Teng, et al. 1991. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. *N. Engl. J. Med. 324:429.*
6. Heidecke, C. D., T. Hensler, H. Weighardt, N. Zantl, H. Wagner, J. R. Siewert, and B. Holzmann. 1999. Selective defects of T lymphocyte function in patients with lethal intraabdominal infection. *Am. J. Surg. 178:288.*
7. Weighardt, H., C. D. Heidecke, K. Emmanuilidis, S. Maier, H. Bartels, J. R. Siewert, and B. Holzmann. 2000. Sepsis after major visceral surgery is associated with sustained and interferon-γ-resistant defects of monocyte cytokine production. *Surgery 127:309.*
8. Munoz, C., J. Carlet, C. Fitting, B. Misset, J.-P. Blériot, and J.-M. Cavaillon. 1991. Dysregulation of in vitro cytokine production by monocytes during sepsis. *J. Clin. Invest. 88:1747.*

9. Hensler, T., C. D. Heidecke, H. Hecker, K. Heeg, H. Bartels, N. Zantl, H. Wagner, J. R. Siewert, and B. Holzmann. 1998. Increased susceptibility to postoperative sepsis in patients with impaired monocyte IL-12 production. *J. Immunol. 161:2655*.
10. Goebel, A., E. Kavanagh, A. Lyons, I. B. Saporoschetz, C. Soberg, J. A. Lederer, J. A. Mannick, and M. L. Rodrick. 2000. Injury induces deficient interleukin-12 production, but interleukin-12 therapy after injury restores resistance to infection. *Ann. Surg. 231:253*.
11. Döcke, W. D., F. Randow, U. Syrbe, D. Krausch, K. Asadullah, P. Reinke, H. D. Volk, and W. Kox. 1997. Monocyte deactivation in septic patients: restoration by IFN-γ treatment. *Nat. Med. 3:678*.
12. Krieg, A. M., A. K. Yi, and G. Hartmann. 1999. Mechanisms and therapeutic applications of immune stimulatory CpG DNA. *Pharmacol. Ther. 84:113*.
13. Lipford, G. B., K. Heeg, and H. Wagner. 1998. Bacterial DNA as immune cell activator. *Trends Microbiol. 6:496*.
14. Wagner, H. 1999. Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv. Immunol. 73:329*.
15. Sparwasser, T., T. Miethke, G. Lipford, K. Borschert, H. Häcker, K. Heeg, and H. Wagner. 1997. Bacterial DNA causes septic shock. *Nature 386:336*.
16. Sparwasser, T., T. Miethke, G. Lipford, A. Erdmann, H. Hacker, K. Heeg, and H. Wagner. 1997. Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-α-mediated shock. *Eur. J. Immunol. 27:1671*.
17. Stacey, K. J., M. J. Sweet, and D. A. Hume. 1996. Macrophages ingest and are activated by bacterial DNA. *J. Immunol. 157:2116*.
18. Sparwasser, T., E. S. Koch, R. M. Vabulas, K. Heeg, G. B. Lipford, J. W. Ellwart, and H. Wagner. 1998. Bacterial DNA and immunostimulatory CpG oligodeoxynucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol. 28:2045*.
19. Hartmann, G., G. J. Weiner, and A. M. Krieg. 1999. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA 96:9305*.
20. Bauer, M., K. Heeg, H. Wagner, and G. B. Lipford. 1999. DNA activates human immune cells through a CpG sequence-dependent manner. *Immunology 97:699*.
21. Krieg, A. M., A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature 374:546*.
22. Klinman, D. M., A. K. Yi, S. L. Beaucage, J. Conover, and A. M. Krieg. 1996. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin-6, interleukin-12, and interferon-γ. *Proc. Natl. Acad. Sci. USA 93:2879*.
23. Hacker, H., H. Mischak, T. Miethke, S. Liptay, R. Schmid, T. Sparwasser, K. Heeg, G. B. Lipford, and H. Wagner. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J. 17:6230*.
24. Chace, J. H., N. A. Hooker, K. L. Mildenstein, A. M. Krieg, and J. S. Cowdery. 1997. Bacterial DNA-induced NK cell IFN-γ production is dependent on macrophage secretion of IL-12. *Clin. Immunol. Immunopathol. 84:185*.
25. Oxenius, A., M. M. A. Martinic, H. Hengartner, and P. Klenerman. 1999. CpG-containing oligonucleotides are efficient adjuvants for induction of protective antiviral immune responses with T-cell peptide vaccines. *J. Virol. 73:4120*.
26. Elkins, K. L., T. R. Rhinehart-Jones, S. Stibitz, J. S. Conover, and D. M. Klinman. 1999. Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J. Immunol. 162:2291*.
27. Krieg, A. M., L. Love-Homan, A. K. Yi, and J. T. Harty. 1998. CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria* monocytogenes challenge. *J. Immunol. 161:2428*.
28. Klinman, D. M., J. Conover, and C. Coban. 1999. Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. *Infect. Immun. 67:5658*.
29. Zimmermann, S., O. Egeter, S. Hausmann, G. B. Lipford, M. Rocken, H. Wagner, and K. Heeg. 1998. CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol. 160:3627*.
30. Vabulas, R. M., H. Pircher, G. B. Lipford, H. Hacker, and H. Wagner. 2000. CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. *J. Immunol. 164:2372*.
31. Schwartz, D. A., T. J. Quinn, P. S. Thorne, S. Sayeed, A.-K. Yi, and A. M. Krieg. 1997. CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J. Clin. Invest. 100:68*.
32. Lipford, G. B., T. Sparwasser, M. Bauer, S. Zimmermann, E. S. Koch, K. Heeg, and H. Wagner. 1997. Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol. 27:3420*.
33. Sparwasser, T., L. Hültner, E. S. Koch, A. Luz, G. B. Lipford, and H. Wagner. 1999. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hematopoiesis. *J. Immunol. 162:2368*.
34. Zantl, N., A. Uebe, B. Neumann, H. Wagner, J. R. Siewert, B. Holzmann, C. D. Heidecke, and K. Pfeffer. 1998. Essential role of γ interferon in survival of colon ascendens stent peritonitis, a novel murine model of abdominal sepsis. *Infect. Immun. 66:2300*.
35. Neumann, B., N. Zantl, A. Veihelmann, K. Emmanuilidis, K. Pfeffer, C. D. Heidecke, and B. Holzmann. 1999. Mechanisms of acute inflammatory lung injury induced by abdominal sepsis. *Int. Immunol. 11:217*.
36. Lagasse, E., and I. L. Weissman. 1996. Flow cytometric identification of murine neutrophils and monocytes. *J. Immunol. Methods 197:139*.
37. Greer, G. G., and E. T. Rietschel. 1978. Inverse relationship between the susceptibility of lipopolysaccharide (lipid A)-pretreated mice to the hypothermic and lethal effect of lipopolysaccharide. *Infect. Immun. 20:366*.
38. Urbaschek, B., B. Ditter, K.-P. Becker, and R. Urbaschek. 1984. Protective effects and role of endotoxin in experimental septicemia. *Circ. Shock 14:209*.
39. Astiz, M. E., A. Galera, D. C. Saha, C. Carpati, and E. C. Rackow. 1994. Monophosphoryl lipid a protects against gram-positive sepsis and tumor necrosis factor. *Shock 2:271*.
40. Jutila, M. A., L. Rott, E. L. Berg, and E. C. Butcher. 1989. Function and regulation of the neutrophil MEL-14 antigen in vivo: comparison with LFA-1 and MAC-1. *J. Immunol. 143:3318*.
41. Gresham, H. D., I. L. Graham, D. C. Anderson, and E. J. Brown. 1991. Leukocyte adhesion-deficient neutrophils fail to amplify phagocytic function in response to stimulation. *J. Clin. Invest. 88:588*.
42. Hampton, M. B., A. J. Kettle, and C. C. Winterbourn. 1998. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood 92:3007*.
43. Roos, D., M. de Boer, F. Kuribayashi, C. Meischl, R. S. Weening, A. W. Segal, A. Ahlin, K. Nemet, J. P. Hossle, E. Bernatowska-Matuszkiewicz, and H. Middleton-Price. 1996. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. *Blood 87:1663*.
44. Segal, A. W. 1996. The NADPH oxidase and chronic granulomatous disease. *Mol. Med. Today 2:129*.
45. Roos, D. 1994. The genetic basis of chronic granulomatous disease. *Immunol. Rev. 138:121*.
46. Jackson, S. H., J. I. Gallin, and S. M. Holland. 1995. The $p47^{phox}$ mouse knockout model of chronic granulomatous disease. *J. Exp. Med. 182:751*.
47. Hacker, H., H. Mischak, G. Hacker, S. Eser, N. Prenzel, A. Ullrich, and H. Wagner. 1999. Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells. *EMBO J. 18:6973*.
48. Ulevitch, R. J. 1999. Endotoxin opens the *Toll*gates to innate immunity. *Nat. Med. 5:144*.
49. Miethke, T., C. Wahl, K. Heeg, B. Echtenacher, P. H. Krammer, and H. Wagner. 1992. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J. Exp. Med. 175:91*.
50. Beutler, B., I. W. Milsark, and A. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science 229:869*.
51. Randow, F., U. Syrbe, C. Meisel, D. Krausch, H. Zuckermann, C. Platzer, and H. D. Volk. 1995. Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor β. *J. Exp. Med. 181:1887*.
52. Echtenacher, B., W. Falk, D. N. Männel, and P. H. Krammer. 1990. Requirement of endogenous tumor necrosis factor/cachectin for recovery from experimental peritonitis. *J. Immunol. 145:3762*.
53. Echtenacher, B., D. N. Männel, and L. Hültner. 1996. Critical protective role of mast cells in a model of acute septic peritonits. *Nature 381:75*.
54. O'Reilly, M., G. M. Silver, J. H. Davis, R. L. Gamelli, and J. C. Hebert. 1992. Interleukin 1β improves survival following cecal ligation and puncture. *J. Surg. Res. 52:518*.
55. Steinhauser, M. L., C. M. Hogaboam, N. W. Lukacs, R. M. Strieter, and S. L. Kunkel. 1999. Multiple roles for IL-12 in a model of acute septic peritonitis. *J. Immunol. 162:5437*.
56. Zisman, D. A., S. L. Kunkel, R. M. Strieter, J. Gauldie, W. C. Tsai, J. Bramson, J. M. Wilkowski, K. A. Bucknell, and T. J. Standiford. 1997. Anti-interleukin-12 therapy protects mice in lethal endotoxemia but impairs clearance in murine *Escherichia coli* peritoneal sepsis. *Shock 8:349*.