



Swansea University  
Prifysgol Abertawe



## Cronfa - Swansea University Open Access Repository

---

This is an author produced version of a paper published in :  
*International Journal of Medical Microbiology*

Cronfa URL for this paper:

<http://cronfa.swan.ac.uk/Record/cronfa23338>

---

### Paper:

AL-Ishaq, R., Armstrong, J., Gregory, M., O'Hara, M., Phiri, K., Harris, L., Rohde, H., Siemssen, N., Frommelt, L., Mack, D. & Wilkinson, T. (2015). Effects of polysaccharide intercellular adhesin (PIA) in an ex vivo model of whole blood killing and in prosthetic joint infection (PJI): A role for C5a. *International Journal of Medical Microbiology*

<http://dx.doi.org/10.1016/j.ijmm.2015.08.005>

---

This article is brought to you by Swansea University. Any person downloading material is agreeing to abide by the terms of the repository licence. Authors are personally responsible for adhering to publisher restrictions or conditions. When uploading content they are required to comply with their publisher agreement and the SHERPA RoMEO database to judge whether or not it is copyright safe to add this version of the paper to this repository.

<http://www.swansea.ac.uk/iss/researchsupport/cronfa-support/>

## Accepted Manuscript

Title: Effects of Polysaccharide Intercellular Adhesin (PIA) in an *ex vivo* model of whole blood killing and in prosthetic joint infection (PJI): A role for C5a

Author: Rand AL-Ishaq Jayne Armstrong Martin Gregory  
Miriam O'Hara Kudzai Phiri Llinos G. Harris Holger Rohde  
Nicolaus Siemssen Lars Frommelt Dietrich Mack Thomas S.  
Wilkinson



PII: S1438-4221(15)30004-7  
DOI: <http://dx.doi.org/doi:10.1016/j.ijmm.2015.08.005>  
Reference: IJMM 50967

To appear in:

Received date: 25-7-2014  
Revised date: 5-8-2015  
Accepted date: 16-8-2015

Please cite this article as: AL-Ishaq, R., Armstrong, J., Gregory, M., O'Hara, M., Phiri, K., Harris, L.G., Rohde, H., Siemssen, N., Frommelt, L., Mack, D., Wilkinson, T.S., Effects of Polysaccharide Intercellular Adhesin (PIA) in an *ex vivo* model of whole blood killing and in prosthetic joint infection (PJI): A role for C5a, *International Journal of Medical Microbiology* (2015), <http://dx.doi.org/10.1016/j.ijmm.2015.08.005>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Effects of Polysaccharide Intercellular Adhesin (PIA) in an *ex vivo* model of whole blood**  
2 **killing and in prosthetic joint infection (PJI): a role for C5a**

3  
4 <sup>1</sup>Rand AL-Ishaq, <sup>1</sup>Jayne Armstrong, <sup>1</sup>Martin Gregory, <sup>1</sup>Miriam O'Hara, <sup>1</sup>Kudzai Phiri, <sup>1</sup>Llinos G.  
5 Harris, Holger Rohde, Nicolaus Siemssen, Lars Frommelt <sup>1,2</sup>Dietrich Mack, <sup>1</sup>Thomas S.  
6 Wilkinson

7  
8 <sup>1</sup>Institute of Life Science, First Floor, Room 137, Microbiology and Infectious Disease,  
9 Swansea University, Singleton Park, SA2 8PP

10 <sup>2</sup>Bioscientia Labor Ingelheim, Institut für Medizinische Diagnostik GmbH, Mikrobiologie  
11 Konrad-Adenauer-Straße 17, 55218, Ingelheim

12  
13  
14 Running title : Role for C5a in prosthetic joint infection

15 Key Words : Polysaccharide Intercellular Adhesin, Prosthetic Joint Infection, Biofilm, Whole  
16 Blood, Complement C5a, *Staphylococcus epidermidis*

17 Word Count : Introduction to the end of Discussion is 2824

18

18

19 **Footnote page**

20 **Conflict of Interest**

21 To the best of our knowledge ALL the authors confirm that they do not have any commercial  
22 or other association that might pose a conflict of interest.  
23

24 **Financial Support**

25 This work was funded by a fellowship awarded to Dr Rand AL-Ishaq by the Iraqi  
26 Government.  
27

28 **Previous meetings**

29 Part of the work was presented at the Staph GBI 2011 Conference in Edinburgh in an oral  
30 presentation by RAI ([www.roslin.ed.ac.uk/staph-gbi2011/programme/](http://www.roslin.ed.ac.uk/staph-gbi2011/programme/))  
31

32 **Corresponding author details**

33 Thomas S Wilkinson  
34 Institute of Life Science  
35 Fifth Floor, Room 503  
36 Microbiology and Infectious Disease  
37 Swansea University  
38 Singleton Park  
39 SA2 8PP  
40  
41 E-mail: [t.s.wilkinson@swansea.ac.uk](mailto:t.s.wilkinson@swansea.ac.uk)  
42 Phone: 0044-(0)1792-295018

43

44 **Abstract**

45 Background: A major complication of using medical devices is the development of biofilm-  
46 associated infection caused by *Staphylococcus epidermidis* where Polysaccharide  
47 Intercellular Adhesin (PIA) is a major mechanism of biofilm accumulation. PIA affects innate  
48 and humoral immunity in isolated cells and animal models. Few studies have examined  
49 these effects in prosthetic joint infection (PJI).

50 Methods: This study used *ex vivo* whole blood modelling in controls together with matched-  
51 serum and staphylococcal isolates from patients with PJI.

52 Results: Whole blood killing of PIA positive *S. epidermidis* and its isogenic negative mutant  
53 was identical. Differences were unmasked in immunosuppressed whole blood pre-treated  
54 with dexamethasone where PIA positive bacteria showed a more resistant phenotype. PIA  
55 expression was identified in three unique patterns associated with bacteria and leukocytes,  
56 implicating a soluble form of PIA. Purified PIA reduced whole blood killing while increasing  
57 C5a levels. In clinically relevant staphylococcal isolates and serum samples from PJI  
58 patients; firstly complement C5a was increased 3-fold compared to controls; secondly, the  
59 C5a levels were significantly higher in serum from PJI patients whose isolates preferentially  
60 formed PIA-associated biofilms.

61 Conclusions: These data demonstrate for the first time that the biological effects of PIA are  
62 mediated through C5a in patients with PJI.

63

64 **Introduction**

65

66 Current estimates suggest that the number of implanted joint prostheses will continue to  
67 increase significantly over the next 20 years (Kurtz et al., 2007). Joint prostheses reduce  
68 pain, replace lost function and improve quality of life. In striking contrast prosthetic joint  
69 infection (PJI) is a serious complication which occurs at a frequency of 1-2% after joint  
70 replacement, with a mortality rate of 1-3% resulting in an increased financial burden to the  
71 healthcare system. The major causative organisms in PJI are *Staphylococcus epidermidis* and  
72 *S. aureus*, accounting for 30-43% and 12-23% respectively (Trampuz and Zimmerli, 2005).  
73 The ability of staphylococci to adhere and grow on biomaterial surfaces to form a biofilm is  
74 of mechanistic importance for the development of a PJI.

75

76 Polysaccharide intercellular adhesin (PIA) is important for biofilm accumulation in *S.*  
77 *epidermidis* biomaterial associated infection (Rohde et al., 2010). PIA is a linear  
78 polysaccharide of  $\beta$ -1,6-N-acetylglucosamine containing positive charges due to  
79 deacetylated amino groups and negative charges due to O-succinoyl ester residues (Mack et  
80 al., 1996). PIA is produced by the *icaABDC* locus which is composed of the operon encoding  
81 a membrane bound enzyme complex (Gerke et al., 1998; Heilmann et al., 1996; Ziebuhr et  
82 al., 1997). PIA has been shown to have effects on innate immunity. In isolated cellular  
83 models PIA inhibits phagocyte killing (Barrio et al., 2000; Vuong et al., 2004), by mechanisms  
84 thought to involve the combined inhibition of C3b and IgG deposition on the bacterial  
85 surface (Kristian et al., 2008) while also decreasing antimicrobial peptide action (Vuong et  
86 al., 2004). In addition, PIA modulates cytokine production (Schommer et al., 2011; Stevens

87 et al., 2009) through mechanisms that may partly involve TLR-2. Consistent with this, *in vivo*  
88 models, suggest that PIA biofilm formation was related to persistent bacteremia in neonates  
89 in intensive-care units (Dimitriou et al., 2011) and appeared as a major virulence factor in  
90 biomaterial-associated infection model in rats and mice (Rupp et al., 2001; Rupp et al.,  
91 1999a; Rupp et al., 1999b) and in *Caenorhabditis elegans* (Begun et al., 2007).

92

93 Recently, the importance of complement activation in staphylococcal infections has become  
94 apparent in experimental infection (von Kockritz-Blickwede et al., 2010) and in human  
95 whole blood (Skjeflo et al., 2014). More specifically, staphylococcal biofilm matrices have  
96 been implicated in complement activation. Here PIA from *S. epidermidis* has been shown to  
97 modulate complement binding and activation in opsonised human neutrophils (Kristian et  
98 al., 2008), and induction of complement C5a has been demonstrated in whole blood  
99 (Fredheim et al., 2011), human serum (Satorius et al., 2013) and neonatal whole blood  
100 (Granslo et al., 2013).

101

102 To date however, it is unclear i) what the necessary infecting doses are for whole blood  
103 production of complement C5a; ii) whether similar responses exist in patients with PJI; iii)  
104 how PIA interacts with host cells; iv) whether such responses may be useful for early  
105 diagnosis. Here we confirm the importance of immunosuppression for PIA induced  
106 complement fragment C5a production. We identify novel PIA structures that contribute to  
107 interactions with leukocytes. Furthermore we identify high levels of C5a in sera from PJI  
108 patients exposed to isolates of *S. epidermidis* producing PIA dependent biofilms.

109

110 **Methods**

111

112 *Bacterial strains and culture conditions*

113 Archived and sequenced *S. epidermidis* strains were isolated as described in previous studies  
114 (Rohde et al., 2007) and form part of our online staphylococcal database (Sheppard, 2012).  
115 Specifically, *S. epidermidis* 1457 was isolated from a central venous catheter infection (Mack  
116 et al., 1992). The isogenic mutant *S. epidermidis* 1457-M10 (M10) was produced by  
117 transposon mutagenesis of the *icaADBC* locus as described previously (Mack et al., 1999).

118

119 *Bacterial culture*

120 One colony of *S. epidermidis* was inoculated into tryptic soy broth (TSB) and incubated  
121 overnight at 37°C. Then 1 ml of overnight culture was centrifuged at 9447g and the  
122 supernatant removed. Pellets were resuspended by flicking and 1 ml of Iscove's Modified  
123 Dulbecco's Medium (IMDM) added prior to one further wash. Optical density was measured  
124 at 600nm and adjusted to OD<sub>600</sub> = 0.1 giving an *S. epidermidis* stock concentration of ~1 x  
125 10<sup>7</sup> cfu/ml.

126

127 *Serum samples*

128 Serum from healthy volunteers was isolated using the vacuette blood collection system  
129 (5ml-9ml) on the day of the experiment. Volunteers gave their consent and the work is one  
130 of the projects (13/WA/0190) assessed by the local ethics committee (Wales Rec 6) at  
131 College of Medicine, Swansea University. Samples from PJI patients included sixty five  
132 matched serum and staphylococcal isolate pairs together with 4 study matched controls



133 that received a prosthetic joint but did not contract a PJI. Patients gave written informed  
134 consent to participate in the study in accordance with the requirements at ENDO clinic,  
135 Hamburg. (Rohde et al., 2007; Rohde et al., 2008.; Rohde et al., 2005).

136

#### 137 *Antibodies*

138 Anti-human CD11b (5µg/ml), anti-human CD18 (20µg/ml), anti-human CD16 (20µg/ml) and  
139 isotype control (20µg/ml) were all purchased from Biolegend.

140

#### 141 *Whole blood killing*

142 Whole blood was collected from healthy volunteers using the vacuette blood collection  
143 system containing sodium heparin (5ml-9ml) on the day of the experiment. Volunteers gave  
144 their consent and the work is one of the projects (13/WA/0190) assessed by the local ethics  
145 committee (Wales Rec 6) at College of Medicine, Swansea University. One milliliter of blood  
146 was added to 1.5ml microcentrifuge tubes before 10µl of *S.epidermidis* 1457 or 1457-M10  
147 stock was added (to give  $\sim 2 \times 10^5$  cfu/ml final). Infected blood was incubated with rotation at  
148 10rpm at 37°C for different time periods according to experiment (0-24 hours). Viable  
149 counts were assessed by gentle lysis of leukocytes in 0.1% Triton X100 for 1 min to release  
150 intracellular bacteria. Then suspensions were diluted and plated on TSB agar. Plates were  
151 incubated at 37°C overnight. The next day colonies were counted and viable counts  
152 estimated. Whole blood remaining at each time point was centrifuged at 9447g for 5 min  
153 and the platelet poor plasma / serum removed and stored at -20°C prior to ELISA analysis.

154

155

155

156 *ELISA*

157 Duoset ELISAs (R and D systems, Abingdon) for human C5a, IL-8, TNF $\alpha$  and IL-1 $\beta$ , were  
158 carried out according to the manufacturers' instructions. Healthy volunteers' sera were  
159 diluted 1/10 and patient sera 1/20.

160

161 *Immunocytochemistry*

162 One colony of *S. epidermidis* 1457 was used to establish an overnight pre-culture in TSB  
163 (without glucose). The following day a 1:100 dilution was made into either; i) untreated  
164 whole blood iii) whole blood pre-treated with dexamethasone (0.1-1 $\mu$ M) for 18 hours; iii)  
165 whole blood pre-treated with cytochalasin D (5 $\mu$ g/ml) for 30 minutes. Cultures were  
166 incubated for 0-24 hours. Then, 200 $\mu$ l of whole blood was added to 3ml of red blood cell  
167 (RBC) lysis buffer (15mM ammonium chloride in 0.1M TrisHCl, pH, 7.5) for up to 10min.  
168 Cytospin preparations were prepared and blocked with 200 $\mu$ l 1% BSA for 1 hour, then  
169 washed X3 with PBS. Then 200 $\mu$ l of rabbit anti-PIA antiserum (diluted 1:50) was applied, the  
170 slides covered and after 30 minutes the slides washed X3 with PBS. Then slides were stained  
171 with fluorescein-conjugated anti-rabbit IgG (Alexa flour 488, diluted 1:100) and propidium  
172 iodide (1 $\mu$ g/ml), covered and incubated for 30 minutes. Slides were washed X3 with PBS  
173 then 25 $\mu$ l of vectashield<sup>®</sup> hardset<sup>™</sup> (Vector Laboratories, Peterborough) was added, a  
174 coverslip added gently and the preparation left in the fridge to harden. Slides were  
175 examined using an Axiovert epifluorescent microscope.

176

177 Structural analysis of WB leukocytes, PIA and *S. epidermidis* were analysed using confocal  
178 microscopy. Here slight modifications to the whole blood killing assays and

179 immunocytochemistry were needed to produce labelled cells. Firstly, *S. epidermidis* 1457  
180 pre-cultures were prepared as above and were stained with syto-9 (20 $\mu$ M) for 1 hour.  
181 Excess syto-9 was removed by centrifugation at 9447g for 5 minutes then resuspended in  
182 PBS. The bacterial suspension was washed a further 5 times. Then 10 $\mu$ l of stained *S.*  
183 *epidermidis* 1457 was used to infect whole blood as previously described. Following whole  
184 blood killing and RBC lysis leukocyte membranes were stained with Cell Mask™ deep red  
185 (Life Technologies, diluted 1:100) for 10 minutes. Then cytopins were prepared as  
186 previously described above. Slides were stained as above with two modifications; i)  
187 propidium iodide was removed and ii) anti-rabbit IgG (alexa 594) was used to detect the PIA  
188 antibodies. Once slides were complete confocal laser scanning microscopy (Zeiss) analysed  
189 3-5 fields per slide. Each field imaged 8-20 sections of 1-1.5 $\mu$ m thickness. To maintain  
190 consistency with the previous immunocytochemistry colours, images were pseudo-coloured  
191 purple for cell mask (cell membranes), red for syto-9 (Bacteria) and green for alexa 594  
192 (PIA).

193

#### 194 *Biofilm assay*

195 A single bacterial colony was picked from a blood plate and suspended in 5 ml of TSB broth  
196 and then incubated at 37°C for 18 hours with shaking at 200rpm. The next day this pre-  
197 culture was diluted 1:100 with fresh TSB, then 200 $\mu$ l aliquoted into each well of a NUNC 96-  
198 well plate. The plate was incubated for 18 hours at 37°C without shaking. On the next day  
199 the media was carefully removed and the wells washed 3X with 200 $\mu$ l PBS and 150 $\mu$ l of  
200 Bouin's fixative was added to each well prior to incubation for 15 minutes. Then Bouin's  
201 fixative was removed and the wells washed once with PBS and left to air dry. Adherent  
202 biofilms were then stained with 150 $\mu$ l of crystal violet for 5 minutes, and then washed X5

203 under running tap water and left it to air dry. The optical density of biofilms was measured  
204 at 570 nm using an Omega Fluo Star plate reader. Biofilm mechanism was investigated by  
205 treating mature biofilms with proteinase K (1mg/ml) or sodium periodate (40mM), to digest  
206 protein dependent and PIA dependent biofilms respectively, for 24 hours prior to washing.

207

#### 208 *Statistical Analysis*

209 Pairwise comparisons were calculated using the unpaired Student's t-test. Multiple dataset  
210 comparisons were subjected to a non-parametric Kruskal-Wallis test and included a Dunn's  
211 post-hoc test. Results were considered significant if  $P < 0.05$ .

212

212

213 **Results**

214 To investigate the effects of PIA on immune cells we developed an *ex vivo* model of whole  
215 blood (WB) infection. Here stationary-phase *S.epidermidis* 1457 which produce a PIA  
216 dependent biofilm, or an isogenic mutant 1457-M10 which does not form biofilm were  
217 added to freshly drawn WB. Dose and time course analysis over the first 3 hours of infection  
218 suggested to us that there was very little difference in the ability of WB to kill *S. epidermidis*  
219 1457 compared to 1457-M10 (Data not shown). Similarly at 6 hours post infection there was  
220 no significant difference in killing response between *S. epidermidis* 1457 and 1457-M10  
221 (Figure 1). Antibody blocking studies (Figure 1) demonstrated the killing was CR-3  
222 dependent as antibodies against CD11b and CD18 could completely inhibit killing, unlike an  
223 antibody against CD16.

224

225 In contrast under immunosuppressive conditions, pre-incubation of whole blood with  
226 dexamethasone resulted in a dose dependent decrease in the killing of *S. epidermidis* 1457  
227 compared to its isogenic mutant which reached significance at 1nM dexamethasone (Figure  
228 2A). Thus immunosuppression unmasks resistance to killing in PIA positive *S. epidermidis*.  
229 Killing of *S. epidermidis* was dependent on actin assembly during phagocytosis (Goddette  
230 and Frieden, 1986; Shoji et al., 2012) as treatment with cytochalasin D demonstrated could  
231 block killing of both strains (Figure 2A). To confirm the presence of PIA under  
232 immunosuppressive conditions we used immunocytochemistry. PIA expression in *S.*  
233 *epidermidis* 1457 over the first 6 hours of growth demonstrated no growth or PIA in WB  
234 alone. In contrast, WB pre-incubated with dexamethasone showed bacterial growth and PIA  
235 production. Complete inhibition of leukocyte phagocytosis with cytochalasin D resulted in

236 dramatic growth and PIA expression. These experiments clearly demonstrate that PIA  
237 expression could be detected in immunosuppressed WB but not in healthy untreated WB.  
238 However bacterial growth and PIA expression in immunosuppressed WB is markedly  
239 reduced compared to PIA positive cultures of *S. epidermidis* 1457 incubated in TSB at similar  
240 times (Supplementary Figure 1A-D).

241

242 We further investigated the localisation of PIA expression in immunosuppressed WB using  
243 immunocytochemistry and confocal microscopy (Figure 3). This work revealed three  
244 patterns of PIA expression and localisation (Figure 3B-G) including; i) 'beads on a  
245 string/bridges' defined by single bacteria interspaced by PIA cable or string-like structures  
246 (Figure 3B and C); ii) 'clumps' defined by dense aggregates of PIA associated with bacteria  
247 (Figure 3D and E); and iii) 'caps' defined by small aggregates of PIA associated with smaller  
248 bacterial numbers expressed on one side of a leukocyte (Figure 3F and G).

249

250 Further investigation of temporal changes at later time-points proved difficult due to  
251 decreases in leukocyte viability. We were intrigued by the intricate patterning of PIA (Figure  
252 3) and we therefore modelled the effects of later time-points by using PIA purified from *S.*  
253 *epidermidis* 1457 in a low endotoxin environment (Supplementary methods). Then, WB was  
254 incubated with PIA prior to infection with PIA deficient *S. epidermidis* 1457-M10 and  
255 determination of killing and cytokine production (Figure 4A-D). At 6 hours post infection  
256 1457-M10 were cleared vigorously from untreated blood (Figure 4A). In striking contrast WB  
257 exposed to PIA could not kill to the same degree, unlike blood incubated with negative  
258 vehicle control (M10-see supplementary methods) (Figure 4A). Thus PIA inhibited killing of  
259 *S.epidermidis* 1457-M10 by WB. Humoral responses in these samples showed that

260 complement fragment C5a was significantly increased in PIA treated WB compared to  
261 control M10 and untreated control (Figure 4B). Cytokine analysis showed similar increases  
262 in IL-8 (Figure 4C) but not IL-1 $\beta$  (Figure 4D) or TNF $\alpha$  and IL-10 (data not shown). In  
263 uninfected controls, PIA induced C5a in the absence of *S. epidermidis* 1457 and was not  
264 significantly different from infected responses (Black bars, Figure 4B). PIA produced a small  
265 but not significant induction of IL-8 and IL-1 $\beta$  responses in the absence of *S. epidermidis*  
266 1457 compared to control M10 or blood alone. Thus at this time point C5a was dependent  
267 on PIA whereas the cytokines were dependent on PIA and infection. Consistent with this,  
268 serum isolated from healthy volunteers showed a similar spectrum of responses (Figure 5A)  
269 with PIA inducing C5a production unlike control M10 and untreated control. To investigate  
270 the specificity of the response we digested PIA (or M10) with dispersin B, a hexosaminidase  
271 shown to breakdown PIA (Figure 5B-D). Here dispersin B could completely inhibit PIA  
272 induced C5a to the levels of control M10 confirming the specificity of the effect (Figure 5B).

273  
274 Finally we investigated the relationship between C5a levels in serum and biofilm producing  
275 staphylococcal isolates from patients with prosthetic hip and knee joint infections archived  
276 from a previous study (Rohde et al., 2007). Firstly we confirmed that there was no  
277 difference in the C5a levels in healthy volunteers and the 4 non-PJI age matched patients.  
278 We therefore included all these in our control group when comparing the 65 PJI patients.  
279 Here C5a levels were higher (3-fold) in patient samples compared to controls (Figure 6A).  
280 This difference was independent of the causative organism as patients infected with *S.*  
281 *epidermidis*, *S. capitis*, *S. aureus* and *S. lugdunensis* all showed similar C5a serum levels  
282 compared to controls (Figure 6B). We performed further experiments on the larger group of  
283 *S. epidermidis* isolates to investigate the influence of biofilm (n=43, Figure 7A). We found

284 that 26 of the 43 *S. epidermidis* isolates formed biofilm (OD570 > 0.1) and their mechanism  
285 of biofilm formation was investigated (Figure 7A) by digesting mature biofilms with  
286 proteinase K or sodium periodate that digest protein and PIA dependent biofilms  
287 respectively. These digestions demonstrated that of 26 biofilm positive isolates, 6 were PIA  
288 dependent, 7 were protein dependent and 13 were dependent on PIA and protein (Figure  
289 7A). Then the C5a data (Figure 6B) was organised according to biofilm mechanism of the  
290 infecting isolate (Figure 7B). Strikingly, C5a levels in serum from patients exposed to biofilm  
291 forming isolates was higher compared to serum from patients exposed to biofilm negative  
292 isolates. Furthermore, C5a levels were significantly higher in serum samples from patients  
293 exposed to isolates producing PIA dependent biofilms compared to serum from patients  
294 exposed to biofilm negative isolates (Figure 7B).



295

296 **Discussion**

297

298 The current study extends our previous observations on the importance of C5a (Conway  
299 Morris et al., 2009; Morris et al., 2011) in medical device related infection, namely ventilator  
300 associated pneumonia, to PIA dependent biofilm formation in PJI. Here we demonstrate; i)  
301 the advantage of an 'immunosuppressed' host for PIA production in WB; ii) clear  
302 interactions between *S. epidermidis*-derived PIA and WB leukocytes, with three distinct  
303 morphological patterns; iii) PIA-induced C5a in human WB and serum; iv) increased C5a in  
304 serum from PJI compared to controls; and finally v) Increased C5a in serum from PIA  
305 dependent PJI compared to biofilm negative PJI. To our knowledge this is the first  
306 demonstration of a link between PIA dependent biofilms and C5a in clinical samples.

307

308 Comparison of whole blood killing of PIA positive and PIA negative *S. epidermidis* could not  
309 detect differences between strains. This was rather surprising considering similar  
310 experiments in isolated cell systems showing that PIA could protect against antimicrobial  
311 peptide killing and neutrophil killing (Vuong et al., 2004). Clearly killing in the whole blood  
312 environment is so rapid that PIA production is delayed. Only when phagocyte efficiency was  
313 reduced with dexamethasone was a more resistant phenotype unmasked in PIA positive *S.*  
314 *epidermidis*. In this model dexamethasone produced a global reduction in cytokines  
315 including IL-8 (data not shown). This has previously been shown to control neutrophil  
316 activation and is a strong candidate for the mechanism of suppression in this model (Hartl et  
317 al., 2007). Others have suggested that dexamethasone causes suppression of neutrophil  
318 phagocytosis (Bober et al., 1995) and free radical release (Liu et al., 2014). These results are

319 consistent with the increased risk of biofilm infections in immunocompromised patients  
320 (Weisser et al., 2010) and the immunosuppressive conditions produced during the foreign  
321 body response following the implantation of a medical device (Higgins et al., 2009; Wagner  
322 et al., 2004; Wagner et al., 2003).

323

324 Our current model of the temporal changes that occur during biofilm infection consists of  
325 two stages. In the first stage 'immunosuppression' leads to a survival advantage in *S.*  
326 *epidermidis* expressing PIA. Here we suggest that PIA is bound to both *S. epidermidis* and  
327 leukocytes which may further reduce phagocytic efficiency as demonstrated previously  
328 (Kristian et al., 2008; Schommer et al., 2011). The first stage is immunosuppression and PIA  
329 dependent but independent of C5a. Then, in the second stage, having established  
330 colonisation of the host, PIA expressing *S. epidermidis* further produce PIA resulting in  
331 increased PIA levels also capable of binding leukocytes and serum components at sufficient  
332 concentration to activate C5a. We have previously demonstrated the importance of C5a in  
333 promoting a state of leukocyte dysfunction defined by decreased neutrophil phagocytosis  
334 that is phosphoinositide-3-kinase and CD88 dependent (Conway Morris et al., 2009; Morris  
335 et al., 2011).

336

337 We identified three populations of PIA; i) 'beads on a string/bridges' defined by single  
338 bacteria interspaced by PIA cable or string-like structures ii) 'clumps' defined by dense  
339 aggregates of PIA associated with bacteria and iii) 'caps' defined by small aggregates of PIA  
340 associated with smaller bacterial numbers expressed on one side of a leukocyte. Such  
341 morphologies have not been identified in contact with leukocytes previously but are  
342 consistent with structures produced in PIA-dependent biofilm formation and remain distinct

343 from structures produced in biofilms produced by accumulation associated protein (Aap)  
344 and extracellular matrix binding protein (Ebmp) (Schommer et al., 2011). Support for these  
345 structures is strengthened by a recent publication confirming the ability of PIA to form self-  
346 associations and entanglements in addition to binding to other proteins (e.g albumin) in  
347 biological fluids (Ganesan et al., 2013).

348

349 Purified PIA had intriguing biological properties that could induce C5a and inhibit WB killing.  
350 Purified PIA from *S. epidermidis* has previously been identified as modulating complement  
351 binding and activation in opsonised human neutrophils (Kristian et al., 2008), whole blood  
352 (Fredheim et al., 2011), human serum (Satorius et al., 2013) and neonatal whole blood  
353 (Granslo et al., 2013). Our data is consistent and extends these findings demonstrating that  
354 PIA may disintegrate from or for a 'bridge' between the bacterial biofilm leukocytes which is  
355 consistent with inhibition of opsonisation shown previously (Kristian et al., 2008).

356

357 The constitutive levels of C5a in healthy volunteers are consistent with other studies (Kunkel  
358 et al., 1983; Stove et al., 1996; Tayman et al., 2011). However, addition of PIA to serum from  
359 healthy volunteers generated a threefold induction of C5a (from 20-60ng/ml) compared to  
360 control. The amount of C5a induced by PIA appears more variable and model dependent as  
361 Freidheim and co workers generated 8 fold increases (23-162ng/ml (2-16nM)) in C5a in  
362 response to 2µg/ml PIA for 30 minutes whereas Satorius et al generated 1 fmol/cm<sup>2</sup>/s in  
363 response to PIA biofilms (Fredheim et al., 2011; Satorius et al., 2013). This current study  
364 generated 60ng/ml (~6nM) in 90 minutes. Taken together these studies and our own  
365 suggest that 60-480ng/ml (6-48nM) of C5a may be produced in 90 minutes in a whole blood  
366 / serum environment confirming the potential for C5a generation in response to PIA. Our

367 serum data in patients with PJI confirmed this potential demonstrating similar 3-fold  
368 increases in C5a levels over healthy volunteer controls (20-55ng/ml). Indeed there was a  
369 striking consistency between C5a levels in the WB killing model (Figure 4B), healthy  
370 volunteer serum induced by PIA (Figure 5) and patient infected with *S. epidermidis*  
371 producing PIA dependent biofilms (Figure 6) alluding to the relevance of the effect.  
372 However, PIA levels are unlikely to rise to biologically active levels (10-30µg/ml) in healthy  
373 volunteers due to rapid clearance of *S. epidermidis* in whole blood.

374

375 Sub-group analysis of PJI serum organised by biofilm mechanism showed a significant  
376 difference between C5a levels from patients whose isolates were biofilm negative (42ng/ml)  
377 compared to PIA dependent (55ng/ml). To our knowledge this is the first study to  
378 demonstrate that C5a may differentiate between non-biofilm and different types of biofilm  
379 in PJI. However, pharmacokinetic simulations exploring the role of C5a in central venous  
380 catheter infection suggest that sufficient C5a could not be generated despite complete  
381 coverage of the catheter with biofilm (Satorius et al., 2013) or could be limited by diffusion  
382 distances (Conrad et al., 2013). Clearly the amount of C5a generated in the PJI patients in  
383 this study is more than sufficient to cause a biological response, such as the inhibition of  
384 killing demonstrated here (Figure 3A). It remains unclear in our study about the proportional  
385 contribution by PIA [to C5a production] although there at least a 13ng/ml increase in serum  
386 C5a between patients with PIA dependent biofilm isolates compared to biofilm negative  
387 isolates. This is consistent with results from Granslo and colleagues who showed that PIA  
388 biofilm produced stronger complement activation than non-PIA biofilm in neonatal late-  
389 onset sepsis (Granslo et al., 2013). More generally, C5a is elevated in Pneumococcal  
390 meningitis (Woehrl et al., 2011), Sepsis (Nakae et al., 1994; Yan and Gao, 2012), Dengue

391 hemorrhagic fever (Wang et al., 2006), pneumonia (Kiehl et al., 1997), and spontaneous  
392 bacterial peritonitis (Frances et al., 2007).

393

394 Finally we identify three limitations that guard against over-interpretation of our results.

395 Firstly we were unable to block PIA induced injury with a C5a blocking antibody (data not  
396 shown) suggesting that other mediators may be involved (e.g. C3a, C4a). Secondly, attempts

397 to measure PIA in the patient sera through an in house ELISA were unsuccessful however

398 our previous work on the same sera did confirm higher titers in patients infected with

399 *icaABDC* positive strains (1:20000-36000) compared to *icaABDC* negative and controls

400 (1:2000-6000)(Rohde et al., 2008.; Rohde et al., 2005). In addition the presence of PIA could

401 be masked by naturally occurring anti-PIA antibodies and the sensitivity of our ELISA. Finally

402 C5a is a particularly labile molecule and its degradation product C5a-DES-ARG might be a

403 more realistic biomarker as we have suggested in previous work (Conway Morris et al.,

404 2009). Our observation that levels of C5a in healthy volunteer controls and controls from

405 patients who had received a prosthetic joint suggests that stability is likely not a problem

406 here.

407

407

408 **Figure Legends**

409

410 **Figure 1: Whole blood killing of *S. epidermidis* 1457 and 1457-M10 in untreated whole**  
411 **blood**

412 One millilitre of whole blood was incubated with *S. epidermidis* (final concentration  $2 \times 10^5$ /ml) 1457 or 1457-M10 for 6 hours prior to release of intracellular bacteria by gentle  
413 lysis, serial dilution and plating. Colony counts were determined following incubation at  
414 37°C overnight. Hatched lines compare treatments in 1457-M10 and solid lines compare  
415 treatments in 1457. Results are expressed as mean  $\pm$  SEM (n=4) of *S. epidermidis* (cfu/ml). \*  
416 represents significant differences at  $P < 0.05$

418

419

420 **Figure 2: Whole blood killing and PIA expression from *S. epidermidis* incubated in**  
421 **dexamethasone treated whole blood**

422 (A) Whole blood was pre-incubated for 18 hours with dexamethasone (0-1nM), pre-  
423 incubated for 30 minutes with cytochalasin D (5 $\mu$ g/ml) or left untreated prior to adding *S.*  
424 *epidermidis* (final concentration  $2 \times 10^5$ /ml) 1457 or 1457-M10. Results are expressed as  
425 mean  $\pm$  SEM (n=4) of *S. epidermidis* (cfu/ml). \* represents significant differences at  $P < 0.05$ .

426 (B-D) *S. epidermidis* 1457 was incubated with; (B) untreated whole blood, (C) whole blood  
427 pre-treated with dexamethasone (1 $\mu$ M), or (D) whole blood pre-treated with cytochalasin D  
428 (5 $\mu$ g/ml) for 6 hours. Samples were cytospun onto microscope slides and stained with rabbit  
429 anti-PIA antibody. Preparations were washed before an Alexa fluor 488-conjugated anti-  
430 rabbit IgG was added with propidium iodide counterstain. Slides were visualised under an

431 Axiovert epifluorescent microscope. Results are representative images from at least 3  
432 experiments. Here PIA is stained green and DNA from bacteria or leukocyte nuclei stained  
433 red with original magnification (X1000).

434

435 **Supplementary Figure 1: PIA expression from *S. epidermidis* in tryptic soy broth**

436 One colony of *S. epidermidis* 1457 was incubated in TSB-glucose overnight (t=0). Then a  
437 1:100 dilution was made into; TSB – glucose (A-B) or TSB + glucose (C-D), and incubated for  
438 3 and 5 hours. Samples were cytospun onto microscope slides and stained with rabbit anti-  
439 PIA antibody. Preparations were washed before an Alexa fluor 488-conjugated anti-rabbit  
440 IgG was added with propidium iodide counterstain. Slides were visualised under an Axiovert  
441 fluorescent microscope. Results are representative images from at least 3 experiments  
442 where PIA is green and *S. epidermidis* bacteria are red with original magnification (X1000).

443

444 **Figure 3: PIA expression from *S. epidermidis* incubated in dexamethasone treated whole  
445 blood**

446 Structural analysis of WB leukocytes, PIA and *S. epidermidis* were analysed using confocal  
447 microscopy. Here whole blood killing assays were carried out with labelled *S. epidermidis*  
448 1457. Firstly, *S. epidermidis* 1457 pre-cultures were prepared and were stained with syto-9  
449 (20µM) for 1 hour with the excess syto-9 removed by multiple centrifugation and washing.  
450 Stained *S. epidermidis* 1457 was used to infect whole blood as previously described.  
451 Following whole blood killing and RBC lysis, leukocyte membranes were stained with Cell  
452 Mask™ deep red (Life Technologies, diluted 1:100) for 10 minutes. Then cytopspins were  
453 prepared as previously described in materials and methods. Slides were stained with rabbit  
454 anti-PIA for 1 hour followed by anti-rabbit IgG (alexa 594) for a further hour. Slides were

455 washed, mounted in vectashield® hardset™ and allowed to harden overnight. Then confocal  
456 laser scanning microscopy (Zeiss) analysed 3-5 fields per slide. Each field images 8-20  
457 sections of 1-1.5µm thickness. Results are representative images from at least 3  
458 experiments. To maintain consistency with the previous immunocytochemistry colours,  
459 images were pseudo-coloured purple for cell mask (cell membranes), red for syto-9  
460 (Bacteria) and green for alexa 594 (PIA).

461

462 **Figure 4: Effect of purified PIA on whole blood parameters**

463 Healthy volunteer whole blood was pre-incubated with PIA (10-60µg/ml) or negative control  
464 (M10) for 3 hours prior to infection with *S. epidermidis* (final concentration  $2 \times 10^5$ /ml) for 6  
465 hours. Whole blood suspensions were subjected to gentle RBC lysis, serial dilution and  
466 plating. (A) Colony counts were determined following incubation at 37°C overnight. Results  
467 are expressed as mean  $\pm$  SEM. Remaining suspensions were centrifuged at 9447g for 5 min  
468 and the supernatants analysed for (B) C5a, (C) IL-8, (D) IL-1 $\beta$ . Results are expressed as mean  
469  $\pm$  SEM of the cytokine measured (n=4). \* represents significant differences at  $P < 0.05$

470

471 **Figure 5: Effect of purified PIA on C5a levels in healthy volunteer serum**

472 (A) Serum from healthy volunteers was incubated with PIA (60µg/ml) for 90 minutes before  
473 being stored immediately at -80°C prior to C5a ELISA. (B) Specificity was investigated by pre-  
474 incubating PIA or vehicle control (M10) with dispersin B (10µg/ml) on rotation at 10rpm for  
475 30 minutes at 37°C prior to incubation with serum. Confirmation of Dispersin B activity was  
476 investigated by immunocytochemistry in cytospin preparations of *S. epidermidis* 1457 which  
477 were untreated (C) or treated with dispersin B at 10µg/ml (D) for 30 minutes prior to  
478 staining for PIA (Green) and bacteria (red).



479 Results are expressed as the mean  $\pm$  SEM (n=6-10) C5a response in healthy volunteer sera. \*  
480 and \*\* represent significant differences at P<0.05 and P< 0.01 respectively.

481

482 **Figure 6: C5a levels in serum from controls and patients with PJI**

483 Archived serum from controls and patients with PJI were assayed for C5a levels. (A) C5a  
484 levels in controls and patients with PJI. (B) C5a levels in controls and patients with PJI  
485 catagorised by species of the infecting isolate. Results are expressed as the mean  $\pm$  SEM C5a  
486 response in healthy volunteer (n=10) and sera from PJI patients (n=65). \*\*, \*\*\* and \*\*\*\*  
487 represent significant differences at P<0.01, P< 0.001 and P<0.0001 respectively.

488

489 **Figure 7: C5a levels in patients with PJI determined by mechanism of biofilm accumulation**

490 Archived serum from healthy volunteers and patients with PJI were assayed for C5a levels.  
491 (A) Schematic representation of biofilm forming properties of *S. epidermidis* PJI isolates  
492 (n=43). All *S. epidermidis* isolates were grown in NUNC 96 well microtiter plates for 24  
493 hours. Then wells were washed three times in PBS prior to picric acid fix and crystal violet  
494 staining. Biofilm mechanism was investigated by treating mature biofilms with proteinase K  
495 (1mg/ml) or sodium periodate (40mM) for 24 hours prior to washing which digest protein  
496 and PIA dependent biofilms respectively..

497 (B) C5a levels in controls and patients with PJI catagorised by biofilm accumulation  
498 mechanism. Results are expressed as the mean  $\pm$  SEM C5a levels in sera from *S. epidermidis*  
499 PJI isolates (n=43). \* represents a significant difference at P<0.05.

500

501 **Acknowledgements**

502 The work was funded by a grant awarded to RAI from the Iraqi Government.

503

504 **References**

- 505 Barrio, B., Vangroenweghe, F., Dosogne, H., Burvenich, C., 2000. Decreased neutrophil bactericidal  
 506 activity during phagocytosis of a slime-producing *Staphylococcus aureus* strain. *Vet Res* 31, 603-609.
- 507 Begun, J., Gaiani, J.M., Rohde, H., Mack, D., Calderwood, S.B., Ausubel, F.M., Sifri, C.D., 2007.  
 508 *Staphylococcal* biofilm exopolysaccharide protects against *Caenorhabditis elegans* immune defenses.  
 509 *PLoS Pathog* 3, e57.
- 510 Bober, L.A., Grace, M.J., Pugliese-Sivo, C., Rojas-Triana, A., Waters, T., Sullivan, L.M., Narula, S.K.,  
 511 1995. The effect of GM-CSF and G-CSF on human neutrophil function. *Immunopharmacology* 29,  
 512 111-119.
- 513 Conrad, E.C., Hsu, Y.Y., Bortz, D.M., Younger, J.G., 2013. Spatiotemporal dynamics of complement  
 514 C5a production within bacterial extracellular polymeric substance. *J Innate Immun* 5, 114-123.
- 515 Conway Morris, A., Kefala, K., Wilkinson, T.S., Dhaliwal, K., Farrell, L., Walsh, T., Mackenzie, S.J., Reid,  
 516 H., Davidson, D.J., Haslett, C., Rossi, A.G., Sallenave, J.M., Simpson, A.J., 2009. C5a mediates  
 517 peripheral blood neutrophil dysfunction in critically ill patients. *Am J Respir Crit Care Med* 180, 19-  
 518 28.
- 519 Dimitriou, G., Fouzas, S., Giormezis, N., Giannakopoulos, I., Tzifas, S., Foka, A., Anastassiou, D.E.,  
 520 Spiliopoulou, I., Mantagos, S., 2011. Clinical and microbiological profile of persistent coagulase-  
 521 negative staphylococcal bacteraemia in neonates. *Clin Microbiol Infect* 17, 1684-1690.
- 522 Frances, R., Gonzalez-Navajas, J.M., Zapater, P., Munoz, C., Cano, R., Pascual, S., Marquez, D.,  
 523 Santana, F., Perez-Mateo, M., Such, J., 2007. Bacterial DNA induces the complement system  
 524 activation in serum and ascitic fluid from patients with advanced cirrhosis. *J Clin Immunol* 27, 438-  
 525 444.
- 526 Fredheim, E.G., Granslo, H.N., Flaegstad, T., Figenschau, Y., Rohde, H., Sadovskaya, I., Mollnes, T.E.,  
 527 Klingenberg, C., 2011. *Staphylococcus epidermidis* polysaccharide intercellular adhesin activates  
 528 complement. *FEMS Immunol Med Microbiol* 63, 269-280.
- 529 Ganesan, M., Stewart, E.J., Szafranski, J., Satorius, A.E., Younger, J.G., Solomon, M.J., 2013. Molar  
 530 mass, entanglement, and associations of the biofilm polysaccharide of *Staphylococcus epidermidis*.  
 531 *Biomacromolecules* 14, 1474-1481.
- 532 Gerke, C., Kraft, A., Sussmuth, R., Schweitzer, O., Gotz, F., 1998. Characterization of the N-  
 533 acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus*  
 534 *epidermidis* polysaccharide intercellular adhesin. *J Biol Chem* 273, 18586-18593.
- 535 Goddette, D.W., Frieden, C., 1986. Actin polymerization. The mechanism of action of cytochalasin D.  
 536 *J Biol Chem* 261, 15974-15980.
- 537 Granslo, H.N., Klingenberg, C., Fredheim, E.A., Acharya, G., Mollnes, T.E., Flaegstad, T., 2013.  
 538 *Staphylococcus epidermidis* biofilms induce lower complement activation in neonates as compared  
 539 with adults. *Pediatr Res* 73, 294-300.
- 540 Hartl, D., Latzin, P., Hordijk, P., Marcos, V., Rudolph, C., Woischnik, M., Krauss-Etschmann, S., Koller,  
 541 B., Reinhardt, D., Roscher, A.A., Roos, D., Griese, M., 2007. Cleavage of CXCR1 on neutrophils  
 542 disables bacterial killing in cystic fibrosis lung disease. *Nat Med* 13, 1423-1430.
- 543 Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., Gotz, F., 1996. Molecular basis  
 544 of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol Microbiol* 20, 1083-  
 545 1091.
- 546 Higgins, D.M., Basaraba, R.J., Hohnbaum, A.C., Lee, E.J., Grainger, D.W., Gonzalez-Juarrero, M., 2009.  
 547 Localized immunosuppressive environment in the foreign body response to implanted biomaterials.  
 548 *Am J Pathol* 175, 161-170.
- 549 Kiehl, M.G., Ostermann, H., Thomas, M., Birkfellner, T., Kienast, J., 1997. Inflammatory mediators in  
 550 BAL fluid as markers of evolving pneumonia in leukocytopenic patients. *Chest* 112, 1214-1220.

- 551 Kristian, S.A., Birkenstock, T.A., Sauder, U., Mack, D., Gotz, F., Landmann, R., 2008. Biofilm formation  
552 induces C3a release and protects *Staphylococcus epidermidis* from IgG and complement deposition  
553 and from neutrophil-dependent killing. *J Infect Dis* 197, 1028-1035.
- 554 Kunkel, S.L., Manderino, G.L., Marasco, W., Kaercher, K., Hirata, A.A., Ward, P.A., 1983. A specific  
555 enzyme-linked immunosorbent assay (ELISA) for the determination of human C5a antigen. *J*  
556 *Immunol Methods* 62, 305-314.
- 557 Kurtz, S., Ong, K., Lau, E., Mowat, F., Halpern, M., 2007. Projections of primary and revision hip and  
558 knee arthroplasty in the United States from 2005 to 2030. *J Bone Joint Surg Am* 89, 780-785.
- 559 Liu, D., Xiong, R., Chen, X., Li, P., Ning, Y., Peng, Y., Zhao, Y., Yang, N., Zhou, Y., 2014. The  
560 glucocorticoid dexamethasone inhibits U937 cell adhesion and neutrophil release via RhoA/ROCK1-  
561 dependent and independent pathways. *Cell Physiol Biochem* 33, 1654-1662.
- 562 Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., Laufs, R., 1996. The  
563 intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-  
564 1,6-linked glucosaminoglycan: purification and structural analysis. *J Bacteriol* 178, 175-183.
- 565 Mack, D., Riedewald, J., Rohde, H., Magnus, T., Feucht, H.H., Elsner, H.A., Laufs, R., Rupp, M.E., 1999.  
566 Essential functional role of the polysaccharide intercellular adhesin of *Staphylococcus epidermidis* in  
567 hemagglutination. *Infect Immun* 67, 1004-1008.
- 568 Mack, D., Siemssen, N., Laufs, R., 1992. Parallel induction by glucose of adherence and a  
569 polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for  
570 functional relation to intercellular adhesion. *Infect Immun* 60, 2048-2057.
- 571 Morris, A.C., Brittan, M., Wilkinson, T.S., McAuley, D.F., Antonelli, J., McCulloch, C., Barr, L.C.,  
572 McDonald, N.A., Dhaliwal, K., Jones, R.O., Mackellar, A., Haslett, C., Hay, A.W., Swann, D.G.,  
573 Anderson, N., Laurenson, I.F., Davidson, D.J., Rossi, A.G., Walsh, T.S., Simpson, A.J., 2011. C5a-  
574 mediated neutrophil dysfunction is RhoA-dependent and predicts infection in critically ill patients.  
575 *Blood* 117, 5178-5188.
- 576 Nakae, H., Endo, S., Inada, K., Takakuwa, T., Kasai, T., Yoshida, M., 1994. Serum complement levels  
577 and severity of sepsis. *Res Commun Chem Pathol Pharmacol* 84, 189-195.
- 578 Rohde, H., Burandt, E.C., Siemssen, N., Frommelt, L., Burdelski, C., Wurster, S., Scherpe, S., Davies,  
579 A.P., Harris, L.G., Horstkotte, M.A., Knobloch, J.K., Ragunath, C., Kaplan, J.B., Mack, D., 2007.  
580 Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus*  
581 *epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections.  
582 *Biomaterials* 28, 1711-1720.
- 583 Rohde, H., Frankenberger, S., Zahringer, U., Mack, D., 2010. Structure, function and contribution of  
584 polysaccharide intercellular adhesin (PIA) to *Staphylococcus epidermidis* biofilm formation and  
585 pathogenesis of biomaterial-associated infections. *Eur J Cell Biol* 89, 103-111.
- 586 Rohde, H., Klettke, J., Burandt, E., Wurster, S., Mack, D., 2008. Evaluation of anti-polysaccharide  
587 intercellular adhesin (PIA) antibodies as a marker for prosthetic joint infections. *Int. J. Med.*  
588 *Microbiol.*, Dresden, p. 14.
- 589 Rohde, H., Klettke, J., Siemssen, N., Frommelt, L., Horstkotte, M.A., Burdelski, C., Knobloch, J.K.M.,  
590 Mack, D., 2005. Development of an enzyme-linked immuno sorbent assay (ELISA) specifically  
591 detecting anti-polysaccharide intercellular-adhesin (PIA) antibodies, 15th European Congress of  
592 Clinical Microbiology and Infectious Diseases (ECCMID). *Clin. Microbiol. Infect.*, p. 486.
- 593 Rupp, M.E., Fey, P.D., Heilmann, C., Gotz, F., 2001. Characterization of the importance of  
594 *Staphylococcus epidermidis* autolysin and polysaccharide intercellular adhesin in the pathogenesis of  
595 intravascular catheter-associated infection in a rat model. *J Infect Dis* 183, 1038-1042.
- 596 Rupp, M.E., Ulphani, J.S., Fey, P.D., Bartscht, K., Mack, D., 1999a. Characterization of the importance  
597 of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the  
598 pathogenesis of biomaterial-based infection in a mouse foreign body infection model. *Infect Immun*  
599 67, 2627-2632.

- 600 Rupp, M.E., Ulphani, J.S., Fey, P.D., Mack, D., 1999b. Characterization of *Staphylococcus epidermidis*  
601 polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-  
602 associated infection in a rat model. *Infect Immun* 67, 2656-2659.
- 603 Satorius, A.E., Szafranski, J., Pyne, D., Ganesan, M., Solomon, M.J., Newton, D.W., Bortz, D.M.,  
604 Younger, J.G., 2013. Complement c5a generation by staphylococcal biofilms. *Shock* 39, 336-342.
- 605 Schommer, N.N., Christner, M., Hentschke, M., Ruckdeschel, K., Aepfelbacher, M., Rohde, H., 2011.  
606 *Staphylococcus epidermidis* uses distinct mechanisms of biofilm formation to interfere with  
607 phagocytosis and activation of mouse macrophage-like cells 774A.1. *Infect Immun* 79, 2267-2276.
- 608 Sheppard, S.K., 2012. Bigsdb *Staphylococcus*.
- 609 Shoji, K., Ohashi, K., Sampei, K., Oikawa, M., Mizuno, K., 2012. Cytochalasin D acts as an inhibitor of  
610 the actin-cofilin interaction. *Biochem Biophys Res Commun* 424, 52-57.
- 611 Skjeflo, E.W., Christiansen, D., Espevik, T., Nielsen, E.W., Mollnes, T.E., 2014. Combined inhibition of  
612 complement and CD14 efficiently attenuated the inflammatory response induced by *Staphylococcus*  
613 *aureus* in a human whole blood model. *J Immunol* 192, 2857-2864.
- 614 Stevens, N.T., Sadovskaya, I., Jabbouri, S., Sattar, T., O'Gara, J.P., Humphreys, H., Greene, C.M., 2009.  
615 *Staphylococcus epidermidis* polysaccharide intercellular adhesin induces IL-8 expression in human  
616 astrocytes via a mechanism involving TLR2. *Cell Microbiol* 11, 421-432.
- 617 Stove, S., Welte, T., Wagner, T.O., Kola, A., Klos, A., Bautsch, W., Kohl, J., 1996. Circulating  
618 complement proteins in patients with sepsis or systemic inflammatory response syndrome. *Clin*  
619 *Diagn Lab Immunol* 3, 175-183.
- 620 Tayman, C., Tonbul, A., Kahveci, H., Uysal, S., Koseoglu, B., Tatli, M.M., Dilmen, U., 2011. C5a, a  
621 complement activation product, is a useful marker in predicting the severity of necrotizing  
622 enterocolitis. *Tohoku J Exp Med* 224, 143-150.
- 623 Trampuz, A., Zimmerli, W., 2005. New strategies for the treatment of infections associated with  
624 prosthetic joints. *Curr Opin Investig Drugs* 6, 185-190.
- 625 von Kockritz-Blickwede, M., Konrad, S., Foster, S., Gessner, J.E., Medina, E., 2010. Protective role of  
626 complement C5a in an experimental model of *Staphylococcus aureus* bacteremia. *J Innate Immun* 2,  
627 87-92.
- 628 Vuong, C., Voyich, J.M., Fischer, E.R., Braughton, K.R., Whitney, A.R., DeLeo, F.R., Otto, M., 2004.  
629 Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major  
630 components of the human innate immune system. *Cell Microbiol* 6, 269-275.
- 631 Wagner, C., Kaksa, A., Muller, W., Denefleh, B., Heppert, V., Wentzensen, A., Hansch, G.M., 2004.  
632 Polymorphonuclear neutrophils in posttraumatic osteomyelitis: cells recovered from the inflamed  
633 site lack chemotactic activity but generate superoxides. *Shock* 22, 108-115.
- 634 Wagner, C., Kondella, K., Bernschneider, T., Heppert, V., Wentzensen, A., Hansch, G.M., 2003. Post-  
635 traumatic osteomyelitis: analysis of inflammatory cells recruited into the site of infection. *Shock* 20,  
636 503-510.
- 637 Wang, W.K., Chen, H.L., Yang, C.F., Hsieh, S.C., Juan, C.C., Chang, S.M., Yu, C.C., Lin, L.H., Huang, J.H.,  
638 King, C.C., 2006. Slower rates of clearance of viral load and virus-containing immune complexes in  
639 patients with dengue hemorrhagic fever. *Clin Infect Dis* 43, 1023-1030.
- 640 Weisser, M., Schoenfelder, S.M., Orasch, C., Arber, C., Gratwohl, A., Frei, R., Eckart, M., Fluckiger, U.,  
641 Ziebuhr, W., 2010. Hypervariability of biofilm formation and oxacillin resistance in a *Staphylococcus*  
642 *epidermidis* strain causing persistent severe infection in an immunocompromised patient. *J Clin*  
643 *Microbiol* 48, 2407-2412.
- 644 Woehrl, B., Brouwer, M.C., Murr, C., Heckenberg, S.G., Baas, F., Pfister, H.W., Zwinderman, A.H.,  
645 Morgan, B.P., Barnum, S.R., van der Ende, A., Koedel, U., van de Beek, D., 2011. Complement  
646 component 5 contributes to poor disease outcome in humans and mice with pneumococcal  
647 meningitis. *J Clin Invest* 121, 3943-3953.
- 648 Yan, C., Gao, H., 2012. New insights for C5a and C5a receptors in sepsis. *Front Immunol* 3, 368.

649 Ziebuhr, W., Heilmann, C., Gotz, F., Meyer, P., Wilms, K., Straube, E., Hacker, J., 1997. Detection of  
650 the intercellular adhesion gene cluster (ica) and phase variation in *Staphylococcus epidermidis* blood  
651 culture strains and mucosal isolates. *Infect Immun* 65, 890-896.  
652  
653

Accepted Manuscript