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

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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. Skjøveldo,†* Dorte Christiansen,* Terje Espevik, ‡ Erik W. Nielsen, ‡,† and Tom E. Mølnes,*,†,‡,*

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 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. Combined inhibition 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: 000–000.
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 Nalgene (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 (Refludan) was obtained from Hoechst (Frankfurt am Main, Germany) or Schering (Cambridge, U.K.). Flow cytometry was performed using an LSR II flow cytometer (Becton Dickinson, San Jose, CA). Syto BC, LDS-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 Ca++ and Mg++ (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 counted on the flow cytometer using Trucount tubes 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).

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 Diatec AS (Oslo, Norway). Comaptin (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]-mlle-NH2 and a corre-
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 inhibition obtained by inhibiting complement activation could be attributed to the C5a effects 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

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\text{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>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>p</td>
<td>%</td>
</tr>
<tr>
<td>IL-1β</td>
<td>573 ± 119</td>
<td>0.0049</td>
<td>420 ± 81</td>
</tr>
<tr>
<td>IL-6</td>
<td>676 ± 96</td>
<td>0.0009</td>
<td>705 ± 213</td>
</tr>
<tr>
<td>IL-8</td>
<td>504 ± 42</td>
<td>&lt;0.0001</td>
<td>505 ± 76</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>229 ± 36</td>
<td>0.0015</td>
<td>195 ± 43</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>692 ± 61</td>
<td>&lt;0.0001</td>
<td>701 ± 205</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>398 ± 55</td>
<td>0.0008</td>
<td>342 ± 57</td>
</tr>
<tr>
<td>VEGF</td>
<td>198 ± 77</td>
<td>0.0493</td>
<td>149 ± 38</td>
</tr>
<tr>
<td>CD11b mono.</td>
<td>286 ± 45</td>
<td>0.0011</td>
<td>277 ± 36</td>
</tr>
<tr>
<td>CD11b PMNs</td>
<td>424 ± 56</td>
<td>0.0009</td>
<td>395 ± 47</td>
</tr>
<tr>
<td>Burst mono.</td>
<td>363 ± 122</td>
<td>0.0315</td>
<td>227 ± 67</td>
</tr>
<tr>
<td>Burst PMNs</td>
<td>364 ± 110</td>
<td>0.0214</td>
<td>246 ± 83</td>
</tr>
<tr>
<td>TCC</td>
<td>587 ± 78</td>
<td>0.0007</td>
<td>967 ± 273</td>
</tr>
</tbody>
</table>

Data are from six different donors examined in consecutive experiments (n = 6) and presented as mean and SEM. mon., monocytes; PMNs, polymorphonuclear leukocytes.

\[
\text{Table II. Downregulation (%) of inflammatory markers by compstatin, anti-CD14, or the combination thereof}
\]

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cowan (% Decrease)</th>
<th>Wood (% Decrease)</th>
<th>LTA (% Decrease)</th>
<th>Cowan (% Decrease)</th>
<th>Wood (% Decrease)</th>
<th>LTA (% Decrease)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>39 ± 21</td>
<td>21 ± 12</td>
<td>43 ± 7</td>
<td>11 ± 22</td>
<td>47 ± 20</td>
<td>61 ± 17</td>
</tr>
<tr>
<td>IL-6</td>
<td>5 ± 8</td>
<td>20 ± 3</td>
<td>18 ± 11</td>
<td>34 ± 6</td>
<td>47 ± 20</td>
<td>61 ± 17</td>
</tr>
<tr>
<td>IL-8</td>
<td>44 ± 8</td>
<td>38 ± 10</td>
<td>50 ± 10</td>
<td>19 ± 13</td>
<td>31 ± 12</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>29 ± 9</td>
<td>6 ± 14</td>
<td>31 ± 6</td>
<td>17 ± 7</td>
<td>12 ± 13</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>41 ± 9</td>
<td>35 ± 29</td>
<td>63 ± 11</td>
<td>7 ± 26</td>
<td>13 ± 26</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>23 ± 12</td>
<td>1 ± 15</td>
<td>26 ± 3</td>
<td>2 ± 6</td>
<td>1 ± 21</td>
<td>30 ± 8</td>
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<tr>
<td>TNF</td>
<td>41 ± 13</td>
<td>15 ± 20</td>
<td>45 ± 11</td>
<td>3 ± 24</td>
<td>2 ± 24</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>VEGF</td>
<td>16 ± 8</td>
<td>5 ± 8</td>
<td>31 ± 7</td>
<td>17 ± 12</td>
<td>12 ± 27</td>
<td>7 ± 6</td>
</tr>
<tr>
<td>CD11b mono.</td>
<td>17 ± 7</td>
<td>8 ± 3</td>
<td>3 ± 9</td>
<td>28 ± 4</td>
<td>31 ± 7</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>CD11b PMNs</td>
<td>65 ± 10</td>
<td>63 ± 7</td>
<td>46 ± 8</td>
<td>7 ± 4</td>
<td>5 ± 1</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Burst mono.</td>
<td>57 ± 17</td>
<td>39 ± 13</td>
<td>22 ± 10</td>
<td>48 ± 30</td>
<td>12 ± 19</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>Burst PMNs</td>
<td>71 ± 19</td>
<td>61 ± 20</td>
<td>31 ± 10</td>
<td>45 ± 29</td>
<td>30 ± 15</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>TCC</td>
<td>98 ± 11</td>
<td>99 ± 25</td>
<td>99 ± 13</td>
<td>5 ± 18</td>
<td>6 ± 6</td>
<td>16 ± 10</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.

mon., monocytes; PMNs, polymorphonuclear leukocytes.
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). In this series of experiments, the cytokines IL-1β, TNF, IL-6, and IL-8 were examined, and the Cowan strain used.

When used alone, both anti-CD14 and anti-TLR2 were inefficient in inhibiting the cytokines (Fig. 3). In combination, compstatin and anti-CD14 tended to be slightly more efficient in reducing the inflammatory response as compared with compstatin combined with anti-TLR2, but the differences were not significant.

Discussion

Because of the uniqueness of the whole-blood model and the multitude of markers for inflammation measured, this study is a novel contribution to the knowledge of the S. aureus–induced acute inflammatory response. We demonstrate that simultaneous upstream inhibition of complement and CD14 efficiently attenuated a Gram-positive, bacteria-induced inflammation, and that the complement-mediated effects of this inflammation were mainly due to C5a–C5aR interaction.

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
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 *S. aureus*. Notably, TLR9 was recently implicated in the activation of host immune responses by *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 *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 *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 *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 *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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**FIGURE 3.** Effect of anti-TLR2 and anti-CD14 on the IL-1β, IL-6, IL-8, and TNF response to *S. aureus* in human whole blood. Heat-inactivated *S. aureus* Cowan induced a significant release of IL-1β, IL-6, IL-8, and TNF when added to human whole blood to a final concentration of 10^9/ml and incubated for 2 h compared with the same regimen with PBS alone. The IL-1β and TNF releases were significantly attenuated by preincubating the whole blood with the C3-blocking peptide compstatin (Comp.), either alone or in combination with anti-CD14 (aCD14) or anti-TLR2 (aTLR2). The IL-6 release was significantly attenuated by anti-CD14, compstatin, or anti-TLR2 alone, or compstatin combined with either anti-CD14 or anti-TLR2. The IL-8 release was significantly attenuated by anti-TLR2 alone, or compstatin combined with either anti-CD14 or anti-TLR2. The tenth column (Control) represents stimulated whole blood preincubated with PBS containing control peptide and control Ab. n = 6–13 (data are obtained from 13 consecutive and independent experiments where all inhibitors were included in at least 6 of them, 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).
Disclosures
The authors have no financial conflicts of interest.

References


