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VITAMIN C, HYDROCORTISONE, AND THE COMBINATION THEREOF SIGNIFICANTLY INHIBITED TWO OF NINE INFLAMMATORY MARKERS INDUCED BY *ESCHERICHIA COLI* BUT NOT BY *STAPHYLOCOCCUS AUREUS* – WHEN INCUBATED IN HUMAN WHOLE BLOOD

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ABSTRACT—Vitamin C combined with hydrocortisone is increasingly being used to treat septic patients, even though this treatment regimen is based on questionable evidence. When used, a marked effect on key players of innate immunity would be expected, as sepsis is featured by a dysregulated immune response. Here, we explored the effect of vitamin C and hydrocortisone alone and combined, in an *ex vivo* human whole-blood model of *Escherichia coli*- or *Staphylococcus aureus*-induced inflammation. Inflammatory markers for activation of complement (terminal C5b-9 complement complex [TCC]), granulocytes (myeloperoxidase), platelets (β-thromboglobulin), cytokines (tumor necrosis factor [TNF], IL-1β, IL6, and IL-8), and leukocytes (CD11b and oxidative burst) were quantified, by enzyme-linked immunosorbent assay, multiplex technology, and flow cytometry. In *E. coli*- and *S. aureus*-stimulated whole blood, a broad dose-titration of vitamin C and hydrocortisone alone did not lead to dose–response effects for the central innate immune mediators TCC and IL-6. Hence, the clinically relevant doses were used further. Compared to the untreated control sample, two of the nine biomarkers induced by *E. coli* were reduced by hydrocortisone and/or vitamin C. TNF was reduced by hydrocortisone alone (19%, $P = 0.01$) and by the combination (31%, $P = 0.01$). The oxidative burst of monocytes and granulocytes was reduced for both drugs alone and their combination, (ranging 8–19%, $P < 0.05$). Using *S. aureus*, neither of the drugs, alone nor in combination, had any effects on the nine biomarkers. In conclusion, despite the limitation of the *ex vivo* model, the effect of vitamin C and hydrocortisone on bacteria-induced inflammatory response in human whole blood is limited and following the clinical data.

KEYWORDS—Bacteria, complement, cytokines, hydrocortisone, inflammation, sepsis, vitamin C

INTRODUCTION

Sepsis is a life-threatening condition caused by a dysregulated host response to infection (1). The hunt for a cure has been ongoing for years, but, except for growing alertness, early identification, and rapid onset with antibiotics and organ supportive care, drugs specifically targeting causal molecules or pathways responsible for the dysregulated immune response inducing sepsis, are still missing (2). As such, clinical trials in sepsis generally lack effectiveness (3, 4), and a need for precision medicine is gaining increasingly more attention since the beginning of the century (5).

Throughout the last few years, an ongoing debate on whether intravenous vitamin C administration to septic patients is beneficial has dominated the field (6). Also known as ascorbic acid, vitamin C is a low molecular weight anti-oxidant, with both anti-inflammatory and immune-enhancing properties due to its capacity to accept and donate electrons in human cells (7). Its role in sepsis is commonly explained through its attenuating effect in oxidative stress and inflammation, among others (6, 8). It has also been shown to suppress tumor necrosis factor (TNF)-induced NF-κB-activation in human cells and mice (9, 10), overturn myeloperoxidase (MPO) expression in lung cells (11), lower histamine levels in patients (12), and decrease high mobility group box 1 secretion in mice (13). Vitamin C acts both as a nuclear transcription factor and a cytokine and is highly concentrated in several immune cells, such as leukocytes, lymphocytes, and macrophages, enhancing their maturation (14), chemotaxis, proliferation, phagocytosis, and interferon production (15). Furthermore, several studies have also demonstrated the antimicrobial effect of vitamin C, both *in vitro* and *in vivo* (16–18), even opening the path to its use in the prevention and treatment of COVID-19 (19, 20).

On the other hand, hydrocortisone is commonly used in clinical sepsis, although the evidence is debated, short- and

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long-term mortality appears to be unaffected, still, its use is recommended for patients with fluid- and vasopressor refractory shock (2, 21). The primary anti-inflammatory action of this and other glucocorticoids is to repress the transcriptional activity of multiple proinflammatory genes such as NF-κB and AP-1. As such, since some of its effects overlap with those of vitamin C, these two drugs are speculated to act synergistically in the setting of bacterial sepsis (22).

The rationale to use vitamin C in sepsis has been highlighted after the retrospective study by Marik and colleagues (23) which demonstrated that a cocktail of hydrocortisone, ascorbic acid, and thiamine reduced sepsis mortality from 40.4% to 8.5% in the treatment group. An absolute and relative reduction in mortality of 32% and 79%, respectively, is biologically implausible, leading to wide critics due to insufficient methodological setup. Furthermore, this finding led to the conduction of randomized controlled trials which demonstrated a lack of effect by this combined treatment regimen (24–29), even undermining its findings by showing higher 90-day mortality in the treatment group (26). However, vitamin C and hydrocortisone are still used in the treatment of sepsis patients as the *in vitro* anti-inflammatory effects are still thought to be of importance for septic patients.

Innate immunity plays a key role in sepsis pathophysiology (30), by activation of upstream pattern recognition receptors resulting in downstream signaling through a multitude of different pathways. The complement system and the Toll-like receptors (TLRs) have proven to be important, independently acting but also interconnected through extensive cross-talk and responsible for a robust and redundant host response (31, 32). Within this complex network featured by a myriad of mediators, evidence suggests an increased activation of radical oxygen species (33). In this situation, anti-oxidants like vitamin C appear as intuitively relevant drugs. However, preclinical data examining the effect of vitamin C and hydrocortisone on bacteria-induced inflammation is limited (16–18).

Thus, the present study aimed to explore the effect of vitamin C, hydrocortisone, and the combination thereof, in a human whole-blood model of inflammation and study key aspects of the innate immune response to Gram-negative and Gram-positive bacteria in the presence and the absence of these drugs.

MATERIALS AND METHODS

Equipment and reagents

Endotoxin-free tubes from Thermo Fischer Scientific NUNC (Roskilde, Denmark), sterile phosphate-buffered saline (PBS) with Ca2+ and Mg2+, and ethylenediaminetetraacetic acid (EDTA) from Sigma–Aldrich (Steinheim, Germany) were used. Lepirudin (Refludan®) from Pharmion (Windsor, UK) was diluted in PBS to a concentration of 2.5 mg/mL and used as an anticoagulant. Ascorbic acid 150 mg/mL (Pascorbin®) was obtained from Pascoe Natural Healthcare (Gießen, Germany), whereas hydrocortisone 50 mg/mL (Solu-Cortef®) was obtained from Pfizer (New York, NY).

Bacteria

Heat-inactivated *Escherichia coli* strain LE392 (ATCC 33572) and live *Staphylococcus aureus* Cowan strain 1 (ATCC 12598) were purchased from the American Type Culture Collection (Manassas, VA). Both bacteria are clinically relevant, representing the most common pathogens of Gram-negative and Gram-positive origin. Previously experiments using the same whole-blood model have demonstrated that live and dead or heat-inactivated strains of *E. coli* and *S. aureus* induced a similar pronounced and broad inflammation (34). Concentrations of both bacteria were adjusted to provoke noticeable inflammation, and differences in concentrations chosen (10^7 bacteria/mL of *E. coli* and 10^8 bacteria/mL of *S. aureus*) to obtain reasonable similar responses.

Complement activation

The soluble terminal C5b-9 complement complex (TCC) was quantified by an established in-house enzyme-linked immunosorbent assay (ELISA) previously described (35) and later modified (36). Briefly, the capture antibody is the monoclonal anti-C9 neoepitope (clone aE11) and the detection antibody is the monoclonal biotinylated anti-C6 (clone 9C4). The results are expressed in ''complement arbitrary units'' (CAU) per milliliter with Stock Complement Standard number 2 as standard (set to 1,000 CAU/mL) (37) and zymosan-activated human serum as the positive control.

Cytokines

Human proinflammatory cytokine interleukin 6 (IL-6) was quantified through an ELISA-based commercial kit from R&D Systems (Minneapolis, MN). The samples were diluted at 1:10 and analyzed according to the manufacturer's provided protocol (catalog number DY206, lot P223708). Proinflammatory cytokines, namely tumor necrosis factor and interleukins 1β (IL-1β), 6 (IL-6), and 8 (IL-8), as well as the anti-inflammatory IL-10, were also measured using a multiplex cytokine panel assay from Bio-Rad Laboratories (Hercules, CA) named Bio-Plex Pro™ Human Cytokine 27-plex Panel (catalog #M500KCAF0Y). The samples were analyzed according to the manufacturer's provided protocol (standard lot # 64301206, control lot # 64347577).

Granulocyte activation

Human granulocyte activation marker MPO was quantified through an ELISA-based commercial kit from R&D Systems (Minneapolis, MN). The samples were diluted 1:80 to 1:4000 and analyzed according to the manufacturer's provided protocol (catalog number DY3174, lot P132450).

Platelet activation

Human platelet activation marker beta-thromboglobulin (β-TG) was quantified through an ELISA-based commercial kit from Diagnostica Stago (Asnières-sur-Seine, France). The samples were diluted 1:500 to 1:2,500 and analyzed according to the manufacturer's provided protocol (Asserachrom, REF 00950, lot 256065).

Ex vivo human whole-blood model of inflammation

The human whole-blood model of inflammation used has previously been described in detail (38). Briefly, fresh human whole blood was collected into 4.5-mL NUNC tubes and anticoagulated with a specific thrombin-inhibitor, lepirudin at a final concentration of 50 μg/mL. Unlike other anticoagulants, lepirudin is inert for complement activation thus allowing cross-talk with complement and the remaining inflammatory network in the whole blood (38). Baseline samples were preincubated with PBS for 5 min at 4°C (samples on slushed ice) after the blood was drawn, then processed immediately by adding EDTA. The rest of the samples was preincubated with PBS or drug for 5 min at 4°C, before adding PBS, *E. coli* to a final concentration of 1×10^7 bacteria/mL or *S. aureus* to a final concentration of 1×10^8 bacteria/mL, then incubated for 120 min at 37 °C. At the end of the incubation, EDTA was added to a final concentration of 20 mM, stopping complement activation. The tubes were centrifuged for 20 min at 3,000*g* at 4 °C. Plasma was collected and stored at −70°C until analyzed.

Vitamin C and hydrocortisone titration and combination experiments

Different concentrations of vitamin C and hydrocortisone were assessed initially by measuring the effect on two different key inflammatory mediators, interleukin 6 (IL-6), and the soluble form of the terminal complement complex (sC5b-9 or TCC). The final concentrations used were equivalent to what is used in the clinical setting, 1.5 g of vitamin C and 50 mg of hydrocortisone (23, 26). Given a blood volume of 5 L, these concentrations of vitamin C and hydrocortisone are equivalent to an instant concentration of 300 μL and 10 μL, respectively.

Incubation of whole blood for CD11b analysis

Whole blood was drawn from six healthy human donors (three males and three females) and treated as described before. Bacterial activation with *E. coli* (adjusted to 1×10^8 bacteria/mL) or *S. aureus*, and PBS (control) was performed for 15 min at 37 °C, after which the blood was fixed with 0.5% (v/v) paraformaldehyde at 37°C

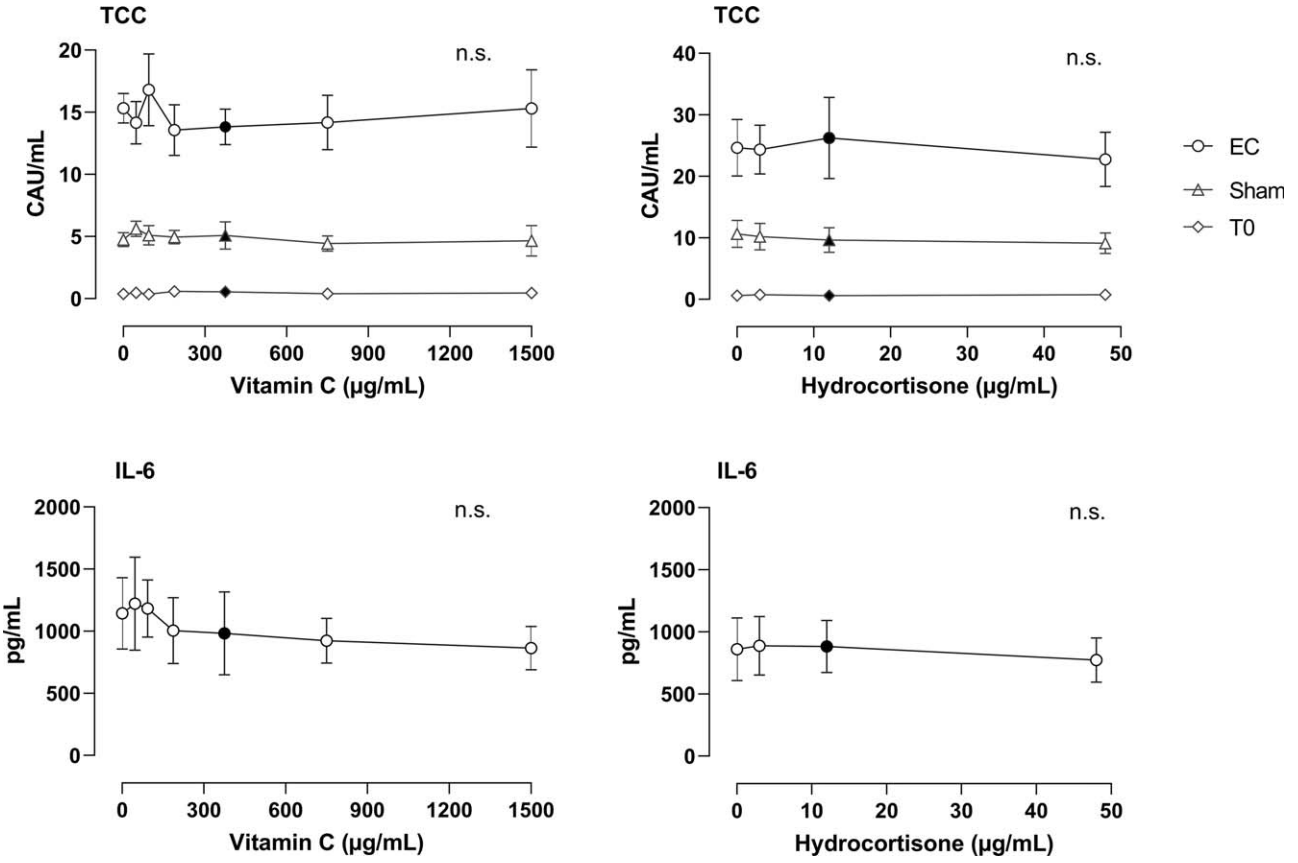

FIG. 1. **Vitamin C and hydrocortisone titration.** Human whole blood was incubated with different concentrations of vitamin C (left panels) or hydrocortisone (right panels) for 120 min (Sham) at 37°C, with or without *E. coli* 10^7 bacteria/mL (EC). The soluble terminal C5b-9 complement complex (TCC) expressed in CAU per milliliter is shown in the upper panels and interleukin 6 (IL-6) in the lower panels. The doses of the drugs closer to those used in the clinical trials (23, 26), and subsequently used in the combination experiments below, are depicted with dark fill. IL-6 levels in the non-incubated samples (T0) or incubated without *E. coli* (Sham) were under the threshold of detection of the ELISA used and therefore not shown in the figure. The data are presented as mean ± SEM (n = 6) for all experiments. Statistical comparisons were performed between the concentrations of vitamin C or hydrocortisone and its absence. ELISA indicates enzyme-linked immunosorbent assay; n.s., not significant.

for 4 min. Fixed blood was stained with anti-CD11b phycoerythrin, anti-CD14 PerCP, and anti-CD45 V450, all antibodies from Becton Dickinson (San Jose, CA), for 15 min at room temperature in the dark. The red cells were lysed with EasyLyse™ (Dako, Agilent, Santa Clara, CA) for 15 min and within 1 h run on a NovoFlow cytometer (ACEA, Agilent), with a threshold on CD45 to exclude debris. Monocytes and granulocytes were gated in a side-scatter/CD14 dot-plot. Expression of the activation marker CD11b (also known as CR3) on granulocytes and monocytes was measured as the mean fluorescence intensity. The data were analyzed by NovoExpress (ACEA, Agilent).

Incubation of whole blood for oxidative burst analysis

Samples were treated as described for CD11b quantification and the oxidative burst was quantified through *Phagoburst*™, a commercial reagent kit for the quantification of the oxidative burst activity of monocytes and granulocytes in heparinized human whole blood from Celonic (Heidelberg, Germany), according to the manufacturer's provided protocol, except for adding DNA-stain. Gating was performed in a forward-scatter/side-scatter dot plot. The flow cytometer and the data analysis software were the same as used for CD11b quantification.

Statistics

The data were analyzed using GraphPad Prism version 8.3.0 (San Diego, CA) for Windows by one- or two-way ANOVA with Geisser-Greenhouse correction and Dunnett's multiple comparisons test for the comparison of multiple columns. A P-value < 0.05 was considered statistically significant.

Ethics

Each blood donor provided informed written consent, in addition to the approval of the study by the Regional Ethical Committee (REK Sør, Ref. number S-04114).

RESULTS

Vitamin C and hydrocortisone titration

To assess the effect of vitamin C and hydrocortisone on complement activation and the formation of IL-6, incremental doses of vitamin C and hydrocortisone, covering the clinical concentrations used, were added to human whole blood (n = 6) and incubated with *E. coli* 10^7 bacteria/mL (EC) or without (Sham), for 120 min at 37°C. Non-incubated baseline samples (T0) were used for comparison (Fig. 1). Neither vitamin C nor hydrocortisone provoked a statistically significant dose–response effect in the formation of TCC or expression of IL-6. The symbols with dark fill in Figure 1 indicate the concentration that was used in the experiments below.

Complement, granulocyte, and platelet activation measured in plasma

The potential effect of vitamin C, hydrocortisone, and the combination thereof on either *E. coli* or *S. aureus* bacterial-induced activation of complement, granulocytes, and platelets were examined in human whole blood (Fig. 2). Neither vitamin C, hydrocortisone, nor the combination thereof had any significant attenuating effect on *E. coli* (Fig. 2, left panels) or *S. aureus* (Fig. 2, right panels) induced complement activation

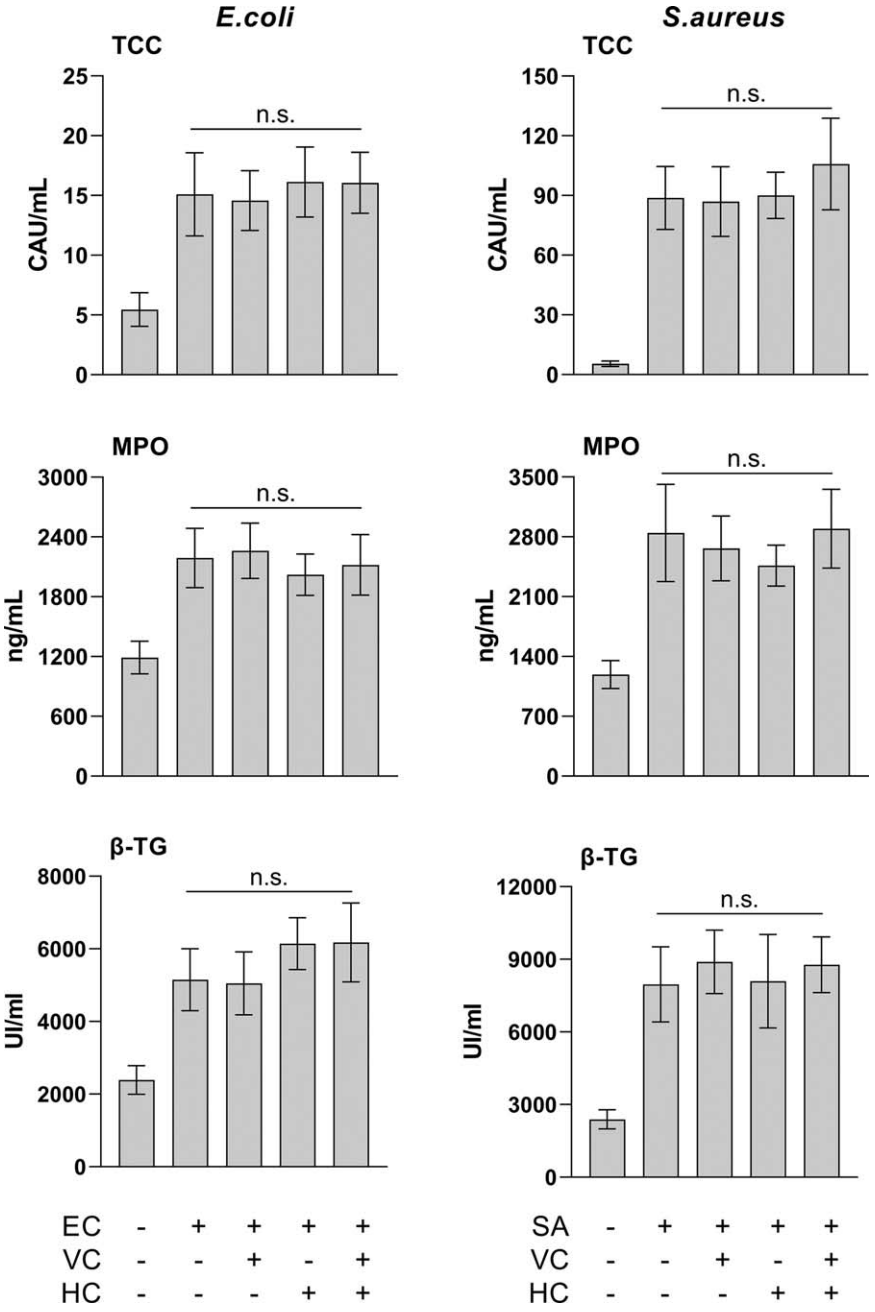

FIG. 2. **Complement, granulocyte, and platelet activation measured in plasma.** Human whole blood was incubated with *E. coli* 10^7 (EC) (left panels) or *S. aureus* 10^8 (SA) (right panels) and the effect of vitamin C 300 μg/mL (VC), hydrocortisone 10 μg/mL (HC), and the combination thereof was evaluated. The soluble terminal C5b-9 complement complex (TCC) expressed in "complement arbitrary units" (CAU) per milliliter is shown in the upper panels and the human granulocyte activation marker myeloperoxidase (MPO) and the human platelet activation marker beta-thromboglobulin (β-TG) in the lower panels. The data are presented as mean ± SEM (n = 6) for all experiments. Statistical comparisons were performed between the groups incubated with bacteria. n.s., not significant.

measured by TCC, granulocyte activation measured by MPO or platelet activation measured by β-TG.

Inflammatory cytokines release measured in plasma

The potential effect of vitamin C, hydrocortisone, and the combination thereof on bacterial-induced cytokine release (IL-1β, IL6, IL-8, and TNF) with *E. coli* (Fig. 3, left panels) or *S. aureus* (Fig. 3, right panels) was examined in human whole blood. *E. coli*-induced TNF formation was attenuated by hydrocortisone alone (19%, $P = 0.01$) and by the combination of vitamin C and hydrocortisone (31%, $P < 0.01$). The release of all other cytokines, induced by either *E. coli* or *S. aureus*, was not affected by vitamin C, hydrocortisone, or the combination thereof (Fig. 3).

Monocyte and granulocyte activation measured by surface markers in flow cytometry

The potential effect of vitamin C, hydrocortisone, and the combination thereof on *E. coli*- (left panels) or *S. aureus*- (right panels) induced upregulation of CD11b on monocytes and granulocytes was examined in human whole blood (Fig. 4).

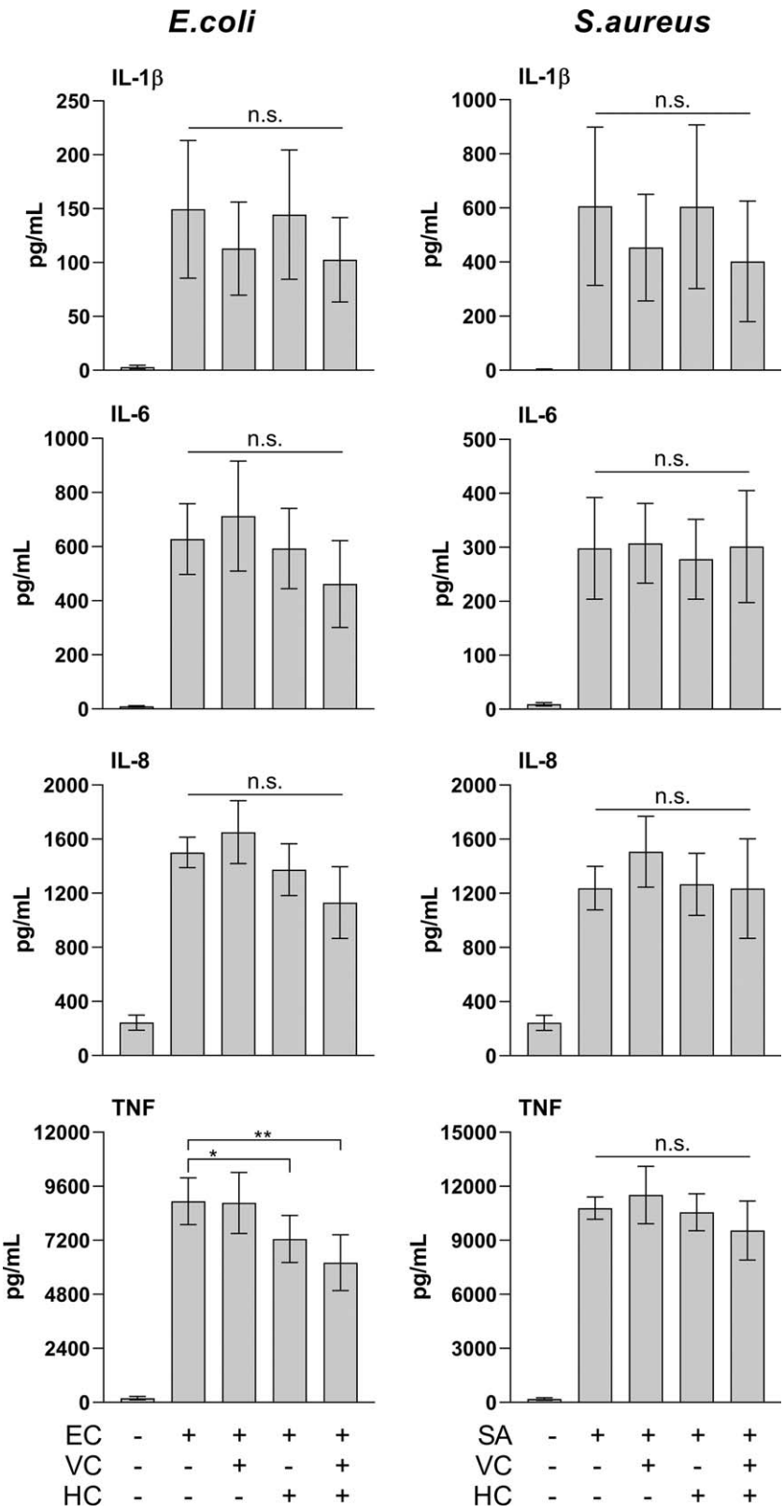

Fig. 3. **Inflammatory cytokines release measured in plasma.** Human whole blood was incubated with *E. coli* 10^7 (EC) (left panels) or *S. aureus* 10^8 (SA) (right panels), and the effect of vitamin C 300 μg/mL (VC), hydrocortisone 10 μg/mL (HC), or the combination thereof on central proinflammatory cytokines, IL-1β, IL-6, IL-8, and TNF, was evaluated. The data are presented as mean ± SEM (n = 6) for all experiments. Statistical comparisons were performed between the groups incubated with bacteria. n.s., not significant. $^{*}P < 0.05$ and $^{**}P < 0.01$.

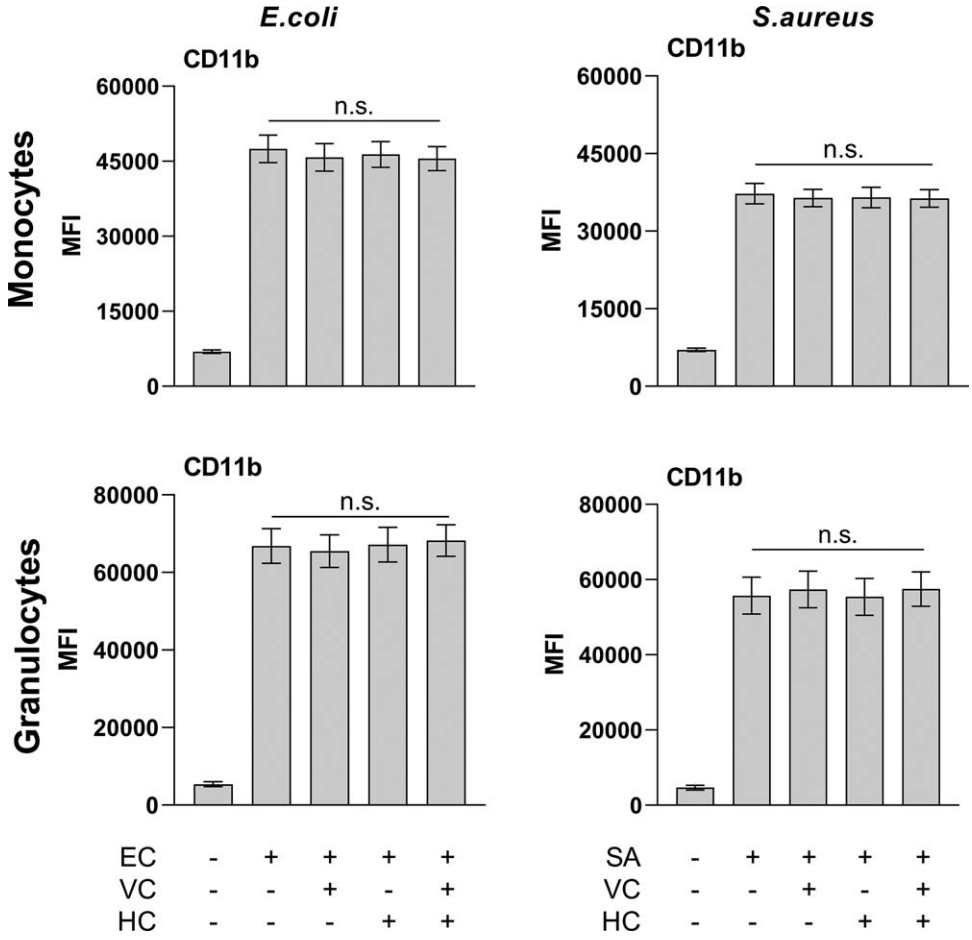

FIG. 4. **Monocyte and granulocyte activation measured by surface markers in flow cytometry.** Human whole blood was incubated with *E. coli* 10^7 (EC) (left panels) or *S. aureus* 10^8 (SA) (right panels), and the effect of vitamin C 300 μg/mL (VC), hydrocortisone 10 μg/mL (HC), or the combination thereof on the activation of monocytes and granulocytes was evaluated. The data are presented as mean ± SEM (n = 6) for all experiments. Statistical comparisons were performed between the several groups incubated with bacteria. n.s., not significant.

Compared to the uninhibited bacteria-induced activation, there were no significant differences in the expression of CD11b on either monocytes or granulocytes (Fig. 4).

Oxidative burst measured by reactive oxygen species in flow cytometry

The release of reactive oxygen species was measured by oxidative burst (Fig. 5). Compared to the *E. coli*-induced activation, vitamin C alone significantly attenuated oxidative burst in monocytes by 14% ($P = 0.04$) and in granulocytes by 19% ($P = 0.02$) (Fig. 5, left panels). Hydrocortisone alone attenuated burst in granulocytes by 16% ($P = 0.02$). The combined inhibition with vitamin C and hydrocortisone reduced the release of reactive oxygen species in monocytes by 8% ($P = 0.04$) and in granulocytes by 15% ($P = 0.03$). *S. aureus*-induced oxidative burst in monocytes and granulocytes were not affected by any inhibition (Fig. 5, right panel).

DISCUSSION

In the present study, we explored the effect of vitamin C and hydrocortisone on *E. coli*- and *S. aureus*-induced inflammation in human whole blood, measuring complement activation, leukocyte activation, platelet activation, and cytokine release. Instead of testing a biological hypothesis by starting at the bench before going to the bedside, we went from bedside to bench, trying to elucidate any rationale for the use of vitamin C in septic patients. In general, hydrocortisone demonstrated anti-inflammatory properties on TNF release, whereas both hydrocortisone and vitamin C showed effects on oxidative burst when *E. coli* was used. No effects were seen by neither of the drugs using *S. aureus*.

Different concentrations of vitamin C and hydrocortisone were assessed initially by measuring the effect on two different key inflammatory mediators, interleukin 6 (IL-6) and the soluble form of TCC (sC5b-9). Both mediators appear early in the sepsis pathophysiology, reflect different inflammatory pathways, and are robust measurable markers (39). However, we found no differences in the measured level of these two markers over a broad concentration range including the doses equivalent to what is used in the clinical practice (Fig. 1) (26). Thus, we decided to continue the experiments with these doses.

As expected, both *E. coli* and *S. aureus* induced a pronounced complement activation and release of both MPO

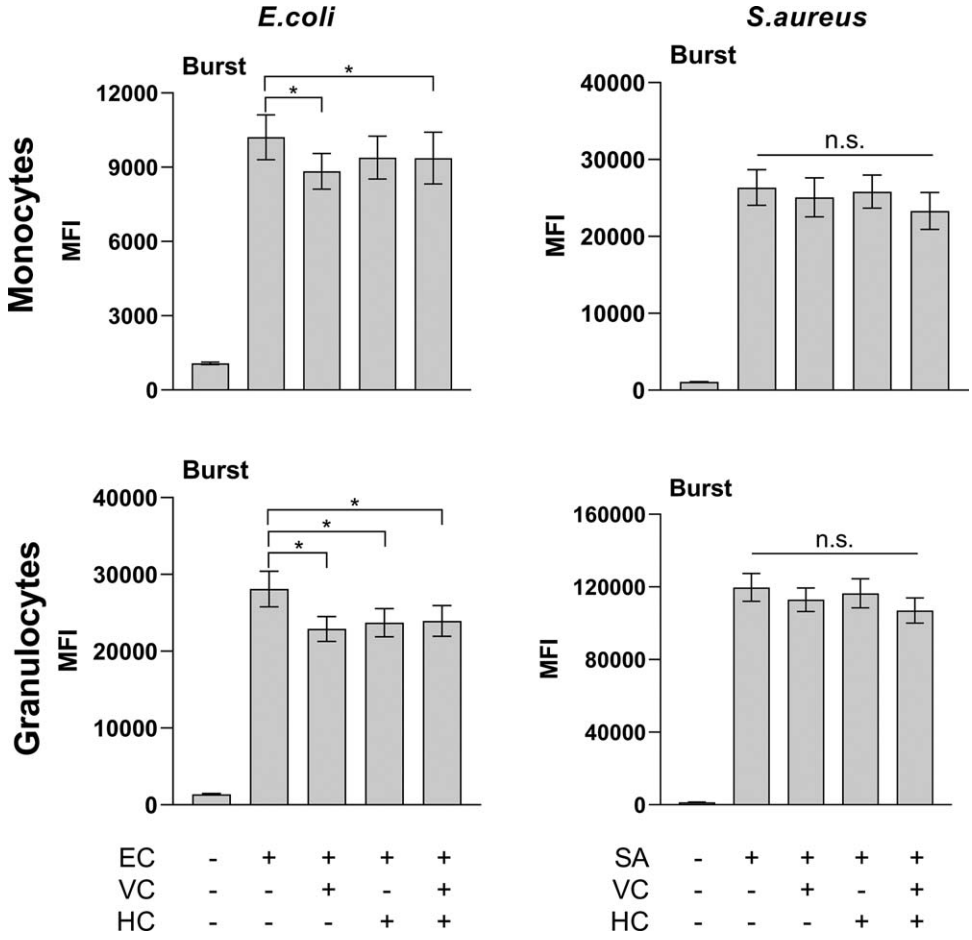

FIG. 5. **Oxidative burst measured by reactive oxygen species in flow cytometry.** Human whole blood was incubated with *E. coli* 10^8 (EC) (left panels) or *S. aureus* 10^8 (SA) (right panels), and the effect of vitamin C 300 μg/mL (VC), hydrocortisone 10 μg/mL (HC), or the combination thereof on the oxidative burst release by monocytes and granulocytes was evaluated. The data are presented as mean ± SEM (n = 6) for all experiments. Statistical comparisons were performed between the several groups incubated with bacteria. n.s., not significant. $^*P < 0.05$.

and β-thromboglobulin. Complement can be regarded as a master alarm system of innate immunity, where in particular the potent anaphylatoxin C5a, appears to be one of the main key players in sepsis (40). Controlled activation of these immunological responses is of prime importance in maintaining homeostasis and immune surveillance. MPO facilitates the neutrophil-mediated killing of bacteria by enhancing the production of oxygen and nitrogen. Uncontrolled MPO release exaggerates inflammation and can also lead to tissue damage in the absence of inflammation (41). Moreover, one previous animal study has described a decrease of MPO release with vitamin C in septic damaged mice lungs (11). Similarly, β-thromboglobulin secreted from platelet granules participates in the inflammation but its excess release will increase sepsis-related impairments (42). However, in our experiments, we could not see attenuating effects of either vitamin C or hydrocortisone on these central markers.

E. coli and *S. aureus* also induced a clear-cut cytokine response assessed by increased formation of the classical proinflammatory cytokines IL-1β, IL6, IL-8, and TNF, reflecting the "cytokine storm." Again, vitamin C alone did not have any attenuating effects on either *E. coli*- or *S. aureus*-induced inflammation. TNF-induced phosphorylation inhibition of NF-κB has been described (9, 10); however, it occurs downstream of the TNF receptor, so a change in the free concentration would not be expected. On the other hand, hydrocortisone significantly decreased *E. coli*-induced TNF formation. This observation is in line with previous studies, describing suppression of TNF formation in human monocytes by hydrocortisone (43). The role of low-dose hydrocortisone in septic patients has been investigated in numerous randomized clinical trials, but its use is still controversial. The Surviving Sepsis Campaign suggests in general against its use, except to those patients with shock not responding to fluid and vasoactive treatment (2). Notably, hydrocortisone did not have any effect on *S. aureus*-induced inflammation.

An essential part of host defense against pathogens is oxidative burst. The NADPH oxidase (NOX2) is the enzyme responsible for the creation of superoxide anions, enabling oxidative damage and destruction of neutrophil- and macrophage-engulfed pathogens (44). Furthermore, it is well known that sepsis and septic shock leads to an augmentation of free radical oxygen species, potentially causing increased damage to the host (33). It is proposed that arachidonic acid and its

derivatives may have antagonistic roles on NOX2 activity (45), advocating the use of vitamin C. In our study, we observed that vitamin C attenuated oxidative burst in monocytes and granulocytes by 14% and 19%, respectively, whereas hydrocortisone attenuated burst in granulocytes by 16%. Among all different inflammatory readouts explored, reduction of oxidative burst by vitamin C was the only significantly affected read-out.

In this *ex vivo* model, we have previously used a completely different strategy to attenuate the inflammatory response to the two bacteria. By targeting upstream pattern recognition molecules of innate immunity, the complement system and the TLR accessory molecule CD14, we have demonstrated a global downstream attenuating effect on all inflammatory mediators investigated (31). This dual inhibition blocked *ex vivo E. coli*-induced inflammation with 80% to 99% (46, 47), whereas *S. aureus*-induced inflammation was attenuated to a similar extent (48). Furthermore, *in vivo* studies showed a reduction of the cytokine storm to the same extent as *ex vivo*, and most important, significantly increased the survival in mice and pigs (49, 50). As compared to these previous observations demonstrating an efficient attenuation of the inflammatory response, it is uncertain whether the effect by vitamin C observed in the present *ex vivo* study would be of biological significance, and if it would be of therapeutical efficacy if translated into the clinic.

Although distant from what happens in clinical sepsis, we staged this bacterial-induced inflammation through a well-used and reliable *ex vivo* whole-blood model allowing cross-talk to occur between all inflammatory branches within human blood (38). Even though our ''clean'' *ex vivo* model is not comparable to the *in vivo* situation, involving the circulatory system, endothelial cells, and organ interaction with companion systemic processes, it is still surprising that vitamin C and its combination with hydrocortisone had only a reduced effect on two of the nine biomarkers induced by *E. coli* and no effect on the inflammatory response induced by *S. aureus*, given the large effect referred in the most optimistic studies (23). When implementing vitamin C to clinical use, one would expect thorough and broad documentation including preclinical studies advocating beneficial effects. Instead, the treatment regimen appears to be based on case reports and sparse references not always sepsis-specific (6–9, 11–18, 23), i.e., not on data justifying the clinical use. Worryingly, this drug keeps being administered to patients and funds being spent in large clinical trials without a solid scientific rationale for its use. To our knowledge, no properly conducted randomized controlled trial has demonstrated that vitamin C increases survival in sepsis and septic shock. On the contrary, several studies recently published document no effect (26, 29).

In conclusion, bacteria-induced inflammation in a physiologically relevant *ex vivo* whole-blood model showed significant attenuation by vitamin C, hydrocortisone, or the combination thereof for two of the nine biomarkers, namely, TNF and oxidative burst. This effect was seen for *E. coli* although no effect was observed with *S. aureus*. The findings of this preclinical study might partly explain the pathophysiological reason for the randomized clinical trials with negative results, linking bedside findings to what we found on the bench.

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, et al.: The third international consensus definitions for sepsis and septic shock (Sepsis-3). *JAMA* 315(8):801–810, 2016.
2. Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, Ferrer R, Kumar A, Sevransky JE, Sprung CL, Nunnally ME, et al.: Surviving sepsis campaign: international guidelines for management of sepsis and septic shock: 2016. *Intensive Care Med* 43(3):304–377, 2017.
3. Sevransky JE, Nour S, Susla GM, Needham DM, Hollenberg S, Pronovost S: Hemodynamic goals in randomized clinical trials in patients with sepsis: a systematic review of the literature. *Crit Care* 11(3):R67, 2007.
4. Macias WL, Nelson DR, Williams M, Garg R, Janes J, Sashegyi A: Lack of evidence for qualitative treatment by disease severity interactions in clinical studies of severe sepsis. *Crit Care* 9(6):R607–R622, 2005.
5. Cecconi M, Evans L, Levy M, Rhodes A: Sepsis and septic shock. *Lancet* 392(10141):75–87, 2018.
6. Kashiouris MG, L'Heureux M, Cable CA, Fisher BJ, Leichtle SW, Fowler AA: The emerging role of vitamin C as a treatment for sepsis. *Nutrients* 12(2):292, 2020.
7. Pohanka M, Pejchal J, Snopkova S, Havlickova K, Karasova JZ, Bostik P, Pikula J: Ascorbic acid: an old player with a broad impact on body physiology including oxidative stress suppression and immunomodulation: a review. *Mini Rev Med Chem* 12(1):35–43, 2012.
8. Kaźmierczak-Barańska J, Boguszewska K, Adamus-Grabicka A, Karwowski BT: Two faces of vitamin C-antioxidative and pro-oxidative agent. *Nutrients* 12(5):1501, 2020.
9. Cárcamo JM, Pedraza A, Bórquez-Ojeda O, Golde DW: Vitamin C suppresses TNF alpha-induced NF kappa B activation by inhibiting I kappa B alpha phosphorylation. *Biochemistry* 41(43):12995–13002, 2002.
10. Qing Z, Xiao-Hui W, Xi-Mei W, Chao-Chun Z: Vitamin C deficiency aggravates tumor necrosis factor α-induced insulin resistance. *Eur J Pharmacol* 829:1–11, 2018.
11. Fisher BJ, Kraskauskas D, Martin EJ, Farkas D, Wegelin JA, Brophy D, Ward KR, Voelkel NF, Fowler AA 3rd, Natarajan R: Mechanisms of attenuation of abdominal sepsis induced acute lung injury by ascorbic acid. *Am J Physiol Lung Cell Mol Physiol* 303(1):L20–L32, 2012.
12. Hagel AF, Layritz CM, Hagel WH, Hagel HJ, Hagel E, Dauth W, Kressel J, Regnet T, Rosenberg A, Neurath MF, et al.: Intravenous infusion of ascorbic acid decreases serum histamine concentrations in patients with allergic and non-allergic diseases. *Naunyn Schmiedebergs Arch Pharmacol* 386(9):789–793, 2013.
13. Kim SR, Ha YM, Kim YM, Park EJ, Kim JW, Park SW, Kim HJ, Chung HT, Chang KC: Ascorbic acid reduces HMGB1 secretion in lipopolysaccharide-activated RAW 264.7 cells and improves survival rate in septic mice by activation of Nrf2/HO-1 signals. *Biochem Pharmacol* 95(4):279–289, 2015.
14. Manning J, Mitchell B, Appadurai A, Shakya A, Pierce LJ, Wang H, Nganga V, Swanson PC, May JM, Tantin D, et al.: Vitamin C promotes maturation of T-cells. *Antioxid Redox Signal* 19(17):2054–2067, 2013.
15. Thomas WR, Holt PG: Vitamin C and immunity: an assessment of the evidence. *Clin Exp Immunol* 32(2):370–379, 1978.
16. Dey S, Bishayi B: Killing of *S. aureus* in murine peritoneal macrophages by Ascorbic acid along with antibiotics chloramphenicol or ofloxacin: correlation with inflammation. *Microb Pathog* 115:239–250, 2018.
17. Kallio J, Jaakkola M, Mäki M, Kilpeläinen P, Virtanen V: Vitamin C inhibits *Staphylococcus aureus* growth and enhances the inhibitory effect of quercetin on growth of *Escherichia coli* in vitro. *Planta Med* 78(17):1824–1830, 2012.
18. Colunga Biancatelli RML, Berrill M, Catravas JD, Marik PE: Quercetin and vitamin C: an experimental, synergistic therapy for the prevention and treatment of SARS-CoV-2 related disease (COVID-19). *Front Immunol* 11:1451, 2020.
19. Carr AC, Rowe S: The emerging role of vitamin C in the prevention and treatment of COVID-19. *Nutrients* 12(11):3286, 2020.
20. Marik PE, Kory P, Varon J, Iglesias J, Meduri GU: MATH+ protocol for the treatment of SARS-CoV-2 infection: the scientific rationale. *Expert Rev Anti Infect Ther* 19(2):129–135, 2021.
21. Rygård SL, Butler E, Granholm A, Møller MH, Cohen J, Finfer S, Perner A, Myburgh J, Venkatesh B, Delaney A: Low-dose corticosteroids for adult patients with septic shock: a systematic review with meta-analysis and trial sequential analysis. *Intensive Care Med* 44(7):1003–1016, 2018.
22. Marik PE: Hydrocortisone, ascorbic acid and thiamine (HAT therapy) for the treatment of sepsis. Focus on ascorbic acid. *Nutrients* 10(11):1762, 2018.
23. Marik PE, Khangoora V, Rivera R, Hooper MH, Catravas J: Hydrocortisone, vitamin C, and thiamine for the treatment of severe sepsis and septic shock: a retrospective before–after study. *Chest* 151(6):1229–1238, 2017.

24. Zabet MH, Mohammadi M, Ramezani M, Khalili H: Effect of high-dose ascorbic acid on vasopressor's requirement in septic shock. *J Res Pharm Pract* 5(2):94–100, 2016.
25. Fowler AA 3rd, Truwit JD, Hite RD, Morris PE, DeWilde C, Priday A, Fisher B, Thacker LR 2nd, Natarajan R, Brophy DF, et al.: The CITRIS-ALI randomized clinical trial. *JAMA* 322(13):1261–1270, 2019.
26. Fujii T, Luethi N, Young PJ, Frei DR, Eastwood GM, French CJ, Deane AM, Shehabi Y, Hajjar LA, Oliveira G, et al.: Effect of vitamin C, hydrocortisone, and thiamine vs hydrocortisone alone on time alive and free of vasopressor support among patients with septic shock: the VITAMINS randomized clinical trial. *JAMA* 323(5):423–431, 2020.
27. Chang P, Liao Y, Guan J, Guo Y, Zhao M, Hu J, Zhou J, Wang H, Cen Z, Tang Y, et al.: Combined treatment with hydrocortisone, vitamin C, and thiamine for sepsis and septic shock: a randomized controlled trial. *Chest* 158(1):174–182, 2020.
28. Iglesias J, Vassallo AV, Patel VV, Sullivan JB, Cavanaugh J, Elbaga Y: Outcomes of metabolic resuscitation using ascorbic acid, thiamine, and glucocorticoids in the early treatment of sepsis: the ORANGES trial. *Chest* 158(1):164–173, 2020.
29. Sevransky JE, Rothman RE, Hager DN, Bernard GR, Brown SM, Buchman TG, Busse LW, Coopersmith CM, DeWilde C, Ely EW, et al.: Effect of vitamin C, thiamine, and hydrocortisone on ventilator- and vasopressor-free days in patients with sepsis: the VICTAS randomized clinical trial. *JAMA* 325(8):742–750, 2021.
30. Kumar V: Sepsis roadmap: what we know, what we learned, and where we are going. *Clin Immunol* 210:108264, 2020.
31. Barratt-Due A, Pischke SE, Nilsson PE, Espevik T, Mollnes TE: Dual inhibition of complement and Toll-like receptors as a novel approach to treat inflammatory diseases – C3 or C5 emerge together with CD14 as promising targets. *J Leukoc Biol* 101(1):193–204, 2017.
32. Barratt-Due A, Pischke SE, Brekke OL, Thorgersen EB, Nielsen EW, Espevik T, Huber-Lang M, Mollnes TE: Bride and groom in systemic inflammation – the bells ring for complement and Toll in cooperation. *Immunobiology* 217(11):1047–1056, 2012.
33. Bar-Or D, Carrick MM, Mains CW, Rael LT, Slone D, Brody EN: Sepsis, oxidative stress, and hypoxia: are there clues to better treatment? *Redox Rep* 20(5):193–197, 2015.
34. Skjeflo EW, Christiansen D, Landsem A, Stenvik J, Woodruff TM, Espevik T, Nielsen EW, Mollnes TE: Phagocytosis of live and dead *Escherichia coli* and *Staphylococcus aureus* in human whole blood is markedly reduced by combined inhibition of C5aR1 and CD14. *Mol Immunol* 112:131–139, 2019.
35. Mollnes TE, Lea T, Frøland SS, Harboe M: Quantification of the terminal complement complex in human plasma by an enzyme-linked immunosorbent assay based on monoclonal antibodies against a neoantigen of the complex. *Scand J Immunol* 22(2):197–202, 1985.
36. Mollnes TE, Redl H, Høgåsen K, Bengtsson A, Garred P, Speilberg L, Lea T, Oppermann M, Götze O, Schlag G: Complement activation in septic baboons detected by neoepitope-specific assays for C3b/iC3b/C3c, C5a and the terminal C5b-9 complement complex (TCC). *Clin Exp Immunol* 91(2):295–300, 1993.
37. Bergseth G, Ludviksen JK, Kirschfink M, Giclas PC, Nilsson B, Mollnes TE: An international serum standard for application in assays to detect human complement activation products. *Mol Immunol* 56(3):232–239, 2013.
38. Mollnes TE, Brekke OL, Fung M, Fure H, Christiansen D, Bergseth G, Videm V, Lappegård KT, Köhl J, Lambris JD: Essential role of the C5a receptor in *E coli*-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* 100(5):1869–1877, 2002.
39. Ricklin D, Barratt-Due A, Mollnes TE: Complement in clinical medicine: clinical trials, case reports and therapy monitoring. *Mol Immunol* 89:10–21, 2017.
40. Ward PA: The harmful role of c5a on innate immunity in sepsis. *J Innate Immun* 2(5):439–445, 2010.
41. Khan AA, Alsahli MA, Rahmani AH: Myeloperoxidase as an active disease biomarker: recent biochemical and pathological perspectives. *Med Sci (Basel)* 6(2):33, 2018.
42. Xu X, Sun B: Platelet granule secretion mechanisms: are they modified in sepsis? *Thromb Res* 136(5):845–850, 2015.
43. Waage A, Bakke O: Glucocorticoids suppress the production of tumour necrosis factor by lipopolysaccharide-stimulated human monocytes. *Immunology* 63(2):299–302, 1988.
44. El-Benna J, Hurtado-Nedelec M, Marzaioli V, Marie JC, Gougerot-Pocidalo MA, Dang PM: Priming of the neutrophil respiratory burst: role in host defense and inflammation. *Immunol Rev* 273(1):180–193, 2016.
45. Gonzalez-Perilli L, Prolo C, Álvarez MN: Arachidonic acid and nitroarachidonic: effects on NADPH oxidase activity. *Adv Exp Med Biol* 1127:85–95, 2019.
46. Brekke OL, Christiansen D, Fure H, Fung M, Mollnes TE: The role of complement C3 opsonization, C5a receptor, and CD14 in *E. coli*-induced up-regulation of granulocyte and monocyte CD11b/CD18 (CR3), phagocytosis, and oxidative burst in human whole blood. *J Leukoc Biol* 81(6):1404–1413, 2007.
47. Brekke OL, Christiansen D, Fure H, Pharo A, Fung M, Riesenfeld J, Mollnes TE: Combined inhibition of complement and CD14 abolish *E. coli*-induced cytokine-, chemokine- and growth factor-synthesis in human whole blood. *Mol Immunol* 45(14):3804–3813, 2008.
48. Skjeflo EW, Christiansen D, Espevik T, Nielsen EW, Mollnes TE: Combined inhibition of complement and CD14 efficiently attenuated the inflammatory response induced by *Staphylococcus aureus* in a human whole blood model. *J Immunol* 192(6):2857–2864, 2014.
49. Huber-Lang M, Barratt-Due A, Pischke SE, Sandanger Ø, Nilsson PH, Nunn MA, Denk S, Gaus W, Espevik T, Mollnes TE: Double blockade of CD14 and complement C5 abolishes the cytokine storm and improves morbidity and survival in polymicrobial sepsis in mice. *J Immunol* 192(11):5324–5331, 2014.
50. Skjeflo EW, Sagatun C, Dybwik K, Aam S, Urving SH, Nunn MA, Fure H, Lau C, Brekke OL, Huber-Lang M, et al.: Combined inhibition of complement and CD14 improved outcome in porcine polymicrobial sepsis. *Crit Care* 19:415, 2015.