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Combined Inhibition of Complement and CD14 Efficiently Attenuated the Inflammatory Response Induced by Staphylococcus aureus in a Human Whole Blood Model

Espen W. Skjeflo,*† Dorte Christiansen,* Terje Espevik,‡§ Erik W. Nielsen,†,* and Tom E. Mollnes*†,‡,§,‖

The complement and TLR systems are activated in sepsis, contributing to an unfavorable inflammatory “storm.” Combined inhibition of these systems has been documented to efficiently attenuate the inflammatory responses induced by Gram-negative bacteria. In this study, we hypothesized that the combined inhibition would attenuate the inflammatory responses induced by Gram-positive bacteria. *Staphylococcus aureus* bacteria (strains Cowan and Wood), as well as *S. aureus* cell wall lipoteichoic acid (LTA), were incubated in thrombin-inhibited human whole blood. Complement was inhibited at the level of C3 and C5, and the TLRs by inhibiting CD14 and TLR2. Thirty-four inflammatory markers were measured by multiplex technology and flow cytometry. Thirteen markers increased significantly in response to Cowan and Wood, and 12 in response to LTA. Combined inhibition with the C3 inhibitor compstatin and the anti-CD14 Ab 18D11 significantly reduced 92% (Cowan, LTA) and 85% (Wood) of these markers. Compstatin alone significantly reduced 54% (Cowan), 38% (Wood), and 83% (LTA), whereas anti-CD14 alone significantly reduced 23, 15, and 67%, respectively. Further experiments showed that the effects of complement inhibition were mainly due to inhibition of C5a interaction with the C5a receptor. The effects on inhibiting CD14 and TLR2 were similar. The combined regimen was more efficient toward the bacterial effects than either complement or anti-CD14 inhibition alone. Complement was responsible for activation of and phagocytosis by both granulocytes and monocytes. Disrupting upstream recognition by inhibiting complement and CD14 efficiently attenuated *S. aureus*–induced inflammation and might be a promising treatment in both Gram-negative and Gram-positive sepsis. *The Journal of Immunology*, 2014, 192: 2857–2864.

Sepsis is partly a result of an overwhelming and damaging immune response to infection (1). The syndrome, ranging from systemic inflammatory response to severe sepsis, is common, expensive, and life-threatening (2). Mortality rates have declined only modestly, and the preferred treatment is still largely supportive, indicating that new therapeutic strategies are needed (3, 4). In >50% of cases, Gram-positive bacteria cause the primary infection leading to sepsis and *Staphylococcus aureus* is the single most common Gram-positive isolate (5–7). Gram-positive bacteria lack LPS in their cell wall but do contain large amounts of peptidoglycan and lipoteichoic acid (LTA), suggested to be involved in the septic pathogenesis (8, 9).

Both the complement and TLR systems are activated in sepsis (10). The complement system is a group of circulating or membrane-bound proteins readily activated by exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns. It is tightly regulated and contributes to the resolution of the initial threat through activation of both innate and adaptive immune responses, opsonization, bacterial killing, and clearance (11, 12). However, this regulation is disturbed in sepsis and the complement system becomes hyperactive, resulting in inflammation and tissue damage through the downstream effects of activation products, particularly the anaphylatoxins C3a and C5a (13, 14).

Similarly, the transmembrane TLRs either perched on the cell membrane of leukocytes (TLRs 1, 2, 4, 5, 6, and 10) or their intracellular compartments (TLRs 3, 7, 8, and 9) can recognize PAMPs. PAMPs often interact with coproteins such as CD14, and this complex is readily combined with the TLR’s extracellular domains of pattern recognition so that intracellular signaling domains are activated (15). TLR signaling activates the nuclear transcription factor κB that results in the release of inflammatory mediators commonly known as cytokines (16).

Separately, the complement and TLR systems represent the main “arms” of early recognition in innate immunity. Together, their simultaneous activation has the potential to induce a synergistic proinflammatory effect because of extensive cross talk (10, 17–19).

We have previously shown that combined inhibition of C3 and CD14 is a powerful tool to attenuate the inflammatory responses (cytokine release, oxidative burst, phagocytosis, and expression of adhesion molecules and tissue factor) induced by Gram-negative bacteria (*Escherichia coli* and *Neisseria meningitidis*), which are mainly triggered through TLR4 stimulation (20). CD14 is a known coreceptor with several of the TLRs, including TLR4 and TLR2 (21). In this study, we hypothesized that blocking CD14 together with complement would attenuate the inflammatory responses induced by Gram-positive bacteria (*S. aureus*), which are potential...
activators of TLR2 (22–25). If so, the combined CD14 and complement inhibition might be a new concept for treatment of Gram-negative and Gram-positive sepsis.

Materials and Methods

Reagents and equipment

All equipment, including tips, tubes, and buffers used in the experiments, were endotoxin free. EDTA and paraformaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Polypropylene tubes were from Nalge Nunc (Roskilde, Denmark). Polystyrene tubes were from Becton, Dickinson and Company (Franklin Lakes, NJ). PBS, with or without calcium and magnesium, was from Life Technologies (Paisley, U.K.). Lepirudin (Refudin) was obtained from Hoechst (Frankfurt am Main, Germany) or Schering (Berlin, Germany). Flow cytometry was performed using an LSR II flow cytometer (Becton Dickinson, San Jose, CA). Syto BC, LPS-751, Alexa 488, and DMSO were obtained from Invitrogen Molecular Probes (Eugene, OR), and anti-CD11b PE and anti-CD14 FITC were from Becton, Dickinson and Company. OD was measured on an MRX microplate reader (Dynex Technologies, Denkendorf, Germany). Cytokines were measured in the Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA).

Bacterial strains and counting

S. aureus Cowan Strain 1 (ATCC 12598) and Wood 46 (ATCC 10832), hereafter Cowan and Wood, respectively, were obtained from American Type Culture Collection (Manassas, VA). For the first series of experiments (presented in Tables I, II), the bacteria were grown in Nutrient Broth, CM0001 (Oxoid Microbiology Products, Basingstoke, U.K.), heat-inactivated for 60 min, and washed once in PBS without Ca2+ and Mg2+ (Sigma-Aldrich). For all the other experiments, the bacteria were grown in DMEM, F12 (Invitrogen, Carlsbad, CA). Both the heat inactivation and the effect of the wash were tested in separate experiments (data not shown).

The bacteria were counted after staining with Syto BC using a bacteria-counting kit (Molecular Probes, Invitrogen). Three aliquots of the bacteria were count on the flow cytometer using Trucount tubes from Becton, Dickinson and Company, and 2500 beads were acquired, to obtain absolute counts. The final concentration of S. aureus in whole blood was 1 × 107/ml. Pneumococci S. aureus LTA was obtained from Invivogen, and a final concentration of 10 μg/ml was used in the experiments. For the phagocytosis assay, heat-inactivated S. aureus were Alexa stained as previously described for E. coli and N. meningitidis (26).

Optimal concentrations were determined in dose–response experiments (data not shown). All agents were tested and found to be endotoxin free in a Limulus amebocyte lysate assay (Lonza, Basel, Switzerland).

Complement and TLR inhibitors

Murine anti-human CD14 F(ab′)2 (clone 18D11), first described in 2001 (27), and a control Ab F(ab′)2, were obtained from Diotec AS (Oslo, Norway). Compatstatin (CD20), a cyclic peptide that inhibits cleavage of complement factor 3, comprised of the following sequence Ac-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-mle-NH2 and a corresponding peptide were synthesized as previously described (28). The cyclic hexapeptide Ac[OPdChaWR] was used as the C5a receptor antagonist (CSARag) (29). Compatstatin, control peptide, and CSARag were a kind gift from Prof, John D. Lambris. Eculizumab (Soliris) was obtained from Alexion Pharmaceuticals (Cheshire, CT). The T2.5 monoclonal anti-TLR2 Ab was obtained from Hycult Biotech (Uden, the Netherlands).

The optimal inhibitor concentrations were determined in separate dose–response experiments (data not shown).

Whole-blood model of sepsis

Blood from healthy donors was obtained after informed consent according to guidelines from the local ethics committee. Experiments with the blood of each donor were performed as single experiments at different time points as described earlier (30). In brief, fresh human whole blood was collected in polypropylene tubes containing lepirudin (50 μg/ml). Aliquots of whole blood in sterile polypropylene tubes were then preincubated for 9 min in a Stuart block heater at 37°C with PBS, Abs, or inhibitors in PBS (14.3% of total volume, v/v) as indicated. S. aureus, LTA, or PBS (14.3% of total volume, v/v) was then added, and samples were incubated for 15 min. After incubation, 125 μl whole blood was immediately processed for flow cytometry whereas the remaining blood was left to incubate for a total of 2 h on a Rock-n-roller as previously described (30). Aliquots for assay of complement activation products were then immediately supplemented with EDTA (10 mM final concentration) and placed on ice (0°C) to stop further activation. Samples were centrifuged (3220 × g, 15 min at 4°C), and plasma was stored at −80°C until further analysis.

Flow cytometry

The oxidative burst was measured using a Burst test kit (Orpegen Pharma, Heidelberg, Germany). Immediately after 15 min of incubation, 100 μl blood was added to sterile polystyrene tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ) and treated according to the kit procedures, albeit with the minor practical modification of omitting DNA stain to the sample. The cells were resuspended in PBS and analyzed on the flow cytometer. Gates were set on granulocytes and monocytes to analyze each population with regard to mean fluorescence intensity in a forward/侧 scatter dot plot. CD11b was measured after 15 min of incubation. The cells were fixed with 0.5% (v/v) paraformaldehyde in an equal volume for 4 min at 37°C and then stained with anti-CD11b PE, anti-CD14 FITC, and the nuclear dye LDS-751. Samples were run on the flow cytometer with threshold at LDS-751 to exclude red cells and debris. Granulocytes were gated in a side scatter/CD14 dot plot, and CD11b expression was measured as median fluorescence intensity. Phagocytosis of Alexa-stained S. aureus in whole blood was assessed after 15 min according to kit instructions (ORPEGEN Pharma) and otherwise processed as previously described for E. coli and N. meningitidis (26).

ELISA

ELISA plates were prepared by centrifugation, as described earlier.

The cytokine levels in the plasma were analyzed using the microsphere-based Bio-Plex Human Cytokine 27-plex (27 different cytokines including chemokines and growth factors) Assay (Bio-Rad, Hercules, CA). The assay was performed according to instructions from the manufacturer and comprised the following: IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor, G-CSF, GM-CSF, IFN-γ, IFN-γ-induced peptide-10, MCP-1, MIP-1α, MIP-1β, platelet-derived growth factor-BB, RANTES, TNF, and vascular endothelial growth factor (VEGF). The intra-assay coefficient of variation was <10% for all ELISA and multiplex assays.

Statistics

Statistics were calculated by ordinary or repeated-measures one-way ANOVA with Dunnett’s multiple-comparison posttest for the comparison of multiple columns, paired t tests for the comparison of two columns, and the χ2 test for trend using GraphPad Prism 5 and 6 for Mac. A p value < 0.05 was considered significant. Graphs are presented as means with SEM.

Results

Inflammatory effects induced by S. aureus and LTA

Initially, a total of 34 inflammatory markers or mediators was measured (see Materials and Methods). First, the responses to whole bacteria and LTA were examined in the whole-blood model. Cowan and Wood induced a statistically significant increase in 13 inflammatory markers, and exactly the same markers increased for both strains; IL-1β, IL-6, IL-8, eotaxin, MIP-1α, MIP-1β, TNF and VEGF, CD11b in monocytes and granulocytes, oxidative burst in monocytes and granulocytes, and TCC (Table I). LTA induced a statistically significant increase in 12 of the 13 markers induced by whole bacteria, with only VEGF not being significantly increased (Table I). None of the mediators that were not significantly induced by whole bacteria was induced by LTA.

Inhibitory effects of compstatin, anti-CD14, and the combination thereof

We then investigated whether the C3 inhibitor compstatin, a neutralizing anti-CD14 Ab, or the combination of the two could
inhibit the inflammatory responses induced by the bacteria or LTA, that is, reduce the levels of the inflammatory mediators. Compstatin inhibited 7 and 5 of the 13 mediators induced by Cowan and Wood, respectively, and 10 of the 12 induced by LTA. Anti-CD14 inhibited 3 and 2 of the 13 mediators induced by Cowan and Wood, respectively, and 8 of the 12 induced by LTA.

Compstatin and anti-CD14 in combination inhibited 12 and 11 of the 13 mediators induced by Cowan and Wood, respectively, and 11 of the 12 induced by LTA. With respect to the number of mediators inhibited, the combined inhibition was statistically more efficient as compared with single inhibition for both the Cowan and Wood strains (\(p < 0.05\)). This was not the case for LTA, consistent with the relatively efficient effect of the single inhibition on LTA as compared with whole bacteria. The quantitative effects of the different treatment regimens are detailed in Table II. Mean reduction of all mediators using compstatin was 42, 28, and 38% for Cowan, Wood, and LTA, respectively. Mean reduction by anti-CD14 for all mediators was 18, 13, and 27% for Cowan, Wood, and LTA, respectively. The corresponding values using compstatin and anti-CD14 combined were 62% for Cowan, 55% for Wood, and 62% for LTA.

### The mechanism of the complement-mediated inhibitory effect

To explore by which mechanism complement induced the inflammatory responses, we extended the panel of inhibitors by including eculizumab (an Ab blocking C5 cleavage and thus inhibiting both C5a release and C5b-9 formation), and a C5a receptor antagonist, and compared the effect with these with that of compstatin. The cytokines IL-1β, TNF, IL-6, and IL-8, and the granulocyte and monocyte activation markers CD11b, phagocytosis, and oxidative burst were selected as readouts in response to the Cowan strain in these experiments.

All complement inhibitors (compstatin, eculizumab, and the C5aR antagonist) inhibited the cytokine levels in a similar fashion, and the inhibition was statistically significant for all four cytokines when either of the inhibitors was combined with anti-CD14 (Fig. 1). The differences between the inhibitors were not statistically significant. Thus, most of the effects obtained by inhibiting complement activation could be attributed to the C5a effects mediated through the C5aR.

Similarly, the complement-mediated effects on leukocyte activation were investigated by the three complement inhibitors and their combination with anti-CD14 (Fig. 2). All complement inhibitors, as well as their combination with anti-CD14, significantly and substantially inhibited monocyte and granulocyte expression of CD11b (Fig. 2, upper panel) and phagocytosis (Fig. 2, lower panel). Oxidative burst showed a pattern virtually identical to the phagocytosis results (data not shown). Thus, most of the bacteria-induced leukocyte activation was complement

### Table I. Percentage upregulation of inflammatory markers by S. aureus and LTA in human whole blood compared with PBS in human whole blood

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cowan %</th>
<th>Wood %</th>
<th>LTA %</th>
</tr>
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<tbody>
<tr>
<td>IL-1β</td>
<td>573 ± 119</td>
<td>420 ± 81</td>
<td>342 ± 47</td>
</tr>
<tr>
<td>IL-6</td>
<td>676 ± 96</td>
<td>705 ± 213</td>
<td>357 ± 56</td>
</tr>
<tr>
<td>IL-8</td>
<td>504 ± 42</td>
<td>505 ± 76</td>
<td>303 ± 48</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>229 ± 36</td>
<td>195 ± 43</td>
<td>187 ± 47</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>692 ± 61</td>
<td>701 ± 205</td>
<td>502 ± 67</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>398 ± 55</td>
<td>342 ± 57</td>
<td>303 ± 50</td>
</tr>
<tr>
<td>TNF</td>
<td>700 ± 32</td>
<td>556 ± 163</td>
<td>308 ± 43</td>
</tr>
<tr>
<td>VEGF</td>
<td>198 ± 77</td>
<td>149 ± 38</td>
<td>198 ± 84</td>
</tr>
<tr>
<td>CD11b mono.</td>
<td>286 ± 45</td>
<td>277 ± 36</td>
<td>291 ± 44</td>
</tr>
<tr>
<td>CD11b PMNs</td>
<td>424 ± 56</td>
<td>395 ± 47</td>
<td>412 ± 54</td>
</tr>
<tr>
<td>Burst mono.</td>
<td>363 ± 122</td>
<td>227 ± 67</td>
<td>159 ± 45</td>
</tr>
<tr>
<td>Burst PMNs</td>
<td>364 ± 110</td>
<td>246 ± 83</td>
<td>143 ± 46</td>
</tr>
<tr>
<td>TCC</td>
<td>587 ± 78</td>
<td>967 ± 273</td>
<td>1198 ± 169</td>
</tr>
</tbody>
</table>

Data are from six different donors examined in consecutive experiments (\(n = 6\)) and presented as mean and SEM.

### Table II. Downregulation (%) of inflammatory markers by compstatin, anti-CD14, or the combination thereof

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Compstatin (% Decrease)</th>
<th>Anti-CD14 (% Decrease)</th>
<th>Compstatin + anti-CD14 (% Decrease)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>39 ± 21</td>
<td>-4 ± 10</td>
<td>26 ± 8</td>
</tr>
<tr>
<td>IL-6</td>
<td>5 ± 8</td>
<td>47 ± 20</td>
<td>61 ± 17</td>
</tr>
<tr>
<td>IL-8</td>
<td>44 ± 8</td>
<td>31 ± 12</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>29 ± 9</td>
<td>12 ± 13</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>41 ± 9</td>
<td>13 ± 26</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>23 ± 12</td>
<td>1 ± 21</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>TNF</td>
<td>41 ± 13</td>
<td>-3 ± 24</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>VEGF</td>
<td>16 ± 8</td>
<td>-12 ± 27</td>
<td>7 ± 6</td>
</tr>
<tr>
<td>CD11b</td>
<td>17 ± 7</td>
<td>31 ± 7</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>CD11b PMNs</td>
<td>65 ± 10</td>
<td>5 ± 10</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Burst mono.</td>
<td>57 ± 20</td>
<td>122 ± 48</td>
<td>19 ± 10</td>
</tr>
<tr>
<td>Burst PMNs</td>
<td>71 ± 19</td>
<td>31 ± 10</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>TCC</td>
<td>98 ± 11</td>
<td>99 ± 25</td>
<td>99 ± 13</td>
</tr>
</tbody>
</table>

Data are from six different donors examined in consecutive experiments (\(n = 6\)) and presented as mean and SEM.

\(p < 0.05\), repeated-measures one-way ANOVA.

<table>
<thead>
<tr>
<th></th>
<th>mon., monocytes; PMNs, polymorphonuclear leukocytes.</th>
<th>mon., monocytes; PMNs, polymorphonuclear leukocytes.</th>
<th>mon., monocytes; PMNs, polymorphonuclear leukocytes.</th>
</tr>
</thead>
</table>
dependent, and the effect was again attributed to the C5a–C5aR interaction.

Comparison of anti-CD14 and anti-TLR2

Because TLR2 is one of the main pattern recognition molecules of Gram-positive bacteria, the effects of compstatin and anti-CD14 in combination led us to examine the relative contribution of anti-CD14 versus a TLR2-neutralizing mAb, either alone or in combination with an anti-CD14 Ab (aCD14). The IL-6 release was significantly attenuated by anti-CD14 and compstatin alone, and with anti-CD14 in combination with either of the complement inhibitors. The TNF release was significantly attenuated by compstatin and eculizumab alone, and by all three complement inhibitors when combined with anti-CD14. The tenth column (control) represents stimulated whole blood preincubated with PBS containing control peptide and control Ab. n = 6 (data are obtained from six consecutive and independent experiments using six different donors, mean ± SEM). *p < 0.05, Student t test for PBS versus PBS + S. aureus. #p < 0.05, repeated measures, one-way ANOVA, where all columns are compared with the second column (S. aureus + PBS).

The two S. aureus strains induced a similar response and increase in the same key markers of inflammation. These were, in turn, reduced substantially by the combined inhibition of CD14 and C3. All cytokines except IL-6 seemed to be relatively more dependent on complement than on CD14. Furthermore, activation of leukocytes, as measured by CD11b expression, phagocytosis, and oxidative burst were largely complement dependent. Combination of a complement inhibitor with anti-CD14 tended to reduce nearly all markers more efficiently, although the reductions of the most complement-dependent markers were modest.

However, not all of the measured cytokines did increase, indicating that they are not involved in the pathogenesis of Gram-positive sepsis or rather, that they did not have time to change. Whereas Gram-negative sepsis is documented to induce cytokine changes within 5 h, Gram-positive sepsis is suggested to peak later (50–75 h) after the initial insult (33). Thus, our data and conclusions are limited to the specific markers that did increase significantly as a response to S. aureus within the 2-h time frame.

No other study has examined the number of cytokines in response to S. aureus as in this study, but some report an increase of IL-6, IL-8, and TNF. However, one study did not detect any IL-1β (34), whereas another claims that S. aureus does not induce TNF (35). Most studies on S. aureus have been done using the isolated cell-wall components peptidoglycan and LTA, rather than whole bacteria. These studies indicate a synergism between the two components triggering the release of IFN-γ and TNF and TNF, IL-6, and IL-10, respectively (36, 37). We have used human whole blood...
and whole bacteria ex vivo, and argue that the model is relevant for a clinical situation of S. aureus infection. The discrepancies with this study’s findings can otherwise be due to the use of different bacterial strains, their concentration, or the type of experimental model. For instance, de Kimpe et al. (36) studied mice and cultured macrophages, in contrast with whole human blood used in this study, whereas Wang et al. (37) used fewer bacteria than we did. An advantage of the whole-blood model we used, compared with work done on isolated cells, is that all cells and plasma components are left able to cross talk. In return, this limits our ability to identify which cell subpopulations produce which cytokines.

The amount of bacteria used in this study appears rather high, but we argue that it is still within a range that is clinically relevant in severe sepsis. Previous studies have used CFUs per milliliter to indicate the number of bacteria. This method usually underestimates the number substantially. First, only live bacteria are detected, whereas the whole bacterial load contributes to the inflammatory reaction. Second, the local concentration of bacteria at an infectious site is much higher than in the blood. Recent patient studies using PCR have detected bacteria in amounts comparable with what we have used (38).

Several studies report that S. aureus components activate complement, and recently elevated TCC values were reported in case reports of S. aureus bacteremia (39). In line with our findings, it has also been reported that S. aureus triggers CD11b upregulation and oxidative burst (40). Furthermore, phagocytosis was recently implicated as a crucial event of the innate immune response to S. aureus (22, 41). Our findings indicate that complement is crucial to phagocytosis, and that inhibition of complement nearly abolishes the process of phagocytosis altogether.

It has been hypothesized that LTA is a Gram-positive LPS analog suggested to bind TLR2 as LPS binds TLR4 (42, 43). In this study, we demonstrate a clear proinflammatory effect of LTA in whole blood. The 10 mg/ml we used is several logs higher than the LPS dose required for similar effects (44), but it is roughly comparable with the concentration of whole bacteria used (43, 45). Accordingly, LTA induced a significant increase of the same mediators as the whole bacteria, except for VEGF. However, the effect of LTA was substantially less pronounced than for the whole bacteria, indicating that LTA is not essential for Gram-positive–induced inflammation. As for the whole bacteria, LTA generally seemed more dependent on complement than on CD14. We therefore argue that the use of whole bacteria is essential when studying the inflammatory response in whole blood. Notably, the binding of LTA to TLR2 has recently been questioned (46). In the present context, the combined inhibition of complement and anti-CD14 efficiently attenuated the increase of all mediators studied, both when induced by LTA or by whole bacteria, highlighting a role of CD14 in the TLR responses to S. aureus.

Thus, we specifically compared the effect of TLR2 inhibition with CD14 inhibition. We found that anti-CD14 was at least as

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Complement-dependent CD11b expression and phagocytosis of S. aureus. Heat-inactivated S. aureus Cowan led to a significant level of both CD11b expression (top) and phagocytosis (bottom) after 15 min in both granulocytes and monocytes when added to human whole blood at a final concentration of 10^8 bacteria/ml. The CD11b expression and phagocytosis were both significantly inhibited toward baseline values by preincubating the whole blood with the C3-inhibitory peptide compstatin (Comp.), the C5-inhibitory Ab eculizumab (Eculiz.), or a C5a-receptor antagonist (C5aRag), either alone or in combination with an anti-CD14 Ab (aCD14). The tenth column (Control) represents stimulated whole blood preincubated with PBS containing control peptide and control Ab. n = 6 (data are obtained from six consecutive and independent experiments using six different donors, mean ± SEM). *
p, 0.05, Student t test for PBS versus PBS + S. aureus. **p < 0.05, repeated-measures, one-way ANOVA, where all columns are compared with the second column (S. aureus + PBS).
efficient as anti-TLR2 when combined with a complement inhibitor, although the differences were modest. The anti-TLR2 Ab used has a documented effect of attenuating innate immune responses of TLR2 agonists of both Gram-positive and Gram-negative bacteria (47, 48). We therefore speculate whether the effect of anti-CD14 and complement inhibition is caused by reducing the TLR2 responses with anti-CD14 or by inhibition of additional TLRs that recognize \textit{S. aureus}. Notably, TLR9 was recently implicated in the activation of host immune responses by \textit{S. aureus} (49), and CD14 is associated with TLR9 (50). Although Kusunoki et al. (44) indicated CD14 as a key recognition molecule of an unidentified \textit{S. aureus} surface component, it was not suggested to be a key target of innate immune regulation. Indeed, several different molecules of the innate immune system recognize \textit{S. aureus} surface component, it was not suggested to be a key target of innate immune regulation. Indeed, several different molecules of the innate immune system recognize \textit{S. aureus} (51), and CD14 is a coreceptor for a majority of these molecules (21). Yet, the use of anti-CD14 exerted very little effect on its own. The effect of combined CD14 and complement inhibition is in line with our previous studies with Gram-negative bacteria (14, 20), although the contribution of complement when compared with CD14 seems to be substantially greater in the response to Gram-positive bacteria than in response to Gram-negative. Thus, identifying complement and CD14 as main triggers of inflammation in response to both Gram-negative and Gram-positive bacteria also make them eligible for a double blockade. This treatment could attenuate the detrimental innate immune response in both monomicrobial and polymicrobial sepsis.

Importantly, this fits our hypothesis of an upstream inhibition of complement and TLR to attenuate the inflammatory response (23). The complement-mediated effects seem to be largely mediated by C5a–C5aR interaction. However, the applicability of the whole-blood model is limited by the neutralized thrombin. Indeed, it has been postulated that thrombin may activate complement directly at the level of C5 (52). Thus, our findings have to be tested in animal models by using species-reactive complement inhibitors and CD14 blockers to better address the complexity of a biological system. Until then, the present model offers important insight into the inflammatory response of \textit{S. aureus} in whole blood. The results are strengthened by use of standardized analyses, validated in our laboratory through many years of similar experiments with Gram-negative bacteria.

In conclusion, complement and CD14 inhibition disrupt upstream recognition of \textit{S. aureus} and efficiently attenuate the inflammatory response. Thus, the double blockade might be a promising treatment in both Gram-negative and Gram-positive sepsis.

**Acknowledgments**

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