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Vancomycin and daptomycin modulate the innate immune response in a murine model of LPS-induced sepsis

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Abstract

Sepsis is a leading cause of death worldwide, despite the use of multimodal therapies. Common antibiotic regimens are being affected by a rising number of multidrug-resistant pathogens, and new therapeutic approaches are therefore needed. Antibiotics have immunomodulatory properties which appear to be beneficial in the treatment of sepsis. We hypothesized that the last-resort antibiotics vancomycin (VAN) and daptomycin (DMC) modulate cell migration, phagocytosis, and protein cytokine levels in a murine model of lipopolysaccharide (LPS)-induced sepsis. Ten to twelve-week-old C57BL/6 mice ($n = 4$–6 animals per group) were stimulated with LPS for 20 h, followed by the administration of VAN or DMC. The outcome parameters were leukocyte accumulation and effector function. Quantification of the immune cells in the peritoneal lavage was performed using flow cytometry analysis. Phagocytosis was measured using pHrodo *E. coli* BioParticles. The response of the cytokines TNFα, IL-6, and IL-10 was measured in vitro using murine peritoneal macrophages stimulated with LPS and VAN or DMC. VAN decreased both the peritoneal macrophage and the dendritic cell populations following LPS stimulation. DMC reduced the dendritic cell population in the peritoneal cavity in LPS-infected mice. Both antibiotics increased the phagocytic activity in peritoneal macrophages, but this effect was diminished in response to LPS. Phagocytosis of dendritic cells was increased in LPS-infected animals treated with VAN. VAN and DMC differently modulated the levels of pro-and anti-inflammatory cytokines. In a murine model of LPS-induced sepsis, VAN and DMC exhibit immunomodulatory effects on cells involved in innate immunity. The question of whether these antibiotics exhibit synergistic effects in the treatment of septic patients, beyond their bactericidal properties, should be further evaluated in future studies.

Keywords

antibiotics, dendritic cells, immunomodulatory properties, macrophages, neutrophils, phagocytosis

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Introduction

Sepsis is a global healthcare burden, and a leading cause of death.[1] The Third International Consensus Conference (Sepsis-3) re-defined sepsis as "a life-threatening organ dysfunction due to a dysregulated host response to infection".[2] The Surviving Sepsis Campaign recommends immediate administration of broad-spectrum antibiotics in patients with suspected sepsis or septic shock, an approach known as the first-hour bundle.[3] This therapeutic

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regimen is based on the traditional knowledge that antibiotic agents exert antimicrobial effects by killing invading pathogens. However, an increasing number of multidrug-resistant bacterial infections may only respond to the so-called "last-resort antibiotics".[4,5] The last-resort antibiotics vancomycin (VAN) and daptomycin (DMC) are frequently used in the treatment of multidrug-resistant gram-positive pathogens.[4]

Data from both experimental and clinical trials suggest that certain antibiotics have pro- or anti-inflammatory properties and therefore directly modulate the immune response of the host.[3,6–8] We reported in earlier studies that a variety of antibiotic agents modulate cytokine production, Toll-like receptor expression, and phagocytosis of human monocytes.[4,7,9] Other authors have demonstrated that antibiotics alter the chemotaxis of polymorphonuclear neutrophils (PMN).[10] These results strengthen the case for the immunomodulatory potential of antibiotics, and point to the underestimated role of antibiotic treatment in selectively altering the immune response of the host. We believe that new treatment strategies which involve the potential immunomodulation effects of already-approved antimicrobial drugs may help to optimize sepsis therapy in the future. The first clinical trials showed that broad-spectrum antibiotics modulated the immune response under septic conditions, and improved the outcome of individual patients.[11–14]

The interplay between the different innate immune cells—macrophages, PMNs, and dendritic cells—is of considerable importance in infectious diseases.[15] Schiwon and co-workers demonstrated that TNF-mediated crosstalk between macrophages and PMNs regulates the migration of neutrophils into sites of infection in tissues.[16] Phagocytosis is a critical part of the innate immune defense for the elimination of invading pathogens.[7] Recent in vitro studies have shown that antibiotics alter the phagocytic activity of monocytes.[4,7,9] The question of whether antibiotic agents such as VAN and DMC alter the migration of monocytes, PMNs and dendritic cells, or phagocytic activity, has not been fully answered. Further research is clearly needed.

Mouse models of sepsis do not reflect all factors associated with sepsis in humans, due to differences between the genomes of humans and mice. The various murine models available can represent different aspects of the condition, according to the focus of the study. Among the established mouse sepsis models, we chose the endotoxin administration (LPS) model, which is known to produce high levels of circulating inflammatory cytokines after the administration of lipopolysaccharide (LPS).[17]

We hypothesized that VAN and DMC alter innate immune cell migration, phagocytic activity, and cytokine responses, in a murine model of LPS-induced sepsis. We found that VAN altered the migration of both peritoneal macrophages and dendritic cells, and their phagocytic activities, in the presence of LPS. DMC affected cell migration and phagocytosis in dendritic cell populations. Both antibiotic agents altered the levels of pro-and anti-inflammatory cytokines.

Materials and methods

Ethical approval

All animal experiments were approved by the governmental ethical review board at the Ministry of Nature, Environment and Consumer Protection of the German state of Northrhine Westphalia (LANUV Recklinghausen, permit number 87-51.04.2010.A152). The present study followed international, national, and institutional guidelines for animal welfare and complied with the relevant legislation. Animal handling was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" which was published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Animal preparation and experimental procedures

We studied 10–12-week-old male C57BL/6 mice purchased from Charles River (Charles River Laboratories Germany GmbH, Cologne, Germany). All mice were kept under specific pathogen–free conditions in isolated, ventilated cages with free access to water and rodent food.

LPS (LPS, *Escherichia coli*, Serotype 055:B5 (L6529), Sigma-Aldrich, Steinheim, Germany) was injected intraperitoneally into the mice at a concentration of 10 mg/kg body weight (B.W.) (Figure 1) to induce sepsis. Antibiotics were administered 20 h after the LPS stimulation was commenced, to mirror a realistic clinical scenario. In most clinical cases, a host is first infected with the pathogen, and subsequently a delay occurs between the onset of sepsis and admission to the hospital to start antimicrobial therapy.

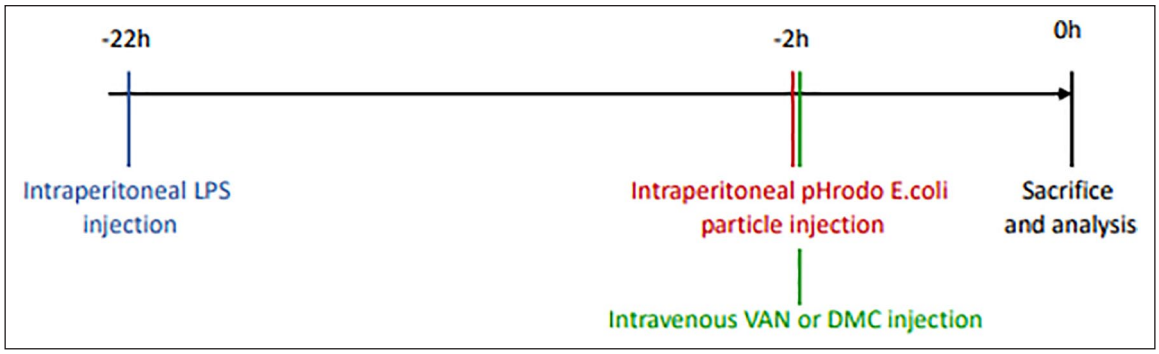

Figure 1. Summary of the experimental procedures.

The antibiotic agent was given intravenously by puncture of the tail vein with a 25 G needle. For VAN (Fresenius Kabi, Bad Homburg, Germany), a concentration of 15 mg/kg B.W. was chosen, whereas DMC (Novartis, Nuremberg, Germany) was given at a dose of 6 mg/kg B.W. These doses reflect the clinically relevant doses of VAN and DMC.[18,19]

Two hours after commencing the antibiotic treatment, and 22 h after LPS injection, the mice were anesthetized with isoflurane (Baxter, Unterschleissheim, Germany) via a face mask, and euthanized in deep isoflurane anesthesia. The peritoneal cavity was carefully prepared, and lavage was performed with 3 ml of an ice-cold PBS solution (Gibco/Invitrogen, Karlsruhe, Germany). After injection, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution. The abdominal fluid was collected with an IV 20 G catheter and a 5 ml syringe.

Flow cytometry

The peritoneal fluid was filtered using a nylon mesh with a pore size of 100 µm. Cell suspensions were preincubated with anti–CD16/CD32 mAb to block FcγRII/III receptors (BD Biosciences, Franklin Lakes, NJ, USA) for 10 min at 4°C. The cells were stained with anti-mouse fluorochrome-conjugated antibodies in panel-appropriate combinations in the dark for 20 min at 4°C with the following antibodies from Thermo Fisher (Waltham, MA, USA): F4/80 (BM-8), CD11c (HL3) and BioLegend (San Diego, CA, USA): CD45 (30-F11), Gr1 (RB6-8C5). The viability of the cells was confirmed using Hoechst 33258 staining (Thermo Fisher Scientific, Waltham, MA, USA) 10 min prior to measurement.

After the incubation period, the cells were washed and resuspended with FACS-buffer after centrifugation at 400 × *g*. Flow cytometry was performed on a FACS Canto II device, and raw data was analyzed using the dedicated Flow-Jo software (BD Bioscience, Franklin Lakes, NJ, USA).

Gating strategy for flow cytometry analyses was performed as illustrated in Figure 2. All cell like events were isolated by using the forward scatter-area versus sideward scatter-area. Doublets were excluded using forward scatter-area versus sideward scatter-with. To isolate the living cells, the forward scatter-area versus the Hoechst staining was used. To determine the immune cells, the forward scatter-area versus a CD45 staining was utilized. Immune cells were further classified by staining the particular surface markers Gr-1 and F4/80. We used CD11c as a function of the autofluorescence channel AF430 for the definition of dendritic cells (for more details on the gating strategy please refer to the corresponding legend of Figure 2(A) and (B)).

Phagocytosis was measured as previously published.[4,7] Briefly, pHrodo Red *E. coli* BioParticles (Invitrogen/Thermo Fisher, Waltham, MA, USA) were injected 20 h after the LPS injection into the peritoneal cavity. After 2 h, the animals were sacrificed, and peritoneal lavage was performed.

In vitro experiments

Peritoneal macrophages were harvested from healthy C57BL/6 mice. Lavage was performed as described previously. The cells were counted and allowed to adhere to the plate by culturing them for 2 h at 37°C. Nonadherent cells were removed by gently washing the plate three times with PBS heated to 37°C.

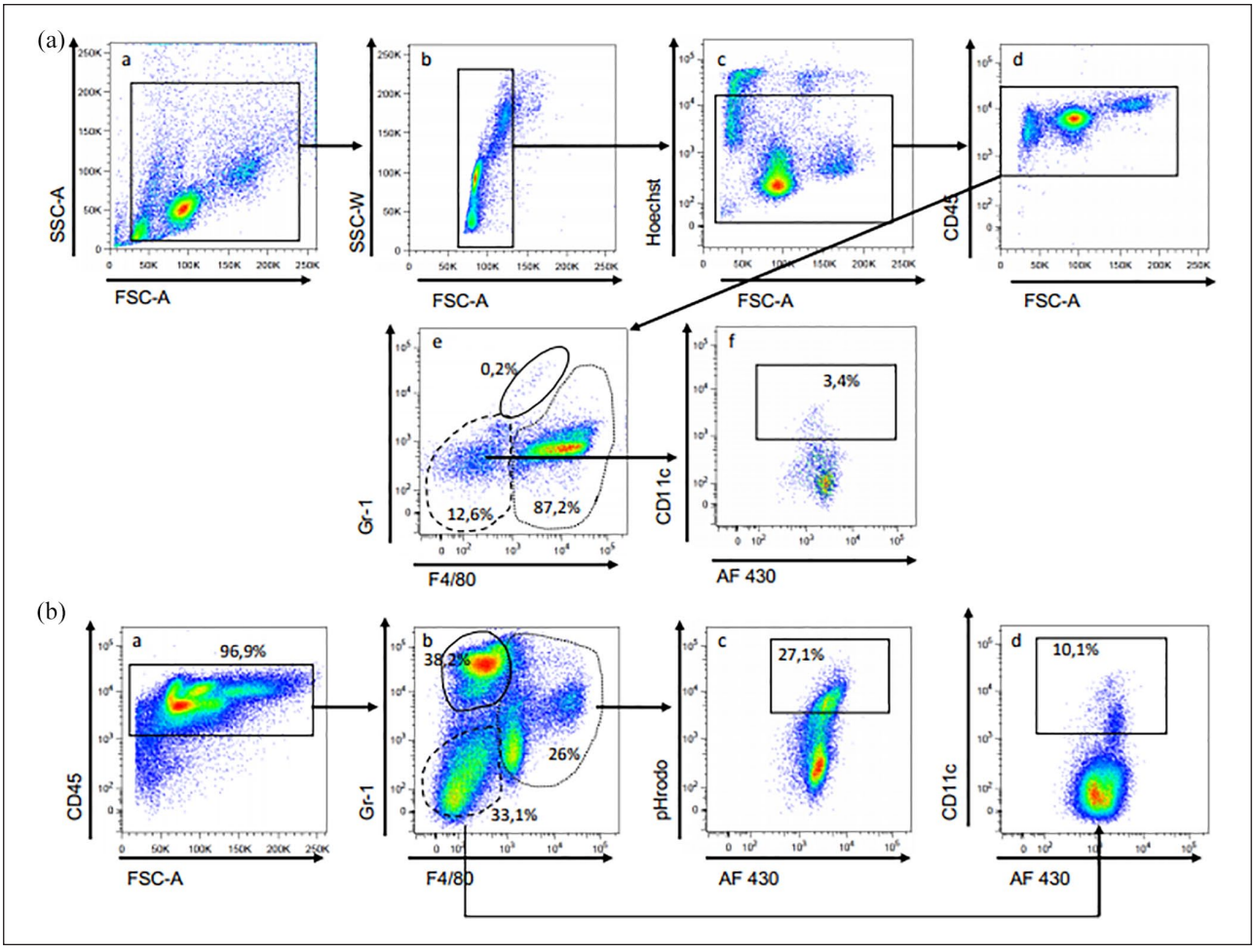

Figure 2. Cell distribution in the peritoneal lavage of healthy and LPS stimulated mice. Exemplary flow cytometry results visualized as pseudo-color dot plots. The gating of the peritoneal lavage was performed as follows (A): firstly, we isolated all cell like events (A, a), using forward scatter-area versus sideward scatter-area. Doublets were excluded (A, b), using forward scatter-area versus sideward scatter-with. Living cells were isolated (A, c) using forward scatter-area versus Hoechst staining. Immune cells were determined (A, d), using forward scatter-area versus CD45 staining. Immune cells were further classified and stained for the particular surface markers Gr-1 and F4/80 (A, e). Determination of peritoneal macrophages (A, d, dotted line: $CD45^+$, $F4/80^+$), neutrophils (A, d, solid line: $CD45^+$, $F4/80^-$, $Gr1^+$) and classical dendritic cells (A, f: $CD45^+$, $F4/80^-$, $Gr1^-$, $CD11c^+$) in the peritoneal lavage of untreated C57BL/6 mice. We used CD11c as a function of the autofluorescence channel AF430 for definition of classical dendritic cells. Gating of pHrodo infected mice (B) was added by determination of pHrodo+ cells (exemplary shown for macrophages (B, c). Gating was performed according to the current guidelines for the use of flow cytometry and cell sorting in immunological studies.[20]

Cytokine quantification

Fluorescent bead-based flow cytometry assays for the cytokines TNFα, IL-6, and IL-10 were performed using FlowCytomix Multiplex Kits (Bender MedSystems GmbH, eBioscience, Life Technologies, Darmstadt, Germany) on the cell culture supernatant of peritoneal macrophages incubated in vitro, according to the manufacturer's protocol. Sample reading was performed using a FACS Canto II Becton Dickinson (BD Bioscience, Franklin Lakes, NJ, USA) cytometer, and analyzed with eFlowCytomix Pro software (eBioscience, Life Technologies, Darmstadt, Germany).

Statistical analysis

All data are expressed as mean ± SD. The variance and effect size were calculated using the Cohen's effect size test, resulting in a power of 80%. The main statistical target was the development of LPS-induced peritonitis. Secondary targets were the pro- and anti-inflammatory immune responses, phagocytosis, cytokine release, and immune cell

migration and accumulation. The statistical analyses were performed using GraphPad Prism 8 Software (GraphPad Software, San Diego, California, USA, www.graphpad.com). Variables were tested for normality using Shapiro-Wilk tests. One-way ANOVAs with Bonferroni post hoc tests were performed, and a *P*-value of less than 0.05 was considered to indicate significant differences.

Results

In the presence of LPS, VAN decreased both the macrophage and dendritic cell population in the abdominal cavity

Several different mouse models allow the investigation of sepsis. In this project, we decided to use a highly standardized method involving peritoneal LPS injection 22 h prior to the sacrifice of the mice, to test our hypothesis about the immunomodulatory influences of VAN and DMC. Twenty hours after LPS injection, the animals received pHrodo *E. coli* particles intravenously, for the analysis of the phagocytic activity of the different leukocyte subsets (Figure 1).

Firstly, we addressed the question of whether intravenous VAN application modulates the accumulation of macrophages, PMNs and dendritic cells in the abdominal cavity, in the presence or absence of LPS. Leukocyte subsets were defined according to the surface expression of CD45 as a pan-immune cell marker; F4/80 as a macrophage specific marker; Gr-1, which is composed of two components, Ly6C and Ly6G, as a neutrophil marker; and CD11c to characterize dendritic cells (Figure 2A). Immune cells were further analyzed with respect to pHrodo uptake, to examine phagocytosis activity (Figure 2B). Under healthy conditions, there are primarily macrophages in the peritoneal cavity, with only a few neutrophils and dendritic cells. After stimulation with LPS, we observed an increase of the numbers of innate immune cells in the lavage fluid (Figures 2A, B and 3(a)–(c)).

Our data revealed that treatment with pHrodo already caused an inflammatory reaction in the peritoneal cavity, which was mirrored by a significant increase in macrophages ($CD45^+$ $F4/80^+$), neutrophils ($CD45^+$ $F4/80^-$ $Gr\text{-}1^+$) and dendritic cells ($CD45^+$ $F4/80^-$ $Gr\text{-}1^-$ $CD11c^+$) (Figure 3(a)–(c), ***P < 0.01)*** compared to the control mice.

Following LPS stimulation, the level of peritoneal macrophages ($CD45^+$ $F4/80^+$) was further elevated when compared with pHrodo-treated mice (Figure 3(a), $P < 0.01$). In LPS-infected mice, VAN administration decreased the proportion of peritoneal macrophages, compared to mice without VAN administration (Figure 3(a), $P < 0.01$). In contrast, antibiotic treatment with VAN did not alter the amount of neutrophils ($CD45^+$ $F4/80^-$ $Gr\text{-}1^+$) in the abdominal cavity in either control-treated or LPS-stimulated mice (Figure 3(b), $P > 0.05$). However, neutrophils also showed a reaction following pHrodo injection, when compared to the controls (Figure 3(b), $P > 0.05$). We also observed this phenomenon in the group of dendritic cells (Figure 3(c), $P < 0.01$). LPS infection increased the number of dendritic cells ($CD45^+$ $F4/80^-$ $Gr\text{-}1^-$ $CD11c^+$) when compared with the control-treated mice (Figure 3(c), $P < 0.01$). When the mice were stimulated with LPS, VAN administration reduced the number of dendritic cells in the peritoneal lavage, compared with LPS-stimulated mice which were not treated with VAN (Figure 3(c), $P < 0.01$).

These data demonstrated that VAN treatment of LPS-stimulated mice ameliorated the LPS-induced increase of both the macrophage and the dendritic cell populations.

In the presence of LPS, DMC decreased the dendritic cell population in the abdominal cavity

We also analyzed the effect of DMC on the cellular immune response. The administration of pHrodo caused significant elevation of leukocyte numbers in the peritoneal cavity, as already described in Figure 3.

Flow cytometry analysis revealed that, in LPS-treated mice, the proportion of peritoneal macrophages was elevated when compared with the control mice, which were treated with pHrodo particles only (Figure 4(a), $P < 0.01$). In contrast, the administration of DMC in control-treated and LPS-stimulated mice did not change the proportion of macrophages or neutrophils in the abdominal cavity, when compared to mice without DMC treatment (Figure 4(a) and (b), $P > 0.05$). LPS stimulation increased the number of dendritic cells when compared with control mice (Figure 4(c), $P < 0.01$). DMC led to a decrease in the number of dendritic cells in the abdominal cavity when

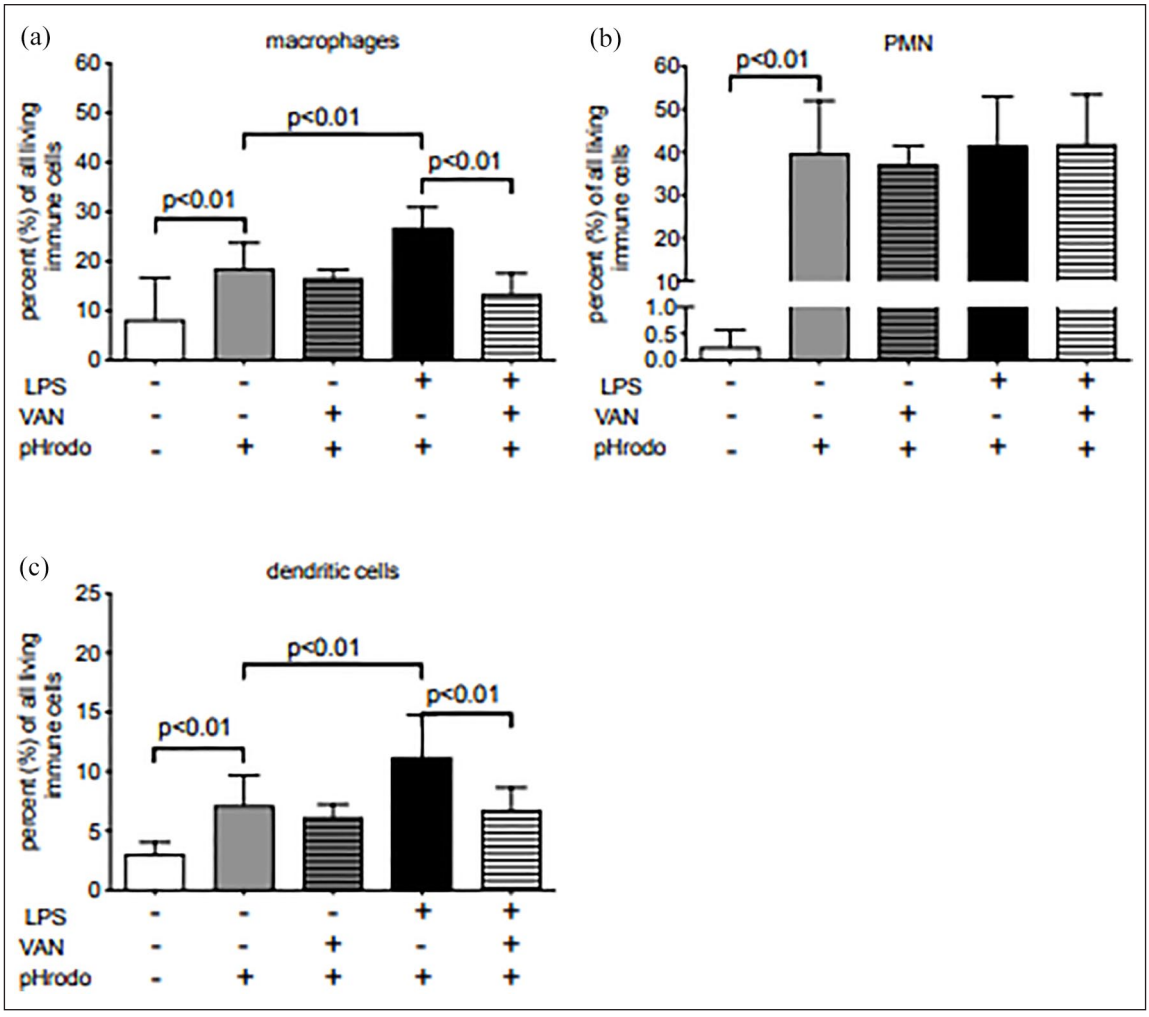

Figure 3. Effects of VAN on migration of macrophages (a), PMNs (b), and dendritic cells (c) in the abdominal cavity in unstimulated and LPS-challenged C57BL/6 mice. 20 h after LPS administration, animals received an intravenous injection of either PBS or VAN. 2 h after antibiotic treatment, mice were sacrificed, and peritoneal lavage was collected for flow cytometry-based cell quantification. Data are depicted as percent of all living immune cells in the lavage fluid. Each column represents the percent of either macrophages (a), PMNs (b), or dendritic cells (c) in unstimulated or LPS-treated mice. $n = 4$–6 mice/group; data are expressed as mean ± SD, $P < 0.01$ is considered significant.

compared to LPS-treated mice without DMC administration (Figure 4(c), $P < 0.01$).

These results indicate that DMC treatment accounted for the decline in macrophage and dendritic cell populations under inflammatory conditions.

VAN modulates the phagocytic activity of both peritoneal macrophages and dendritic cells

Next, we investigated whether VAN injection alters the phagocytic activity of macrophages, neutrophils, and dendritic cells in the peritoneal lavage. Twenty hours after LPS stimulation, we injected fluorescent *E. coli* pHrodo particles intraperitoneally, to monitor phagocytosis concomitantly with the start of the antibiotic treatment.

In LPS-stimulated mice, the level of phagocytic macrophages in the peritoneal cavity increased four-fold when compared to mice without LPS stimulation (Figure 5(a), $P < 0.01$). VAN administration increased the phagocytic activity of macrophages in mice compared to PBS-treated control animals (Figure 5(a), $P < 0.01$). In contrast, the phagocytotic activity did not differ when LPS-stimulated mice were treated with VAN

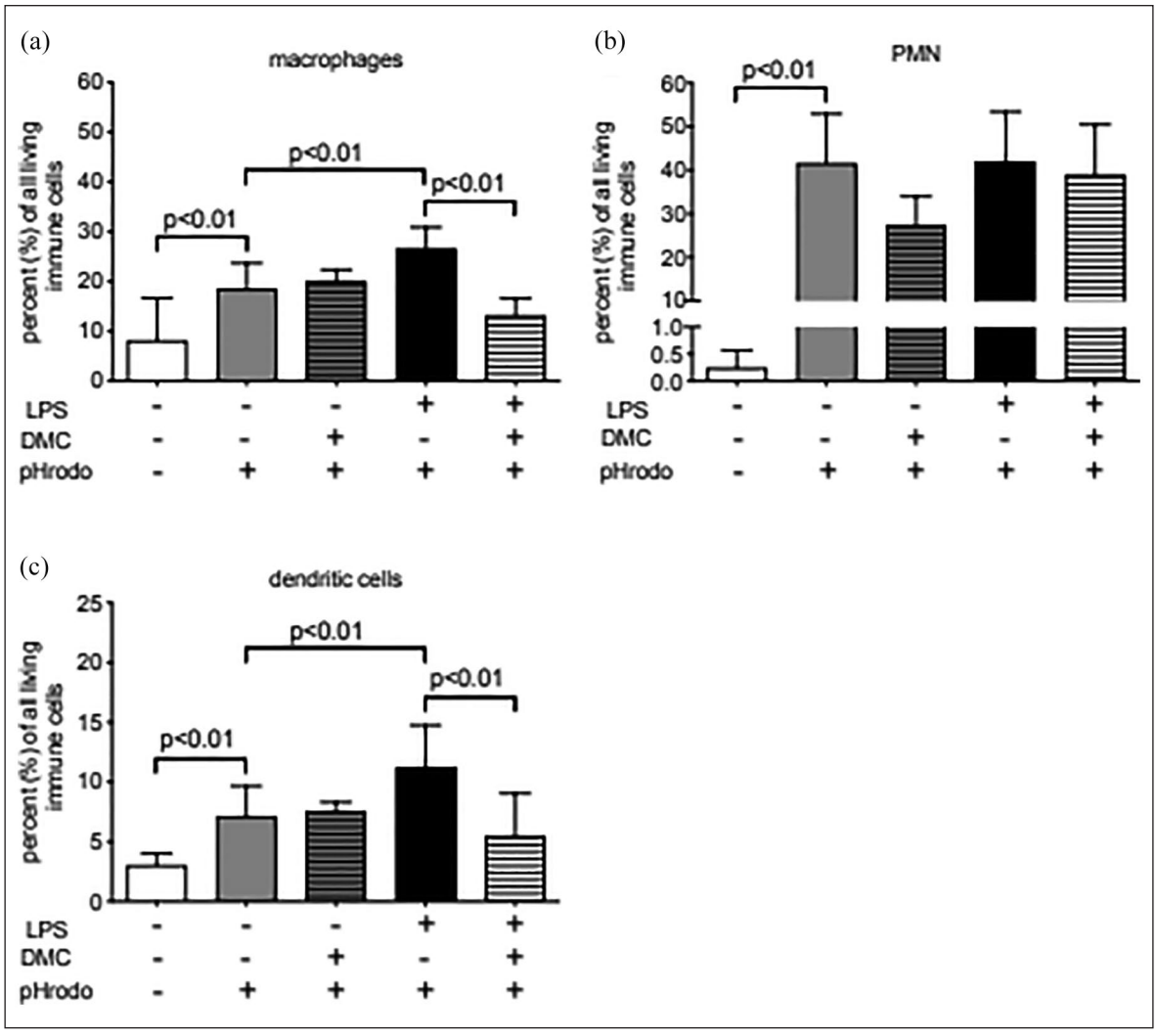

Figure 4. Effects of DMC on migration of macrophages (a), PMNs (b), and dendritic cells (c) in the abdominal cavity in unstimulated and LPS-treated C57BL/6 mice. 20 h after LPS administration, animals received an intravenous injection of either PBS or VAN. 2 h after antibiotic treatment, mice were sacrificed and peritoneal lavage was collected for flow cytometry-based cell quantification. Data are depicted as percent of all living immune cells in the lavage fluid. Each column represents the percent of either macrophages (a), PMNs (b), or dendritic cells (c) in unstimulated or LPS-treated mice. $n = 4$–6 mice/group; data are expressed as mean ± SD, $P < 0.01$ is considered significant.

(Figure 5(a), $P > 0.05$). Treatment with VAN did not change the phagocytotic activity of peritoneal neutrophils in the LPS pre-treated or control-treated mice (Figure 5(b), $P > 0.05$). VAN administration increased the percentage of phagocyting dendritic cells in LPS-stimulated mice (Figure 5(c), $P < 0.01$).

These results indicate that the administration of VAN increased the phagocytic activity of macrophages under non-inflammatory conditions. However, this effect was diminished in the presence of the inflammatory stimulus. VAN treatment increased the phagocytic activity of dendritic cells in LPS-infected mice.

DMC modulated the phagocytotic activity of peritoneal macrophages

We then analyzed the effect of DMC administration on the phagocytic activity of leukocytes. Mono-administration of DMC increased the phagocytic activity of peritoneal macrophages compared with that in control mice (Figure 6(a), $P < 0.01$). This effect was diminished in the presence of LPS. DMC treatment did not change the phagocytic activity in either neutrophils or dendritic cells, compared with the corresponding controls (Figure 6(b) and (c), $P > 0.05$).

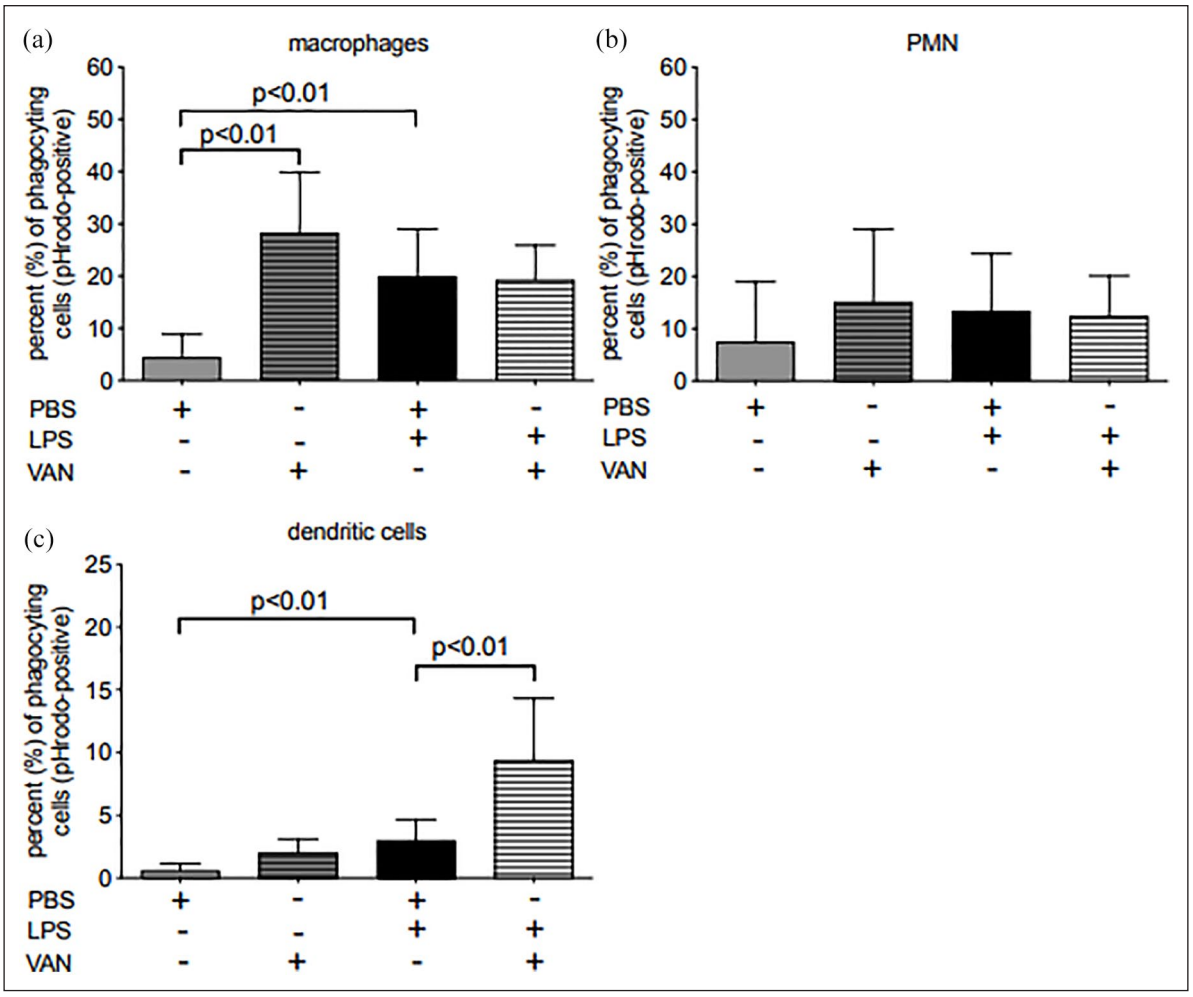

Figure 5. Effects of VAN on the phagocytic activity of macrophages (a), PMNs (b), and dendritic cells (c) in the abdominal cavity in unstimulated and LPS-treated C57BL/6 mice. 20 h after the LPS administration, animals received an intravenous injection of either PBS or VAN and an intraperitoneal injection of fluorescent *E. coli* pHrodo particles. After 2 h, animals were sacrificed and peritoneal lavage was collected for flow cytometry-based quantification of cell specific *E. coli* particle intake. Data are depicted as percent of phagocyting cells in the lavage fluid. Each column represents the percent of either pHrodo positive macrophages (a), PMNs (b), or dendritic cells (c) in unstimulated or LPS-treated mice. $n = 4$–6 mice/group; data are expressed as mean ± SD, $P < 0.01$ is considered significant.

These data demonstrate that DMC treatment enhanced the phagocytic activity of native peritoneal macrophages in the abdominal cavity, in the absence of an inflammatory stimulus.

VAN treatment decreased IL-10 protein levels in LPS-stimulated peritoneal macrophages

Another important effector function of leukocytes is their ability to produce cytokines, which modulate the inflammatory response. We determined whether the levels of TNFα, IL-6, and IL-10 were altered in the supernatant of peritoneal macrophages cultured in vitro with or without LPS stimulation, after VAN administration.

When peritoneal macrophages were stimulated with LPS, TNFα levels increased 10-fold compared to LPS-free controls (Figure 7(a), $P < 0.01$). VAN treatment did not alter TNFα levels in the presence of LPS when compared to LPS-treated macrophages without VAN administration (Figure 7(a), $P > 0.05$). VAN administration exhibited no difference in IL-6 protein levels during septic conditions compared to the respective controls (Figure 7(b), $P > 0.05$). In the presence of LPS, IL-10 protein levels increased 30-fold when compared to unstimulated controls (Figure 7(c), $P < 0.01$). In the presence of VAN, IL-10 cytokine levels decreased in LPS-stimulated macrophages when compared to the cells without antibiotic treatment (Figure 7(c), $P < 0.01$).

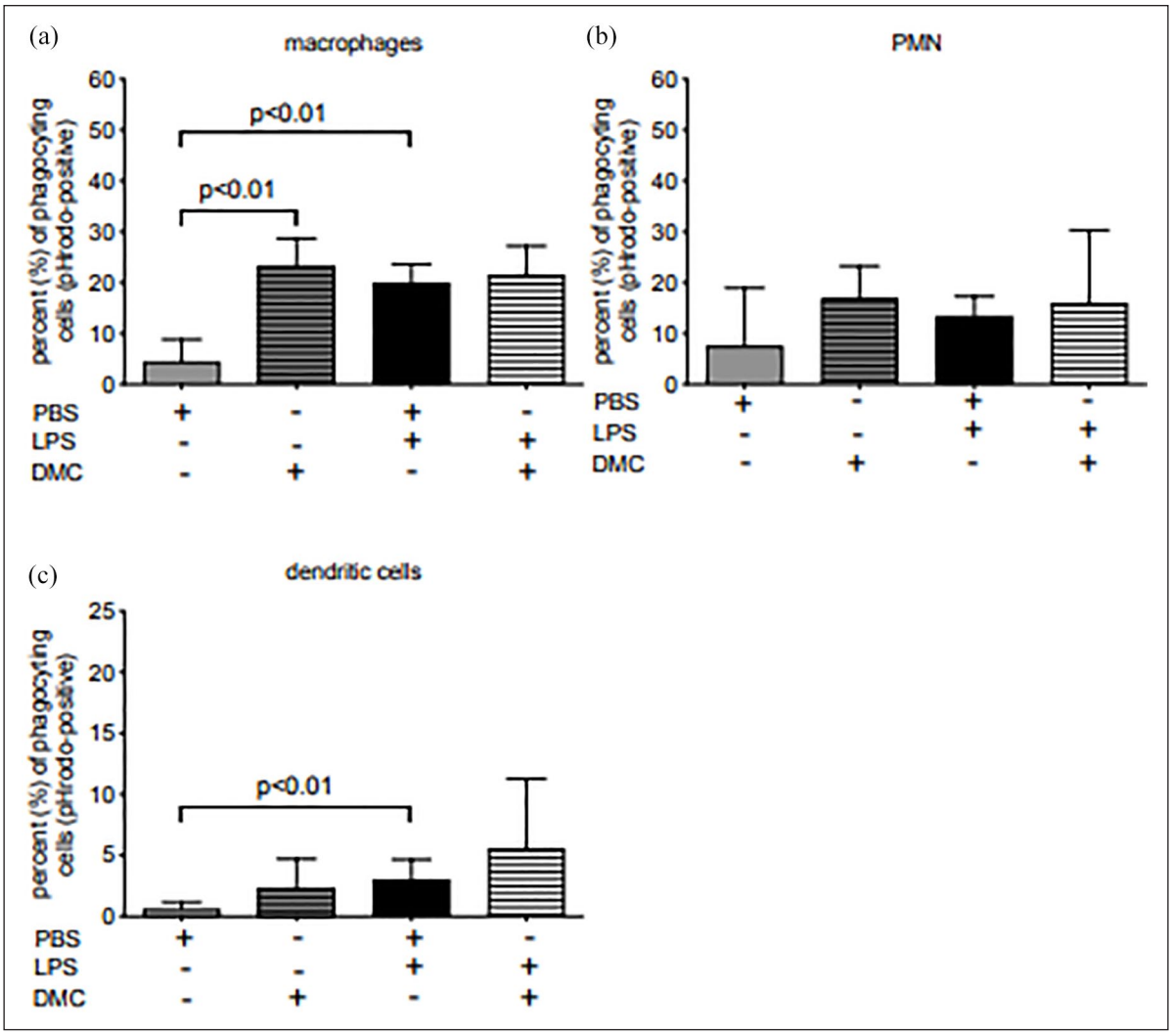

Figure 6. Effects of DMC on the phagocytic activity of macrophages (a), PMNs (b), and dendritic cells (c) in the abdominal cavity in unstimulated and LPS-treated C57BL/6 mice. 20 h after the LPS administration, animals received an intravenous injection of either PBS or DMC and an intraperitoneal injection of fluorescent *E. coli* pHrodo particles. After 2 h, animals were sacrificed and peritoneal lavage was collected for flow cytometry-based quantification of cell specific *E. coli* particle intake. Data are depicted as percent of phagocyting cells in the lavage fluid. Each column represents the percent of either pHrodo positive macrophages (a), PMNs (b), or dendritic cells (c) in unstimulated or LPS-treated mice. $n = 4$–6 mice/group; data are expressed as mean ± SD, $P < 0.01$ is considered significant.

Taken together, VAN decreased the level of the anti-inflammatory cytokine IL-10 in LPS-stimulated macrophages but did not affect the pro-inflammatory cytokine response.

DMC alters protein levels of Il-6 and IL-10 in LPS-stimulated peritoneal macrophages

Having observed the effect of VAN on IL-10 production in our model of LPS-induced sepsis, we analyzed the influence of DMC treatment on cytokine production. Our data show that DMC did not affect TNFα levels in both, LPS-stimulated macrophages and unstimulated samples (Figure 8(a), $P > 0.05$). However, the administration of DMC increased the concentration of IL-6 in non-LPS-stimulated macrophages as well as in LPS-stimulated samples, compared to the respective controls (Figure 8(b), $P < 0.01$). Under inflammatory conditions, DMC treatment further enhanced IL-6 concentrations in the supernatant, compared to LPS mono-stimulation (Figure 8(b), $P < 0.01$). Stimulation with LPS caused an increase in IL-6 levels, compared to the unstimulated controls. Simultaneously, incubation with DMC reduced IL-10 protein levels in the supernatant of LPS-

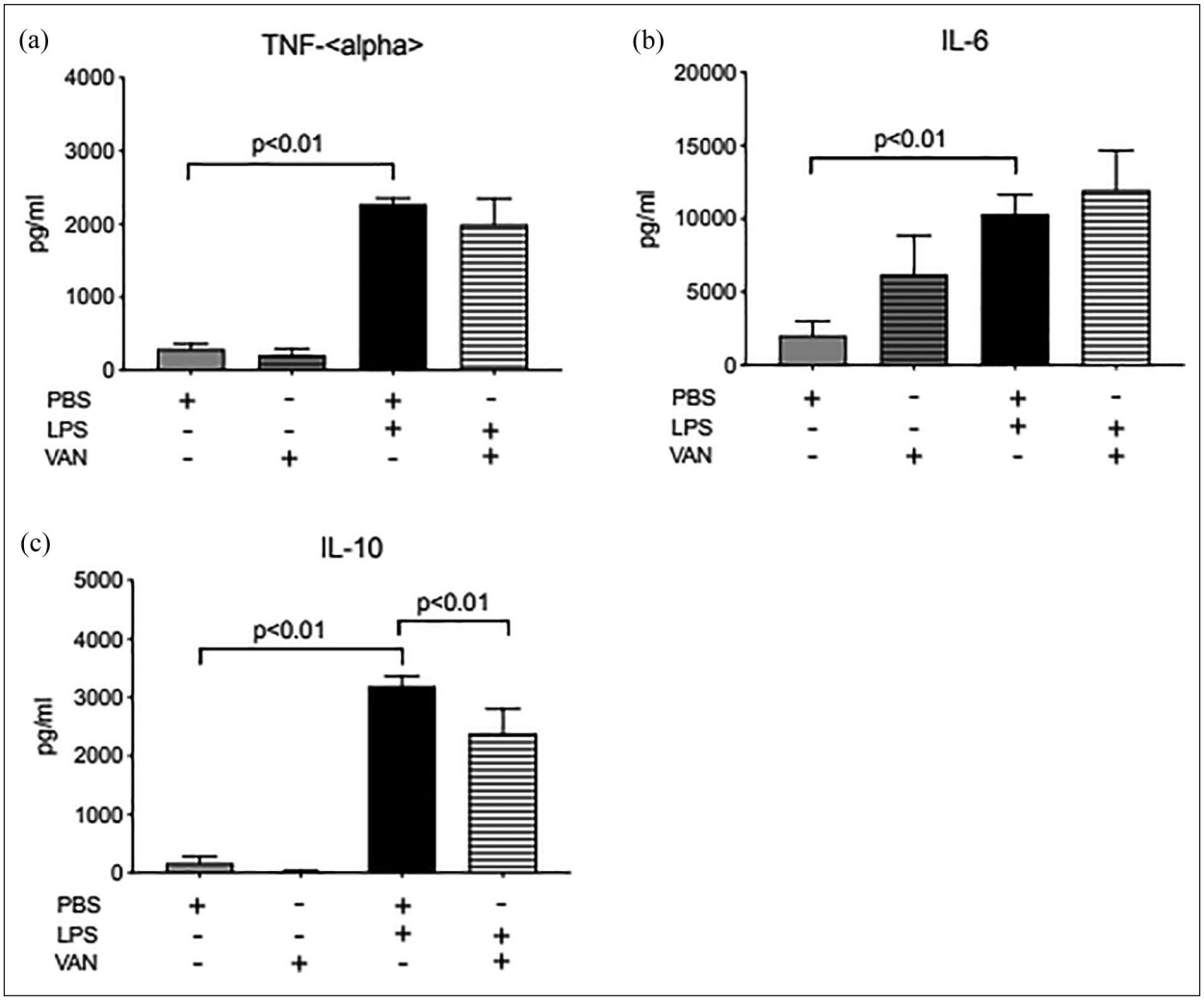

Figure 7. Cytokine levels of (a) TNFα, (b) IL-6, and (c) IL-10 in the supernatant of LPS stimulated and unstimulated peritoneal macrophages. 20 h after PBS or LPS administration, macrophages were treated with VAN. 2 h after antibiotic treatment, supernatant was collected for cytokine measurements. Each column represents the level of the respective cytokines in pg/ml. $n = 4$–8samples/group; data are expressed as mean ± SD, $P < 0.01$ is considered significant.

stimulated macrophages, compared to the respective controls (Figure 8(c), $P < 0.01$).

These results demonstrate that DMC influences both pro- and anti-inflammatory cytokine release in LPS-stimulated macrophages.

Discussion

In this study, we measured the direct immunomodulatory properties on cells of the innate immune response, using two "last-resort" antibiotics in an LPS-induced murine model of sepsis. We found that the administration of VAN led to a decrease in both the peritoneal macrophage and the dendritic cell populations after LPS stimulation. DMC reduced the dendritic cell population in the peritoneal cavity in LPS-challenged mice. Both antibiotics increased the phagocytic activity of peritoneal macrophages. However, this effect was diminished after the administration of endotoxin. In contrast, the phagocytic activity of dendritic cells was increased in LPS-treated animals. Both antibiotics differently modulated the levels of pro- and anti-inflammatory cytokines.

The mouse model of LPS-induced sepsis is well established, and mirrors gram-negative bacterial infections in a standardized manner.[21,22] LPS is a potent activator of inflammatory responses, leading to cytokine release and leukocyte recruitment.[23] In the peritoneal cavity, the resident leukocyte population is composed of 45%–90% macrophages, 2%–6% dendritic cells, and less than 5%

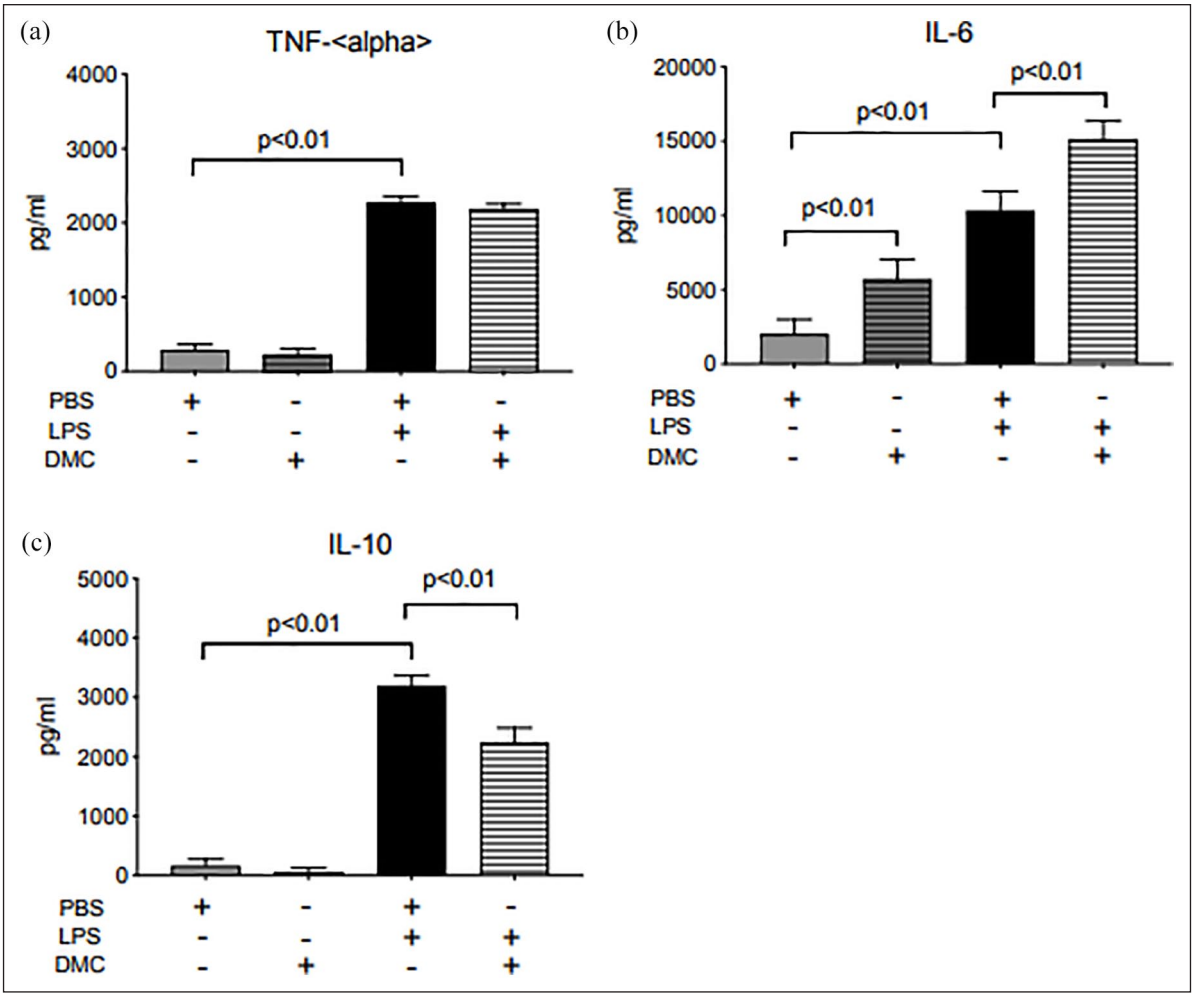

Figure 8. Cytokine levels of (a), TNFα, (b) IL-6, and (c) IL-10 in the supernatant of LPS stimulated and unstimulated peritoneal macrophages. 20 h after PBS or LPS administration, macrophages were treated with DMC. 2 h after antibiotic treatment, supernatant was collected for cytokine measurements. Each column represents the level of the respective cytokines in pg/ml. $n = 4$–8 samples/group; data are expressed as mean ± SD, $P < 0.01$ is considered significant.

neutrophils.[24] These numbers are consistent with the results of our flow cytometry analysis, and mirror the typical cell distribution in the peritoneal lavage of healthy mice, which had received neither LPS nor pHrodo particles. After 20 h of LPS stimulation, we observed an accumulation of macrophages and DCs in the peritoneal lavage. These effects were attenuated when either VAN or DMC was administered. Our findings suggest that both substances have the potential to suppress LPS-mediated immune cell recruitment in inflammation.

When pathogens invade, macrophages initiate an inflammatory response and activate their effector function. This response includes phagocytosis of cellular debris and pathogens, secretion of cytokines, and stimulation of other immune cells.[25] Phagocytosis is one of the most efficient tools for the elimination of pathogens in acute infections, and serves as a marker for leukocyte function.[26] Bode and co-workers reported an increased in vitro phagocytic activity when LPS-stimulated monocytes were co-incubated with VAN.[4] We confirmed these findings in a murine model of LPS-induced sepsis in combination with pHrodo *E. coli*-based phagocytosis assay. In this project we explored this issue further, as we were able to evaluate phagocytosis activity at the individual leukocyte cell population level. We demonstrated that different subpopulations of innate immune cells, the peritoneal macrophages and dendritic cells, had enhanced phagocytic activity in the presence of the antibiotic VAN. Although DMC elevated the killing activity of native macrophages, this phenomenon was

absent in LPS-treated animals. The possibility that this increased killing activity might regulate the internalization of bacterial fragments, possibly by regulating other phagocytic receptors, cannot be excluded, and needs further investigation.

We further investigated the effector function of leukocytes by measuring the in vitro levels of different cytokines in the supernatant of murine peritoneal macrophages. IL-6 is an important mediator of the acute phase response in mammals. Our data revealed that IL-6 levels were increased in response to the administration of DMC. The concentration of IL-6 was even higher when stimulated with LPS. This finding points to an influence of DMC on IL-6 liberation in peritoneal macrophages. IL-6 is a pleiotropic cytokine, and is mainly released by tissue macrophages. Although the application of DMC did not increase the peritoneal macrophage population in our experiments, we observed elevated levels of IL-6. This phenomenon might be explained by an enhanced pro-inflammatory effector function caused by the administration of DMC. A prospective observational study has found that high levels of IL-6 are correlated with the severity of sepsis in critical care patients who suffered from severe injuries.[27] Whether the elevated IL-6 levels that are caused by the administration of DMC may alter the course of sepsis in humans needs further investigation. Lipopeptides such as the antibiotic agent DMC are known immunomodulators, that interact with pattern recognition receptors such as Toll-like receptors.[28] Lipopeptides are also known to promote pro-inflammatory signaling in macrophages and dendritic cells.[28] Our findings support the hypothesis that DMC has immunomodulatory properties in mammals.

Another well-characterized pro-inflammatory cytokine is TNFα, which can be secreted by both resident sentinel macrophages and recruited helper macrophages.[16] In our study, the level of TNFα in the supernatant of LPS-stimulated peritoneal macrophages was not affected by antibiotic treatment with VAN or DMC.

Both dendritic cells and macrophages secrete various cytokines and chemokines that contribute to inflammation and healing. In this study, IL-10 levels were measured, to monitor the anti-inflammatory effects of both antibiotics. Cells of the monocyte lineage represent the major source of IL-10 under septic conditions.[29,30] We observed that both VAN and DMC reduced IL-10 levels in the supernatant of LPS-stimulated peritoneal macrophages. These findings indicate that the last-resort antibiotics VAN and DMC modulate the anti-inflammatory axis of the innate immune response, and might contribute to a shift toward a pro-inflammatory exacerbation of the septic immune response.

IL-6 and IL-10 have been suggested to be biomarkers of the course of sepsis. A decrease in IL-6 was associated with a better prognosis, and overproduction of IL-10 was found to be the main predictor of severity and fatal outcome.[31] Based on these data, our findings indicate that VAN or DMC treatment prevents IL-10 overproduction and may show a protective effect. Further studies are needed to elucidate the underlying mechanism.

Recently, we investigated the impact of VAN and DMC on the innate immune response in an in vitro LPS-model in human monocytes.[4] In the present study, we translated our findings to a small animal model of LPS-induced sepsis, to improve the depth of our clinically relevant observations. This model allowed us to study the effector functions of immune cells in the presence of VAN or DMC, with special focus on cell migration and phagocytosis.

In this study, we used an LPS endotoxemia murine model to induce a severe inflammatory status in mice, resembling septic conditions in humans. Low-dose injection of LPS into healthy human volunteers induces pathophysiologic alterations that are similar to those reported in patients with sepsis.[32] These findings support the value of the use of the endotoxin LPS to study the underlying mechanisms of sepsis. We took advantage of the highly standardized murine model of LPS-induced sepsis, and investigated the clinically and physiologically relevant effects and function of leukocytes in the presence of either VAN or DMC. There are known limitations in clinical translation from mouse to patient, which apply to our study, especially with regard to the direct comparison of leukocyte subsets, which are known to have distinct gene expression profiles in humans and mice respectively.[33]

The approach used to check the phagocytosis activity of leukocyte subsets, using pHrodo *E. coli* particles was an appropriate choice to test our hypothesis, because it allows the quantification of phagocytosis by the use of flow cytometry-based tools. Our data revealed that the administration of

pHrodo *E. coli* particles itself activated the immune response in the peritoneal cavity. For the purpose of our 3-R based experimental setup (replace, reduce, refine), we took this activation into account. To further clarify these circumstances, we added data from healthy and completely untreated animals.

In summary, this study provides new evidence that the last-resort antibiotics VAN and DMC modulate the accumulation and phagocytosis of macrophages and dendritic cells in a mouse model of LPS-induced peritonitis. Combined with a highly standardized in vitro model to detect cytokine secretion using peritoneal macrophages, our data indicate that VAN suppressed leukocyte migration to the peritoneal cavity, and reduced IL-10 levels under inflammatory conditions. VAN enhanced the phagocytosis activity of dendritic cells in a non-inflammatory environment. Treatment with DMC under septic conditions led to a reduced number of dendritic cells and IL-10 levels, but increased the concentration of IL-6.

Conclusions

In a murine model of LPS-induced sepsis, VAN and DMC exhibited immunomodulatory effects on cells of the innate immunity response. The question of whether these antibiotics might exhibit synergistic effects in the treatment of septic patients, beyond their individual bactericidal properties, needs further evaluation to investigate in more detail the mechanisms underlying these effects.

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Author contributions

S.M., V.Z., B.D., S.F., and C.K.W. performed the experiments, S.M., V.Z., S.F., and C.K.W. evaluated the data, S.M., G.B., and C.K.W. designed the study with support from S.F. and C.P., S.M. and C.K.W. wrote the paper in consultation with S.F., G.B., M.C., and C.P.

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Ethics approval

Ethical approval for this study was obtained from the local committee for animal care (LANUV, Recklinghausen, Germany, permit number 87-51.04.2010.A152).

Animal welfare

The present study followed international, national, and institutional guidelines for humane animal treatment and complied with relevant legislation.

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