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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

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Conflict of Interest

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Abstract

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

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

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

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

Introduction

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

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

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

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

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

Methods

Bacterial strains and culture conditions

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

Bacterial culture

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

Serum samples

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

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

Antibodies

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

Whole blood killing

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

155

156 *ELISA*

157 Duoset ELISAs (R and D systems, Abingdon) for human C5a, IL-8, TNFα and IL-1β, 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 (0.1-1µM) for 18 hours; iii)
165 whole blood pre-treated with cytochalasin D (5µg/ml) for 30 minutes. Cultures were
166 incubated for 0-24 hours. Then, 200µl 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 200µl 1% BSA for 1 hour, then
169 washed X3 with PBS. Then 200µl 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 (1µg/ml), covered and incubated for 30 minutes. Slides were washed X3 with PBS
173 then 25µl of vectashield® hardset™ (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

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

Biofilm assay

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

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

Statistical Analysis

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

Results

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Finally we identify three limitations that guard against over-interpretation of our results. Firstly we were unable to block PIA induced injury with a C5a blocking antibody (data not shown) suggesting that other mediators may be involved (e.g. C3a, C4a). Secondly, attempts to measure PIA in the patient sera through an in house ELISA were unsuccessful however our previous work on the same sera did confirm higher titers in patients infected with *icaABDC* positive strains (1:20000-36000) compared to icaABDC negative and controls (1:2000-6000)(Rohde et al., 2008.; Rohde et al., 2005). In addition the presence of PIA could be masked by naturally occuring anti-PIA antibodies and the sensitivity of our ELISA. Finally C5a is a particularly labile molecule and its degradation product C5a-DES-ARG might be a more realistic biomarker as we have suggested in previous work (Conway Morris et al., 2009). Our observation that levels of C5a in healthy volunteer controls and controls from patients who had received a prosthetic joint suggests that stability is likely not a problem here.

Figure Legends

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

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

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

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

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

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

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

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

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

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

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

Figure 4: Effect of purified PIA on whole blood parameters

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

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

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

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

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

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

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

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

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

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