

BRIEF CONCLUSIVE REPORT

Ascorbic acid attenuates activation and cytokine production in sepsis-like monocytes

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Abstract

Sepsis manifests due to the host's dysregulated immune response to infection. High-dose ascorbic acid (AA) has emerged as a potential treatment of sepsis, yet little is known regarding how AA influences the immune system in sepsis, such as monocytes. The objective of this study is to investigate the effects of high-dose AA on monocyte polarization and cytokine production in vitro. Monocytes isolated from healthy donors ($n = 6$) were polarized in vitro for 48 h using LPS or lipoteichoic acid (LTA). Polarization was confirmed by surface marker expression using flow cytometry. In parallel, monocytes from septic patients ($n = 3$) were analyzed for polarization markers as a comparison with the in vitro polarization. The effect of AA on monocyte polarization was then evaluated. Finally, monocytes were analyzed for cytokine production by intracellular staining. Both LPS and LTA induced polarization in healthy monocytes in vitro, with increased expression of both pro (M1) (CD40 and PDL1, $p < 0.05$) and anti-inflammatory (M2) (CD16 and CD163, $p < 0.05$) polarization markers. This pattern resembled that of monocytes from septic patients. Treatment with AA significantly inhibited surface expression of CD16 and CD163 ($p < 0.05$) in a dose-dependent manner. Finally, AA attenuated LPS- or LTA-induced cytokine production of IL-1 β , IL-6, IL-8, and TNF. In conclusion, AA attenuates proinflammatory cytokine production and diminishes up-regulation of CD16 and CD163, but not of CD40 and PDL-1 in LPS- or LTA-polarized monocytes. This study provides important insight into the effects of high-dose AA on monocytes and potential implications in sepsis.

KEYWORDS

inflammation, polarization, surface markers

Abbreviation: AA, Ascorbic acid; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MFI, median fluorescence intensity.

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1 | INTRODUCTION

Sepsis is a condition that occurs due to a dysregulated immune response to infection. Treatment for sepsis currently include anti-infective therapy and supportive care. Yet, to date, no treatment targeting the dysregulated immune system is in clinical use. High-dose ascorbic acid (AA)/vitamin C has emerged as a potential treatment and has showed early promising results.^{1,2} Additionally, AA has displayed anti-inflammatory and antioxidative effects in several animal models of sepsis.³⁻⁶ However, recent randomized controlled studies have failed to demonstrate a general benefit of AA treatment in sepsis and septic shock.⁷⁻¹⁰ Notably, these studies did not thoroughly distinguish among different types of sepsis, pathogens, or immunologic states.

Few studies have addressed the effects of AA on the immune response in sepsis. Monocytes are immune cells with roles in cytokine production, antigen presentation, and phagocytosis. Monocytes can polarize and acquire proinflammatory (M1) and anti-inflammatory (M2) features, which influence their effector functions. In sepsis, monocytes show signs of polarization, expressing markers related to both M1 (CD40 and PDL1) and M2- (CD16 and CD163) polarization.¹¹⁻¹³ Still, it is important to note that monocytes are rarely exclusively M1 or M2, and conflicting data exist regarding surface markers in sepsis, likely reflecting time points and subgroups. Functionally, septic monocytes display a state of exhaustion with immunosuppressive features, increased ROS generation, and intact or increased phagocytosis.^{14,15}

To our knowledge, there has been no studies investigating the effects of AA on monocyte polarization. Here, we aimed to investigate the effects of high-dose AA on monocyte polarization and function in vitro within the setting of sepsis.

2 | MATERIALS AND METHODS

2.1 | Patients

Healthy controls ($n = 6$) and patients with blood-culture confirmed sepsis ($n = 3$) were included in this study. Ethical approval was obtained from the Swedish Ethical Review Authority (2019-05146). Clinical characteristics of the septic patients have previously been described in Kahn et al.¹⁵

2.2 | Surface marker expression in whole blood from patients with sepsis

Blood was collected in EDTA tubes from healthy controls ($n = 3$) or sepsis patients ($n = 3$) upon informed consent. One hundred microliters of EDTA blood was stained with lineage markers and anti-CD40, PDL1, CD16, and CD163 (see Supplementary Materials and Methods

for details). Analysis was performed by a CytoFLEX flow cytometer (Beckman Coulter).

2.3 | Monocyte isolation and culture

Blood was collected in heparin tubes from healthy controls. A density gradient (LymphoPrep; Alere Technologies) was used to collect the PBMCs. Cells were washed once with PBS. Monocytes were further isolated through CD14 positive selection with magnetic beads using the MACS LS columns according to the manufacturer's instructions (Miltenyi Biotech). Monocytes (0.25×10^6) were cultured in 500 μ l of RPMI-1640 medium with 2.05 mM L-glutamine (Gibco Life Technologies) and supplemented with 10% normal human serum (Sigma-Aldrich). The monocytes were cultured at 37°C, 5% CO₂.

L-AA (Sigma-Aldrich) was prepared fresh by dilution in medium prior to each experiment. The possible effects of AA-induced toxicity and pH changes were investigated (see Supplementary Materials and Methods and Figures S1(A) and S1(B)). AA at 125 μ g/ml was used for experiments if not otherwise indicated.

2.4 | Polarization and flow cytometry

Monocytes from healthy controls ($n = 6$) were polarized in vitro by the addition of LPS (10 ng/ml; Invivogen) or LTA (1 μ g/ml; Sigma-Aldrich), simulating Gram-negative and Gram-positive bacterial-induced polarization, respectively. The cells were subsequently cultured for 48 h, with or without AA. In some experiments, AA was alternatively added after 24 h of stimulation with LPS or LTA. Next, cells were detached with PBS/0.5 mM EDTA and gentle pipetting. Monocytes were washed and resuspended in 50 μ l PBS. The monocytes were subsequently stained for anti-CD40, PDL1, CD16, and CD163 (see Supplementary Materials and Methods for details). Cells were finally analyzed by the CytoFLEX.

2.5 | Intracellular cytokine production

Monocytes from healthy controls were isolated as described above. Following isolation, medium was supplemented with 1 μ l/ml of BD GolgiPlug (BD Biosciences) and 1 ng/ml LPS or 100 ng/ml LTA as inducers of cytokine production. When used, AA was added directly, and monocytes were cultured for 5 h before detachment using PBS/0.5 mM EDTA and fixated/permeabilized using the BD CytoFix/Perm kit (BD Biosciences) according to the manufacturer's instructions. The monocytes were subsequently stained for anti-IL-1 β (clone: JK1B-1, Alexa fluor 647; Biolegend), anti-IL-6 (clone: MQ2-13A5, PE-Cy7; Biolegend), anti-TNF (clone: MAb11, BV650; BD), and anti-IL-8 (clone: E8N1, Alexa

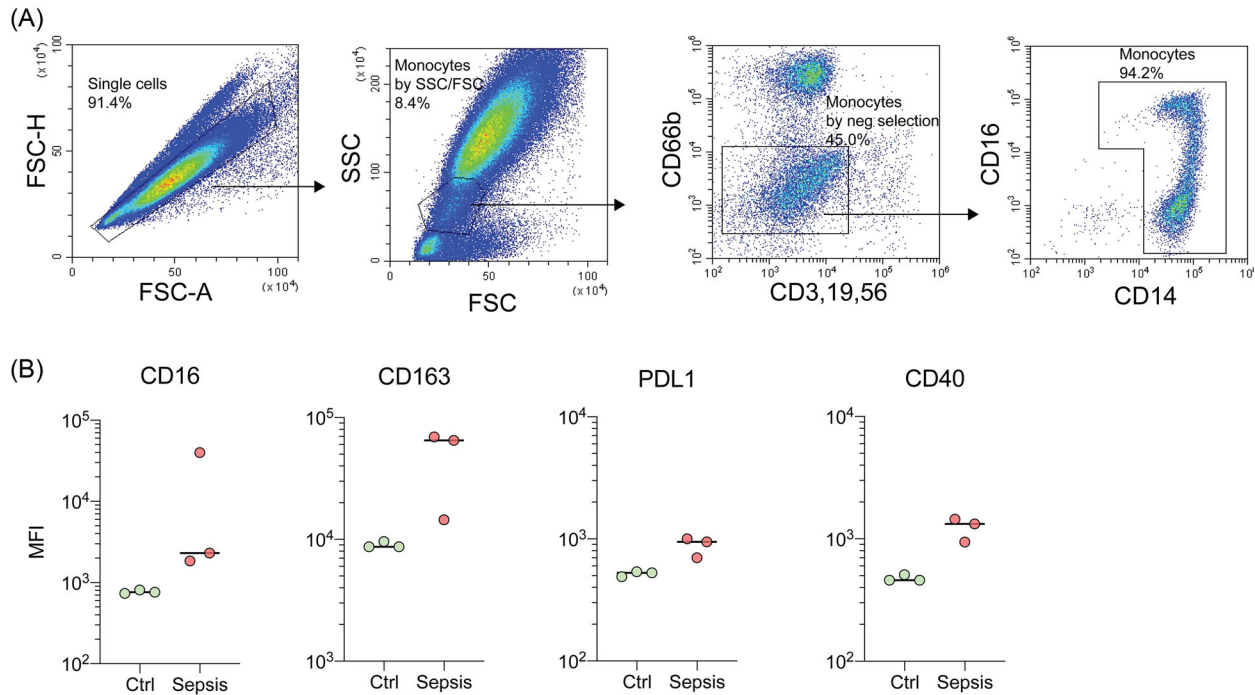


FIGURE 1 Monocytes in blood from patients with sepsis show increased expression of the polarization markers CD16, CD163, CD40, and PDL1. Monocytes in whole blood from sepsis patients ($n = 3$) or healthy controls ($n = 3$) were analyzed for the expression of 4 polarization markers. Panel (A) shows a representative gating strategy in a patient with sepsis. Panel (B) shows the expression of polarization markers in sepsis or healthy controls. Line at median

fluor 488; BD), all diluted 1:50, for 30 min at 4°C and analyzed by a CytoFLEX.

2.6 | Statistics

Statistical analysis was performed using Prism 8. Values are presented as median if not otherwise indicated. Paired data were analyzed using the Wilcoxon matched-pair signed rank test. $p < 0.05$ was considered statistically significant.

3 | RESULTS AND DISCUSSION

Septic monocytes have been previously shown to be polarized as evidenced by increased expression of M1 (CD40 and PDL1) and M2 (CD16 and CD163) polarization markers, amongst others.^{11–13} However, results on the expression of polarization markers are conflicting and likely reflect different time points and subgroups amongst patients. Thus, we sought to confirm previously described changes in monocyte polarization in our setting. First, we observed that monocytes in whole blood from septic patients ($n = 3$) displayed signs of polarization, that is, increased expression of CD16, CD40, CD163, and PDL-1 when compared with controls ($n = 3$) (Figures 1(A) and 1(B)). Next, we sought to explore the influence of 2 bacterial components, LPS and lipoteichoic acid (LTA), on polarization of healthy monocytes. Stimulation of healthy monocytes ($n = 6$) resulted in the significant up-regulation of CD16,

CD40, CD163, and PDL-1 ($p < 0.05$) upon 48 h of LPS stimulation (Figures 2(A) and 2(B)). Histograms of a representative LPS-stimulated sample can be found in Figure 2(A). Additionally, CD16, CD163, and PDL1 were up-regulated following polarization with LTA ($p < 0.05$) (Figure 2(C)). Thus, polarization can be induced by LPS or LTA, with a polarization pattern resembling that of monocytes from patients with sepsis.

AA has been suggested as a treatment for multiple diseases, ranging from cancer to sepsis. Patients with sepsis have lower levels of AA, which can be reversed upon AA treatment.¹ The general effects of AA in sepsis are believed to be anti-inflammatory and antioxidative.¹⁶ Multiple animal and disease models of sepsis have shown the benefits of AA treatment on several organ systems, for example, the vascular and pulmonary systems.^{17–20} An early retrospective before–after study found that AA reduced mortality in patients with sepsis, which was also later observed in a randomized clinical trial.^{2,8} Furthermore, a reduction in organ failure has been observed, which was one of several secondary end points.¹⁰ However, it is important to note that several randomized control trials failed to demonstrate a general beneficial effect of AA in sepsis patients, with an additional failure to meet their primary outcomes.^{7–10,21,22} However, these studies did not thoroughly distinguish among different types of sepsis, pathogens, or immunologic states. Hence, AA may have potential within specific time windows or in certain subgroups.

Few studies have investigated effects of AA treatment on the immune response in sepsis. AA has been shown to restore several effector functions of septic neutrophils *ex vivo*, for example,

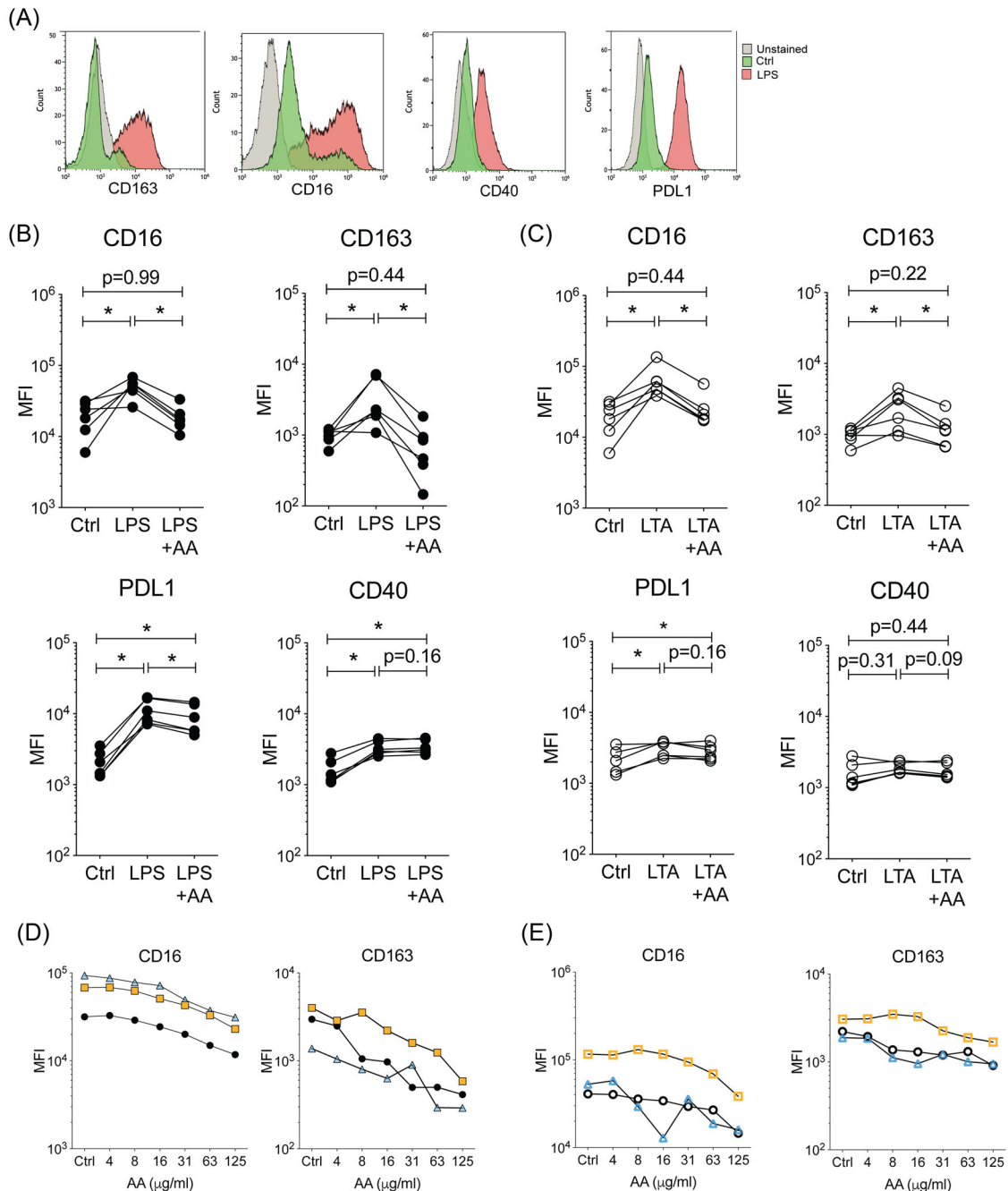


FIGURE 2 Ascorbic acid inhibits up-regulation of CD16 and CD163, but not CD40 and PDL-1, in LPS or LTA-polarized monocytes in vitro. Monocytes were isolated from healthy controls ($n = 6$) and polarized for 48 h or not (ctrl) before analysis of surface markers by flow cytometry. In addition, monocytes were treated or not with $125 \mu\text{g/ml}$ of AA. Panel (A) shows representative histograms of the 4 markers of 1 LPS-induced control. Panel (B) shows LPS-polarized monocytes with or without AA treatment and panel (C) similarly shows LTA-polarized monocytes. The effects of AA were dose dependent in $n = 3$ controls polarized with (D) LPS or (E) LTA. Data are presented as median fluorescence intensity (MFI). Statistics were performed using the Wilcoxon matched-pair signed rank test. * $p < 0.05$. LPS, lipopolysaccharide; LTA, lipoteichoic acid; AA, ascorbic acid

phagocytosis and NETosis.²³ In monocytes, dehydroascorbic acid was shown to protect against LPS-induced oxidative stress.²⁴ AA has also been shown to inhibit ROS production, NF κ B activation, and to inhibit apoptosis.²⁵⁻²⁷ Thus, AA can influence multiple aspects of the immune system, but little is known of its effects in sepsis.

To investigate if AA could modulate monocyte polarization, non-treated and AA-treated monocytes were stimulated by either LPS or LTA. AA concentrations of $125 \mu\text{g/ml}$ attenuated the expression of CD16 and CD163 in both LPS- and LTA-polarized monocytes to that of the controls ($p < 0.05$; Figures 2(B) and 2(C)), with the inhibition

being dose dependent (Figures 2(D) and 2(E)). In LPS-polarized monocytes, AA slightly reduced the expression of PDL-1, but not of CD40 (Figure 2(B)). Changes in the expression of PDL-1 and CD40 in LTA-polarized monocytes were minor (Figure 2(C)). AA also had no significant effect on the markers in unstimulated monocytes (Figure S2). Thus, the expression of the M2-like markers CD16 and CD163 is attenuated by AA, but not of the M1-like markers CD40 and PDL-1, in LPS- and LTA-polarized monocytes.

Traditionally, CD16 and CD163 are markers of anti-inflammation, or M2 polarization, and are up-regulated by various stimulus *in vitro*, such as IL-10. These monocytes, or macrophages, have traditionally been termed suppressive. Septic monocytes typically display a suppressive or exhausted phenotype. For example, when exposed to LPS, septic monocytes respond poorly with production of proinflammatory cytokines, as opposed to monocytes from patients with hyperinflammatory COVID-19.¹⁵ However, it is important to note the complexity of polarization. For example, CD163⁺ monocytes produce more proinflammatory cytokines than CD163⁻ monocytes in sepsis.¹⁴ Collectively, our data suggest that AA might not target only the proinflammatory aspect (inflammatory cytokine production), but also the immunosuppressive phenotype (CD16 and CD163) of monocytes in sepsis.

Furthermore, the dose-dependent decrease in CD16 and CD163 is interesting, as it suggests that AA has an effect at very low concentrations. Patients administered with AA may reach levels above 3000 μ M in the circulation (compared with the concentration of 125 μ g/ml, or 700 μ M, used in this study).¹ Thus, it is possible that the effect of AA on monocytes and the immune system is even greater *in vivo*. However, it is important to note the difference in distribution, degradation, and uptake of AA between *in vitro* and *in vivo*. Hence, the AA concentration necessary to reach the desired effects observed in this study might be significantly higher *in vivo*.

Next, we investigated if the effects of AA are due to a down-regulatory or inhibitory effect. Addition of AA to monocytes 24 h after LPS or LTA stimulation, instead of at the start of culture, resulted in no altered expression of CD16 and CD163 (Figures 3(A) and 3(B)). Thus, AA inhibits up-regulation, rather than down-regulating, the observed surface markers. This observation is important regarding study design, as it suggests that administration of AA is time sensitive. Hence, the effects of AA may vary during the course of the disease. Most studies investigating the use of AA, alone or as HAT (hydrocortisone, ascorbate, and thiamine) in sepsis have a relatively long enrollment time, with most patients enrolled at the ICU.⁷ Prospective studies with short time spans between emergency department arrival and initiation of treatment with AA include the ORANGES study²⁸ and the ATESS study.²¹ In the ORANGES study, there was a significant time reduction in the reversal of shock, whereas the ATESS study did not meet any prespecified outcomes. However, it should be noted that in the ATESS study, 59% of the treatment group had a malignancy, which may have had an impact on the pathologic pathways involved and subsequently, on the treatment effect. Thus, timing may be a crucial factor in the efficacy of AA treatment in sepsis, which is supported by our data, and could have contributed to the lack of benefits in some stud-

ies. Given our finding, it will be interesting to follow an ongoing study, the C-EASIE trial (ClinicalTrials.gov Identifier: NCT04747795), which is investigating early (within 6 h) administration of AA already at the emergency department.

Another aspect of time sensitivity is outlined in the meta-analysis by Scholtz⁷ where treatment continuing for 3–4 days yielded a positive response, whereas shorter or longer treatment showed no benefit on mortality. This could indicate that treatment must continue long enough to inhibit polarization of freshly produced monocytes (since down-regulation may not occur) but not too long as it may enhance the sepsis-induced immunosuppression.

There have also been animal experiments of megadoses of AA in sepsis, in addition to some studies and case reports on megadoses of AA in COVID-19, indicating some potential beneficial effects.^{29,30} In our *ex vivo* system, such doses have the potential of affecting the viability of the monocytes and thereby possibly attenuating the inflammatory response. It is possible that such megadoses could have a similar effect *in vivo* by down-regulating the immune response through decreasing viability. It is therefore possible that megadoses of AA could have beneficial effects when given later in the disease course, where lower doses have less effect. Hence, it would be interesting to investigate the potential effect of megadoses AA given *in vivo* on monocytes. A speculative regimen in line with this would be an initial megadose of AA, followed by ordinary high dosage of AA, thereby first inhibiting the overactivated monocytes already present, followed by a modulation of the polarization of monocytes produced.

To investigate whether AA affected monocyte function, we assessed cytokine production of IL-1 β , IL-6, IL-8, and TNF in healthy monocytes ($n = 6$). Both LPS and LTA induced production of all cytokines, which was attenuated by AA ($p < 0.05$) (Figures 4(A) and 4(B)). This effect was also dose dependent (Figure S3). Thus, AA affects monocytes on a functional level by inhibiting production of several proinflammatory cytokines. Using a similar method, a previous study found that AA attenuates LPS-induced production of IL-6 and TNF, but not IL-8 in monocytes within whole blood.³¹ The use of whole blood, rather than purified monocytes, could be a major contributor to the discrepancy between our results. Nevertheless, we have further confirmed that AA influences monocytes by inhibiting proinflammatory cytokine production.

The use of LPS- and LTA-polarized monocytes, as opposed to monocytes from patients with sepsis, constitutes a limitation in this study. Although LPS- and LTA-polarized monocytes display similarities with septic monocytes, use of these cells could be important to explore in future studies. In addition, we will pursue studies to investigate the mechanism of AA on monocyte polarization to elude the underlying signaling pathways. We will also further explore its effect on monocyte function in relation to sepsis.

In conclusion, we show that high concentrations of AA attenuate LPS- or LTA-induced surface expression of CD16 and CD163, but not CD40 and PDL-1. Furthermore, AA inhibits LPS- or LTA-induced proinflammatory cytokine production. These data provide an

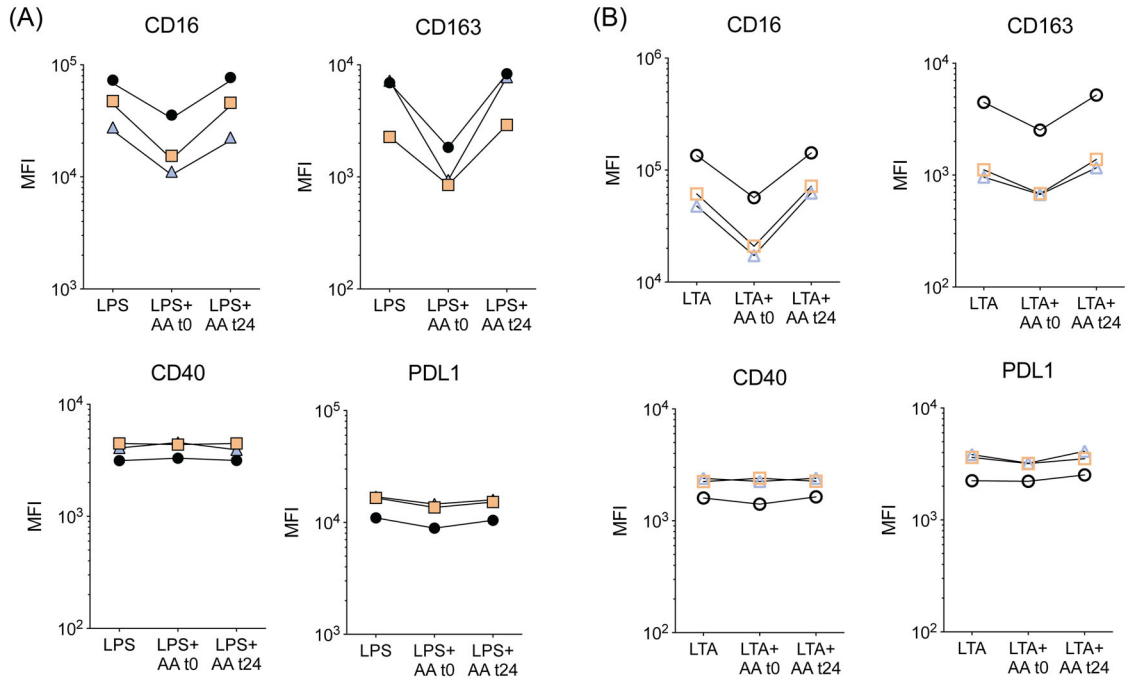


FIGURE 3 Ascorbic acid inhibits up-regulation, rather than down-regulates, CD16 and CD163 expression. Monocytes were isolated from healthy controls ($n = 3$) and polarized for a total of 48 h with (A) LPS or (B) LTA and analyzed for the expression of polarization markers. At the time of polarization (t0), or after 24 h of culture (t24), AA was added at 125 $\mu\text{g}/\text{ml}$. Data are presented as median fluorescence intensity (MFI). LPS, lipopolysaccharide; LTA, lipoteichoic acid; AA, ascorbic acid

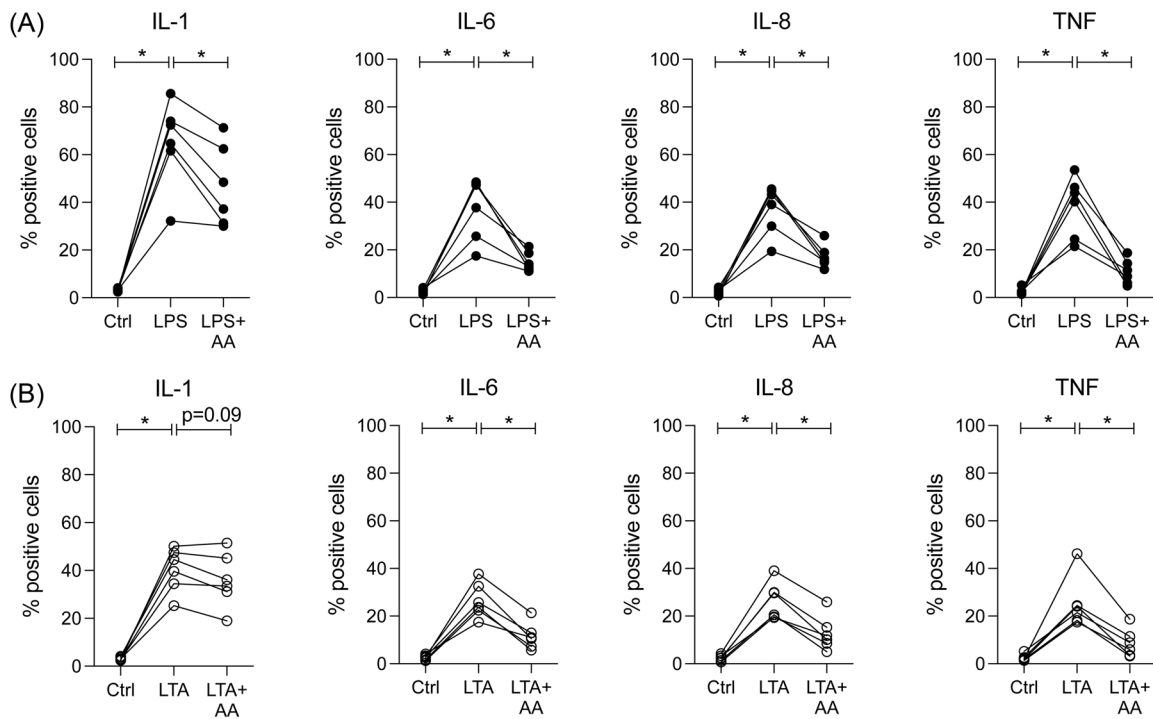


FIGURE 4 Ascorbic acid attenuates production of IL-1 β , IL-6, IL-8, and TNF in LPS or LTA-activated monocytes. Monocytes were isolated from healthy controls ($n = 6$) and treated with brefeldin A followed by LPS or LTA, with or without AA treatment. The cells were cultured for 5 h and analyzed for intracellular accumulation of IL-1 β , IL-6, IL-8, and TNF. Both (A) LPS and (B) LTA significantly induced production of all analyzed cytokines, which was attenuated by treatment with AA. Statistics were performed using the Wilcoxon matched-pair signed rank test. * $p < 0.05$. LPS, lipopolysaccharide; LTA, lipoteichoic acid; AA, ascorbic acid

important insight into the effect of AA on monocytes in relation to sepsis.

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AUTHORSHIP

T. S. carried out the experiments; T. S., R. K., and F. K. interpreted data and wrote the manuscript. F. K. conceptualized and designed the study and collected patient data. All authors have approved the final manuscript, critically revised it, and agreed to be accountable for all aspects of the work.

DISCLOSURE

The authors declare no conflict of interest

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SUPPORTING INFORMATION

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