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Staphylococcus epidermidis polysaccharide intercellular adhesin induces IL-8 expression in human astrocytes via a mechanism involving TLR2.

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- 1 Staphylococcus epidermidis Polysaccharide Intercellular Adhesin induces IL-8
- 2 expression in human astrocytes via a mechanism involving TLR2.
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- 21 drain/device, EVD; intercellular adhesion, ica; IL-1 receptor antagonist, IL-1ra;
- 22 lipopolysaccharide, LPS, lipoteichoic acid, LTA; mean channel fluorescence, MCF;

Summary

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Staphylococcus epidermidis is an opportunistic biofilm-forming pathogen associated with neurosurgical device-related meningitis. Expression of the polysaccharide intercellular adhesin (PIA) on its surface promotes S. epidermidis biofilm formation. Here we investigated the proinflammatory properties of PIA against primary and transformed human astrocytes. PIA induced IL-8 expression in a dose and/or time dependent manner from U373 MG cells and primary normal human astrocytes. This effect was inhibited by depletion of N-acetyl-β-D-glucosamine polymer from the PIA with Lycopersicon esculentum lectin or sodium meta periodate. Expression of dominant negative versions of the TLR2 and TLR4 adaptor proteins MyD88 and Mal in U373 MG cells inhibited PIA-induced IL-8 production. Blocking IL-1 has no effect. PIA failed to induce IL-8 production from HEK293 cells stably expressing TLR4. However, in U373 MG cells which express TLR2, neutralization of TLR2 impaired PIA-induced IL-8 production. In addition to IL-8, PIA also induced expression of other cytokines from U373 MG cells including IL-6 and MCP-1. These data implicate PIA as an important immunogenic component of the S. epidermidis biofilm that can regulate proinflammatory cytokine production from human astrocytes, in part, via TLR2.

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Introduction

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Many neurosurgical procedures require the insertion of medical devices such as external ventricular drains/devices (EVDs) or cerebrospinal fluid (CSF) shunts to relieve intracranial pressure (Key et al., 1995; Yogev et al., 2000). The use of such devices can be associated with infective complications most often as a result of microbial colonisation and the development of biofilm on the surface of the shunt or EVD. Coagulase-negative staphylococci (CoNS), particularly Staphylococcus epidermidis are frequently associated with indwelling medical device infections including neurosurgical shunts and EVDs (Diaz-Mitoma et al., 1987), and there is evidence demonstrating the involvement of biofilm (Diaz-Mitoma et al., 1987; Younger et al., 1987; Kockro et al., 2000). The formation of S. epidermidis biofilm on a device surface involves host and bacterial factors (Costerton et al., 1987; Mack et al., 1992). One of the primary components is an extracellular mucoid polysaccharide, known as polysaccharide intercellular adhesin (PIA), which facilitates cell-cell adhesion and accumulation into a mature biofilm (Mack et al., 1992). PIA is a homoglycan, composed of poly- β -(1 \rightarrow 6)-N-acetylglucosamine, partially O-succinylated and partially de-N-acetylated (Mack et al., 1996; Sadovskaya et al., 2005). Its production is associated with carriage of the intercellular adhesion operon (ica) consisting of four biosynthetic protein encoding genes (icaADBC) (Heilmann et al., 1996; Gerke et al., 1998) and icaR, a negative regulator (Conlon et al., 2002). The poly-N-acetylglucosamine backbone of the polymer is neutral; some negative charges are present due to partial O-succinvlation and the introduction of positive charges along the molecule is dependent on the deacetylating activity of IcaB. Deacetylation of PIA has been reported to be important for both biofilm formation and immune evasion (Vuong *et al.*, 2004).

A major component of the innate immune response is the Toll-like receptor (TLR) family, which are a group of transmembrane receptor proteins expressed on the surface of sentinel cells of the immune system and other cell types (Tosi, 2005). In the central nervous system (CNS) TLRs are expressed on the surface of astrocytes, microglia, and cerebrovascular endothelial cells (CVEs) (Bailey *et al.*, 2006; Lehnardt *et al.*, 2006). Microbial recognition by TLRs is based on their ability to recognise conserved microbial structures and their function is to coordinate an efficient proinflammatory immune response. Both TLR2 and TLR4 can recognise a variety of conserved microbial agonists from both Gram-positive and –negative bacteria. The current dogma being that TLR2 and TLR4 recognise lipopeptides and LPS, respectively. The signalling cascade leading to NFKB activation, and the production of proinflammatory cytokines, from these receptors occurs via the adaptor proteins Myd88 and Mal/TIRAP.

TLR2 has an important functional role in the host response to staphylococcal infection. Whilst it remains a matter for debate TLR2 has been reported to recognise staphylococcal peptidoglycan and lipoteichoic acids (LTA) (Fournier *et al.*, 2005). In the CNS astrocytes are the most abundant cell type that assume a pro-inflammatory functional role. They express TLRs (Bailey *et al.*, 2006; Lehnardt *et al.*, 2006) and TLR-associated signalling proteins (Bsibsi *et al.*, 2002; Kielan *et al.*, 2002; Bowman *et al.*, 2003; Carpentier *et al.*, 2005). It has previously been demonstrated that astrocytes are capable of recognising peptidoglycan via TLR2, and that their stimulation with

Staphylococcus aureus cell-wall material induced IL-1β, TNF-α, MIP-1 and MCP-1 production (Esen et al., 2004). More recently, TLR2 has also been found to be important in the regulation of acute stage brain abscess formation caused by S. aureus and in neurodegeneration induced by Group B Streptococci in microglia (Kielan et al., 2005; Lehnardt et al., 2006). In addition to this, other stimulants such as lipopolysaccharide (LPS), a known TLR4 agonist and the TLR9 agonist uCpG DNA have been shown to induce cytokine production from astrocytes (Lee et al., 2004; Yoo et al., 2008). To date, little is known regarding the host response to S. epidermidis biofilm-related infections, and whether TLR-signalling is involved. In the context of device related infections, it has been previously demonstrated that the glycocalyx produced by CoNS can induce cytokine production from murine peritoneal macrophages (Stout et al., 1994). There is mounting evidence supporting the recognition of carbohydrates by TLRs and that these represent a unique group of TLR agonists with immunogenic properties that are charge dependent (Kalka-Moll et al., 2002; Wang et al., 2005). Considering this, we investigated the immunogenic properties of PIA in human astrocytes and the role of TLRs in these events.

Results

140 PIA induces IL-8 production from human astrocytoma U373 MG cells

PIA is a major component of the *S. epidermidis* biofilm. We investigated PIA's immunogenicity against U373 MG astrocytoma cells. Dose response experiments (0.01-100 μg/ml PIA) performed at 24 h determined 10 μg/ml as the optimum dose of PIA for induction of IL-8 (Figure 1A). IL-1β also induced IL-8 and was used as a positive control. Time course studies confirmed 24h to be the optimal period with no further increase evident in IL-8 levels at 48 h (data not shown). THP-1 monocytic cells were also tested for PIA responsiveness. Compared to control cells (61+/-20 pg/ml IL-8) PIA at 10 or 100 μg induced 1147+/-126 and 1814+/-171 pg/ml IL-8, respectively (P≤0.05 for both)

Lipopeptides, teichoic acids and nucleic acids are not involved in PIA induction of IL-8 in U373 MG cells

PIA is composed of repeating N-acetylglucosamine units. Lectin from $Lycopersicon\ esculentum\ specifically\ binds\ oligomers\ of\ N$ -acetylglucosamine and, as such, is a useful tool for determining the purity of PIA preparations. In order to ensure that the PIA-induced IL-8 response from U373 MG cells was as a result of stimulation with PIA and not contaminating cell-wall material e.g. lipoteichoic acid (LTA), PIA was left untreated or treated with $Lycopersicon\ esculentum\ lectin\ (which\ should\ bind\ to\ and\ remove\ PIA)$ and then added to the cells. Figure 1B shows that the removal of N-acetylglucosamine polymer from the PIA preparations using lectin, caused a significant decrease in IL-8 production ($P \le 0.05$) in comparison to cells that were stimulated with

lectin-untreated PIA. Stimulation of U373 MG cells with LTA led to induction of IL-8, however this response was unaffected by pretreatment with lectin, which does not bind LTA. Furthermore treatment of U373 MG cells with cell wall teichoic acid (CWTA) (0-500 μg/ml) isolated from *S. epidermidis* RP62A led to no detectable increase in IL-8 production (data not shown) indicating that CWTA is unlikely to be a contaminating immunogenic factor in the PIA preparations used here. Hydrogen peroxide can destroy the IL-8 inducing capacity of lipid-containing molecules without affecting PIA. Figure 1C shows that following treatment with H₂O₂ (1%, 37°C for 4 hrs (Zahringer *et al.*, 2008)), PIA retains its ability to induce IL-8. The ability of sodium *meta* periodate-treated PIA to induce IL8 activity was also tested. Degradation of PIA with sodium *meta* periodate should destroy its IL-8 inducing activity. Figure 1D shows that treatment of PIA with sodium *meta* periodate abolishes its IL-8-inducing capacity.

Although we were unable to exclude the possibility of trace amounts of lipopeptides in our PIA preparations, from our GLC fatty acid analysis (Figure 1E) we calculated that no more than 0.005% (w/w) fatty acids could be present. This equates to 0.05ng lipoprotein per $10~\mu g$ PIA. Stimulation of U373 MG cells with a dose of lipopeptide even 10-times higher (0.5 ng) failed to induce IL-8 expression (data not shown). The PIA contained no contaminating nucleic acids as measured by UV spectra or sugar analysis.

Acetylation of PIA is important for its ability to induce IL-8 expression from U373 MG

183 cells

| 184 | We next assessed the role of acetylation in the ability of PIA to induce IL-8 |
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| 185 | production from U373 MG cells. As before stimulation of cells with PIA displaying |
| 186 | ~20% deacetylation, as measured by ¹ H-NMR analysis, resulted in a significant |
| 187 | induction of IL-8 production (P = 0.0003), whereas PIA that was fully deacetylated |
| 188 | failed to induce a response (Figure 1F). |
| 189 | |
| 190 | Induction of IL-8 from U373 MG cells by an adhered biofilm produced by ica- and ica+ |
| 191 | S. epidermidis isolates |
| 192 | Next we investigated whether carriage of the ica operon led to increased IL-8 |
| 193 | production from U373 MG astocytoma cells by intact, adhered S. epidermidis biofilm |
| 194 | (Figure 2). Compared to control cells, BM13, a PIA-producing ica+ isolate, but not |
| 195 | BM3, an ica- isolate, significantly increased IL-8 production (Figure 2A) from U373 |
| 196 | MG cells. The IL-8 induction was similar to that stimulated with an adhered biofilm, |
| 197 | formed by the strong biofilm- producing S. epidermidis strain RP62A. Figure 2B shows |
| 198 | the degree of biofilm produced on the glass surface following staining with crystal |
| 199 | violet. |
| 200 | |
| 201 | $\Delta Myd88$ and ΔMal , but not IL-1ra, inhibit PIA-induced IL-8 expression in U373 MG |
| 202 | cells. |
| 203 | We investigated the mechanism by which PIA induces IL-8 from U373 MG cells by |
| 204 | focusing on the IL-1R/TLR family. Having ruled out the involvement of IL-1RI, by |
| 205 | demonstrating that IL-1ra could not significantly inhibit PIA-induced IL-8 production |
| 206 | whilst effectively blocking IL-1-induced IL-8 expression (Figure 3A), we then |

determined the effect of Δ MyD88 on the PIA effect. Figure 3B shows that expression of a Δ MyD88 transgene significantly inhibits PIA-induced IL-8 expression. IL-1 was used as a positive control as Δ MyD88 is known to inhibit IL-1RI signaling (Muzio *et* al., 1997).

MyD88 is an adaptor protein for the IL-1R/TLR family. Mal is a homologue of MyD88 involved in TLR2 and TLR4 signaling. Figure 3C demonstrates that expression of a functionally inactive version of Mal (Mal P/H) significantly inhibited PIA-induced IL-8 production from U373MG cells. LPS- but not IL-1-induced IL-8 expression was also inhibited. The PIA response in Figure 3C is lower than in 3B due to differences in transfection efficiencies between the two experiments.

We also evaluated the effect of CAY10470, an inhibitor of NFκB, on PIA-induced IL-8 expression. Figure 3D shows that PIA-induced IL-8 is inhibited by CAY10470, further implicating an NFκB signaling cascade in this response. IL-1 was used as a control.

PIA signals via TLR 2 to induce IL-8 production in U373 MG cells

To determine if PIA-induced IL-8 production was TLR4-dependent, we stimulated a stably transfected TLR4-only expressing HEKTLR4 cell line with PIA. In comparison to LPS treatment, which led to a significant increases in IL-8 protein production at doses of 1 or 10 μ g, PIA treatment of HEKTLR4 cells had no measurable effect on IL-8 (Figure 4A), suggesting that TLR2 rather than TLR4 may be the receptor mediating PIA's effect in U373 MG cells. LPS at 1 and 10 μ g maximally induced IL-8 expression from HEKTLR4.

To confirm the role of TLR2, we quantified the effect of TLR2 and TLR4 neutralising antibodies on inhibition of PIA-induced IL-8 production in U373 MG cells. Figure 4B shows that inhibition of TLR2 decreased PIA-induced IL-8 production by ~40% (P≤0.05). An isotype control antibody was used as a control. The TLR2 neutralising antibody also blocked IL-8 production induced by LTA, a known TLR2 agonist. The TLR4 neutralising antibody had no effect on the PIA response but did significantly decrease LPS-induced IL-8.

U373 MG cells express TLR2 on the cell surface

Quantitative fluorescence microscopy was used to detect expression of TLR2 and TLR4 on U373 MG cells. Figure 5A illustrates that these cells express detectable TLR2 on their surface. The median channel fluorescence (MCF) emitted by the FITC-linked anti-TLR2 antibody is significantly greater than that of the isotype antibody (*P*< 0.0011). Expression of TLR4 by these cells was considerably higher. This may explain in part the relatively small inhibition of the LPS response by the TLR4 neutralising antibody seen in Figure 4B.

247 Primary human astrocytes express TLR2 and respond to PIA.

Figure 5B shows that, similar to the transformed astrocytoma cells, primary normal human astrocytes also express cell surface exposed TLR2. Furthermore these cells produce detectable levels of IL-8 and respond to PIA stimulation in a dose-dependent manner (Figure 5C).

PIA regulates expression of multiple cytokines. The effect of stimulation of U373 MG cells with PIA on a range of cytokines was assessed by cytokine array analysis. PIA induced production of a number of cytokines (Figure 6A) and representative examples of those displaying the most obvious increases in production compared to unstimulated cells were selected for further study, namely IL-8, IL-6 and MCP-1. The array findings were validated by performing cytokine-specific ELISAs on the same cell supernatants (Figure 6B). Finally levels of IL-8, IL-6 and MCP-1 were quantified in different CSF samples. Figure 6C shows that CSF isolated from an individual infected with S. epidermidis contained significantly increased levels of IL-8, IL-6 and MCP-1 compared to normal CSF or inflamed, sterile CSF.

Discussion

Here we link PIA, a major component of the *S. epidermidis* biofilm, to activation of TLR2 and expression of IL-8 in human astrocytes.

Biofilm formation is a primary virulence factor of *S. epidermidis*. The production of PIA and the subsequent formation of biofilm have an important role in protecting the bacteria from phagocytosis (Johnson *et al.*, 1986; Falceri *et al.*, 1987). Conversely staphylococcal glycocalyces, including that produced by *S. epidermidis*, have been reported to activate the innate immune response at doses of 10-100 µg/ml leading to production of prostaglandin E₂ (PGE₂), IL-1 and TNF-α in murine peritoneal macrophages (Stout *et al.*, 1994). This is in keeping with our finding that *S. epidermidis* PIA induces a potent IL-8 response from human astrocytes at similar doses and another recent report describing how a capsular polysaccharide from the Gram-negative *Bacteroides fragilis* can activate NFκB in a TLR2-dependent manner (Wang *et al.*, 2006).

Myd88 belongs to a family of TLR adaptor proteins. Whilst MyD88 signals for all TLRs with the exception of TLR3, a second adaptor protein termed Mal/TIRAP (Fitzgerald *et al.*, 2001) is involved only in TLR2 and TLR4 signaling (Horng *et al.*, 2001; Yamamoto *et al.*, 2002). Human astrocytes express mRNA for both Myd88 and Mal/TIRAP (Farina *et al.*, 2007). By blocking signaling through these adaptor proteins, neutralizing TLR2 and 4 and studying the effect of PIA on TLR4-only expressing HEKTLR4 cells we confirmed the likely involvement of TLR2 in the PIA response.

TLR2 has a role in the recognition of structural components of a variety of Gram-positive bacteria, such as *S. epidermidis* and *S. aureus*, as well as fungal and

protozoan structures (Kopp *et al.*, 2003). Kielan *et al.* (2005) have shown that TLR2 recognises *S. aureus* peptidoglycan in a brain abscess model. Furthermore, it was reported that TLR2 expression on astrocytes is necessary for the recognition of Grampositive peptidoglycan, and stimulation of these cells by cell wall material of *S. aureus* has been reported to result in the production of pro-inflammatory mediators (Esen *et al.*, 2004). Recently Zahringer *et al.* (2008) proposed that TLR2 is activated only by lipoproteins or lipopeptides, and that the activity of all other putative compounds so far reported as TLR2 agonists is due to contaminating lipopeptides/proteins. Having thoroughly determined the purity of our PIA preparations via NMR, fatty acid analysis and lectin, NaOH, H₂O₂ and sodium *meta* periodate inhibition studies we are confident that our observed effects represent a true phenomenon.

TLR2 can signal as a heterodimer. In conjunction with TLR1 it confers responsiveness to LTA and triacylated lipopeptides whilst with TLR6 it can respond to diacylated MALP-2. In airway epithelial cells TLR2 and TLR5 are involved in the flagellin response (Soong *et al.*, 2004), thus it is possible that TLR2 may signal cooperatively with another TLR in response to PIA. TLR2's role may not however, be via a direct interaction with PIA. In other cells types CD36 and RP105 act as co-receptors for TLR2 (Nagai *et al.*, 2005, Triantafilou, 2006).

We found that native PIA from *S. epidermidis* CIP 109562, displaying ~20% deacetylation (Sadovskaya *et al.*, 2006) was capable of inducing a significant increase in IL-8 production. However, PIA that was 100% deacetylated, failed to induce the same response. The deacetylation of *N*-acetylglucosamine residues, through the activity of IcaB, introduces positive charges along the length of the molecule, and this appears to

be important in PIA-mediated biofilm formation and immune evasion (Heilmann *et al.*, 1996, Vuong *et al.*, 2004). Using a mouse model of device-related infection, it was shown that a *S. epidermidis* $\Delta icaB$ mutant was significantly impaired in its ability to establish infection compared to a wild type strain (Vuong *et al.*, 2004). Kalka-Moll and co-workers (Kalka-Moll *et al.*, 2002) reported that bacterial polysaccharides from *B. fragilis*, *S. aureus*, and *S. pneumoniae* with zwitterionic charge motifs represent an unusual group of carbohydrates with possibly unique immunogenic properties. Since PIA can be considered as a zwitterionic polymer the immunogenic properties of this polysaccharide may also be charge dependent. Our observation that deacetylation of PIA with NaOH abolishes its IL-8 activating activity support this.

Until recently, there has been much circumstantial evidence supporting TLR expression by astrocytes. This evidence has been based on the ability of known ligands, such as LPS, Gram-positive cell wall antigens and unmethylated CpG DNA to induce cytokine production from the astrocytes (Schumann *et al.*, 1998; Chung *et al.*, 1990; Takeshita *et al.*, 2001). Here we show that both LTA and LPS can induce IL-8 production in U373 MG astrocytoma cells, and provide direct evidence for the presence of TLR2 on these cells. Moreover, we demonstrate the previously unreported expression of TLR2 in primary normal human astrocytes. Our observations support the work of Bowman *et al* (2003), who demonstrated constitutive, low level expression of mRNA-encoding TLR2, as well as TLR4, TLR5, and TLR9 in cultured astrocytes and Lehnardt *et al.* (2006) who reported TLR2 expression by murine astrocytes. Others have reported that cultured human astrocytes display low basal level expression of TLR2, along with TLR3 and TLR4 (Bsibsi *et al.*, 2002). Although the IL-8 response induced by PIA in

the primary NHA cells was less potent than in the transformed cell line it is possible that other *S. epidermidis* factors are likely to enhance cytokine expression by astrocytes *in vivo*. Although *S. epidermidis* CWTA failed to induce IL-8 production from U373 MG cells, other biofilm mediators such as the cell wall proteins Aap (Rohde *et al.*, 2005; Stevens *et al.* 2008), Bap/Bhp (Lasa *et al.*, 2006) or AtlE (Heilmann *et al.*, 1997), that were not present in our PIA preparations could potentially activate TLR2 or other TLRs *in vivo*.

Stimulation of U373 MG cells with an intact *S. epidermidis* biofilm, adhered to a glass surface and produced by known meningitis-causing isolates with variant *ica* genotypes caused different degrees of IL-8 production. The only significant change however was with a known meningitis-causing *S. epidermidis* isolate with an *ica*⁺ genotype. Whilst it is unlikely that the IL-8 production was solely the result of stimulation by PIA present in the biofilm, other cell wall antigens, such as LTA but not CWTA, and peptidoglycan may possibly have been involved. It is therefore possible that other components of *S. epidermidis* biofilm may also contribute to biofilm-related meningitis *in vivo*.

Our cytokine array analysis revealed that in addition to IL-8 production, PIA stimulation of astrocytes also led to increased production of other NF κ B activated genes including IL-6 and MCP-1. *In vivo* astrocytes produce IL-8, IL-6 and MCP-1 as well as other important mediators of astrocyte function, such as RANTES and IL-1 β (Farina *et al.*, 2007). IL-6 has an important immunomodulatory role in the CNS in enhancing the initial local innate immune response. Such activation can have profound effects in the BBB, increasing permeability and allowing for the translocation of peripheral

circulating immune cells into the CNS (Farina *et al.*, 2007). Production of IL-8 and MCP-1 by astrocytes is important in the attraction of immune cells into the CNS and MCP-1 in particular has been reported to have an important role in the recruitment of phagocytes in response to staphylococcal infections (Fournier *et al.*, 2005).

Interestingly, IL-6, IL-8 and MCP-1 were also detected in CSF taken from a patient clinically diagnosed with active meningitis, resulting from *S. epidermidis* infection. The *S. epidermidis* isolate responsible for this infection possessed the genetic determinants (*icaADBC*) necessary for PIA production and was capable of biofilm formation *in vitro* (data not shown). All three cytokines were present at higher concentrations in the CSF from this patient, compared to patient CSF that displayed signs of inflammation due to non-infective causes, and a patient CSF that displayed no signs of inflammation or infection. Additional studies using more patient samples may reveal a real association between the factors.

It is now widely accepted that the primary strategy of *S. epidermidis* immune evasion involves production of a biofilm. Although expression of PIA is necessary for protection against phagocytosis and killing by antibacterial peptides (Vuong *et al.*, 2004; Schroder, 1999), we have demonstrated here that PIA also has pro-inflammatory immunogenic properties.

Experimental procedures

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382 Cell culture and treatments. U373 MG human astrocytoma cells were obtained from the 383 European Collection of Cell Cultures (ECACC), primary normal human astrocytes 384 (NHA) from Lonza, U.K. and HEKTLR4 cells from Invivogen. U373 MG cells were cultured in Eagle's Minimal Essential Medium (MEM) (GIBCO®) supplemented with 385 10% FCS, 1% L-glutamine, 1% sodium-pyruvate, 1% non-essential amino acid 386 (GIBCO®), and 1% penicillin/streptomycin (Invitrogen Life Sciences). NHA cells were 387 cultured in the supplied astrocyte basal medium (Clonetics[®], Lonza) supplemented with 388 389 3% FCS, 0.3% ascorbic acid, 0.1% recombinant human EGF, 0.1% GA-1000, 0.25% Insulin, 1% L-glutamine (Clonetics®, Lonza), and 1% penicillin/streptomycin 390 (Invitrogen Life Sciences). HEKTLR4 cells were cultured in Dulbecco's Modified 391 Eagle's Medium (DMEM) (GIBCO®) supplemented with 10% FCS, 1% 392 393 penicillin/streptomycin (Invitrogen Life Sciences), and 1% blastocidin (10 mg/ml) 394 (Invivogen). Twenty-four hours prior to agonist treatment, cells were placed under 395 serum-free conditions or in media containing 1% FCS for LPS stimulations. Cells were 396 maintained at 37 °C in a humidified atmosphere of 5% CO₂.

Agonist treatments were performed in serum-free conditions or 1% FCS conditions as appropriate. Unless otherwise stated, treatments were performed on 1×10^5 cells per ml.

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Preparation of PIA and Cell Wall Teichoic Acid (CWTA). PIA was prepared from a crude extract of S. epidermidis 5 (CIP 109562), a strain from our collection as described previously by (Sadovskaya et al., 2005). Briefly, the bacteria were grown statically in 750 ml TSB in 3-1 Erlemeyer flasks at 37°C for 24 h. Bacterial biofilms on the bottom

of the flasks were washed twice with NaCl (0.9% w/v) and collected in 0.9% NaCl using 6 mm diameter glass beads. The suspension was then sonicated twice, 30 s each, on ice using an Ikasonic sonicator (IKA Labortechnik) set to 50% amplitude and 50% duty cycle. The sonicate was clarified twice by centrifugation (2,000 x g, 4 °C, 15 min, then 8,700 x g, 4 °C, 10 min). Following this, the supernatant was concentrated using an Amicon 10 kDa cutoff ultrafiltration cell. TCA was added to a concentration of 5% and the precipitate was removed by centrifugation (8,700 x g, 4 °C, 10 min). The now protein-free extract was fractionated on a Sephacryl S-300 column (1 x 80 cm), eluted with water. Amino sugar-rich fractions corresponding to HMW PIA were pooled and lyophilized.

The purity of the PIA was also examined by ¹H-NMR (Varian UNITY INOVA 500 instrument). CWTA was obtained from the SDS-treated *S. epidermidis* RP62A as described previously (Sadovskaya *et al.*, 2004).

Purity of PIA assay. The purity of the PIA was checked by ¹H-NMR (Varian UNITY INOVA 500 instrument) and monosaccharide analysis, as described previously (Sadovskaya *et al*, 2005). Glucosamine was the only monosaccharide present in the PIA preparation. In order to exclude the possible contamination with LTA, *S. epidermidis* LTA and the PIA preparation were analysed for the presence of fatty acids as fatty acids methyl ester (FAME) by gas liquid chromatography (GLC). LTA of *S. epidermidis* RP62A was prepared from defatted cells as described in (Iwasaki *et al.*, 1986). The purified *S. epidermidis* LTA (0.5 mg) was heated in 2 M HCl in absolute methanol at 85 °C for 6 h. The reaction mixture was evaporated to dryness under a stream of nitrogen,

the residue dissolved in 250 μ l of chloroform and analysed by GLC on Shimadzu GC-14 gas chromatograph equipped with a flame ionization detector and a Zebron ZB-5 capillary column (30 m x 0.25 mm), with hydrogen as carrier gas using a temperature gradient 170 °C (3 min), 260 °C at 5°C/min. 5 mg of PIA preparation were treated similarly, and the residue was taken in 10 μ l of chloroform. No peaks corresponding to fatty acids were detected in the PIA preparation (Fig 1E).

UV spectra of the PIA preparation and GlcNAc standard (50 μ g/ml) were detected using a Helios b spectrophotometer in the range of 200-300 nm.

Lycopersicon esculentum Lectin (1 mg/ml) (Sigma) binds *N*-acetyl-β-D-glucosamine oligomers and was used to determine the purity of the PIA preparations. Fifty μg lectin was incubated in a 1:1 ratio with PIA (1 mg/ml) or LTA (1 mg/ml) at 25 °C for 1 h and centrifuged at 14,000 rpm for 30 min. Supernatants were removed and used to stimulate the U373 MG astrocytoma cells.

For fatty acid analysis, 5 mg of PIA preparation were heated in 2 M HCl in absolute methanol at 85 °C for 6 h. The reaction mixture was evaporated to dryness under a stream of nitrogen, the residue dissolved in 10 µl of chloroform and analysed by gas liquid chromatography on Shimadzu GC-14 gas chromatograph equipped with a flame ionization detector and a Zebron ZB-5 capillary column (30 m x 0.25 mm), with hydrogen as carrier gas using a temperature gradient 170 °C (3 min), 260 °C at 5°C/min. Purified *S. epidermidis* LTA (0.5 mg) was treated similarly as a standard. No peaks corresponding to fatty acids were detected in the PIA preparation.

Cytokine protein production. Cells were left untreated or stimulated with agonists or vehicle controls, as indicated. In some experiments cells were pretreated with IL-1R antagonist (IL-1ra, 0.1 μ g/ml) (R & D Systems), or a TLR2 neutralising antibody TLR2.1 (25 μ g/ml) (Bioquote Ltd), or IgG2a (25 μ g/ml) (Bioquote Ltd) for 1 h before agonist treatment.

IL-8, IL-6, and MCP-1 protein concentrations in cell supernatants and patient CSF specimens were determined by sandwich ELISA (R & D Systems). Assays were performed at least three times and in duplicate or triplicate on each occasion.

The expression of 174 different cytokines and growth factors in cell supernatants from untreated and PIA treated U373 MG astrocytoma cells was detected using the RayBio[®] Human Cytokine Antibody Array C series 2000 kit (RayBiotech Inc.), according to the manufacturer's instructions. Signals were detected following exposure to X-ray film (Kodak). Densitometry readings were obtained through illumination on an ultraviolet source (LKB Bromma, 2011 macrovue Transilluminator) and recorded using a gel documentation system (Stratgene Eagle Eye[®] II).

Collection of CSF specimens. CSF was collected from neurosurgical patients with an EVD in situ at Beaumont Hospital, Dublin, by healthcare professionals following informed consent and using the appropriate clinical criteria as approved by the hospital ethics committee, over a three year period as part of routine care. CSF specimens were examined for sterility or for the presence of *S. epidermidis*, following growth on Columbia blood agar. *S. epidermidis* was identified using the API®20 Staph identification system (Biomerieux). CSF specimens were centrifuged at 1000 x g for 10

min and cell free supernatants were immediately transferred into a sterile tube and stored at -80° C until required for experimental analysis. CSF specimens were grouped into the three following defined categories; (i) inflammed CSF, where CSF was sterile and inflammation was due to non-infective cause (n = 1), (ii) normal CSF, where CSF was sterile with no evidence of inflammation as determined by the diagnostic laboratory, that is, normal glucose, protein, and white blood cell count levels (n = 9), and (iii) inflammed and infected CSF, where CSF showed evidence of inflammation, resulting from infection with *S. epidermidis* (n = 1).

Transfection and reporter gene studies. Transfections were performed according to the methods described previously (Greene *et al.*, 2005). Transfections were performed into cells using TransFast Reagent (Promega) in a 1:1 ratio, according to the manufacturer's instructions, using 100 ng of NF-_κB₅-linked luciferase reporter plasmid and 200 ng of either pCDNA3.1 (Invitrogen Life Technologies) or ΔMyD88 expression plasmid (Greene *et al.*, 2005) or pDC304 or Mal P/H expression vector (Carroll *et al.*, 2005). ΔMyD88 contains only a functional TIR domain and lacks the death domain required for downstream signaling. MalP/H is a dominant-negative version of Mal that has a Pro 125 His point mutation in box 2 of the TIR domain. Cells were supplemented with additional growth media for 24 h at 37 °C before being left unstimulated or stimulated as indicated. Cells were lysed with reporter lysis buffer (Promega), and reporter gene activity was quantified by luminometry (PerkinElmer Wallac Victor², 1420 multilabel counter) using the Promega luciferase assay system, according to the manufacturer's instructions.

| 496 | |
|-----|---|
| 497 | Laser-scanning cytometry. U373 MG astrocytoma cells and NHA cells were seeded (1 x |
| 498 | 10 ⁵ cells/well) in four well chamber slides (Nunc, UK), in duplicate. Slides were fixed |
| 499 | in methanol (AnalaR) for 5 min, and then labeled indirectly with goat-anti TLR 2 (Santa |
| 500 | Cruz Biotechnology, USA) and anti-goat FITC, then counter-stained with propidium |
| 501 | iodide (Molecular probes, USA), washed twice in PBS + 1% Tween and allowed to dry. |
| 502 | Fluorescence excitation was provided by a 488 nm laser line. Nuclear (PI) and TLR2 |
| 503 | (FITC) fluorescence were measured at 588 +/- 10 nm or 530 +/- 20 nm, respectively. |
| 504 | Antibody-staining cells were then identified and quantified using CompuCyte software |
| 505 | on the basis of integrated green fluorescence (Kamentsky et al., 1997). |
| 506 | |
| 507 | Data and Statistical analysis. Data was analyzed using GraphPad Prism 4.0 software |
| 508 | package (GraphPad). Data obtained from cytokine array analysis was analyzed using |
| 509 | the Ray®Bio Analysis Tool (Ray®Biotech Inc.) Results are expressed as mean ± SD, |
| 510 | and were compared by One-tailed student t-test. Differences were considered |
| 511 | significant when the P value was ≤ 0.05 . |
| 512 | Acknowledgements |
| 513 | We thank Dr. E. Vinogradov, IBS, NRC Canada for the ¹ H-NMR analysis of PIA and |

dPIA.

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746 Fig. 1. PIA induces IL-8 production from U373 MG astrocytoma cells. U373 MG astrocytoma cells (1 x 10⁵/ml) were left unstimulated or stimulated with (A) 747 748 vehicle, IL-1 (10 ng/ml) or PIA as indicated for 24 h, (B) PIA or LTA (10 μg/ml, 24 h) with or without prior treatment with lectin, (C) PIA or H₂O₂-treated PIA (10 µg/ml, 24 749 h), (D) PIA or NaIO₄-treated PIA (10 µg/ml, 24 h) and production of IL-8 was 750 751 quantified in cell supernatants by ELISA. (E) GLC chromatographic profiles of FAME 752 derived from PIA preparation (a, 50 µg/µl) and S. epidermidis LTA (b, 2 µg/µl). Assignments of FAME was made tentatively using different standards (SupelcoTM 37 753 754 component FAME mix; olive oil FAME). (F) IL-8 ELISA of supernatants from PIA (10 μg/ml), dPIA (10 μg/ml), or IL-1 (10 ng/ml) treated cells (24h). (* vs untreated cells). 755 756 Fig. 2. IL-8 induction from U373 MG cells by S. epidermidis biofilm. 757 758 (A) ΔIL -8 expression (pg/ml) from U373 MG astrocytoma cells (1 x 10⁶ cells/ml) 759 following stimulation fro 24 h with an adhered biofilm on a glass surface, formed by ica (BM3) or ica⁺ (BM13) S. epidermidis isolates. The known ica⁺ biofilm-positive RP62A 760 761 was included as a positive control. (B) Adherent biofilm on the glass surface was stained 762 with crystal violet (0.4% w/v). A glass slip with no adhered biofilm acted as a control. 763 764 Fig. 3 MyD88, Mal and NFKB, but not IL-1, are involved in PIA-induced IL-8 765 expression. U373 MG astrocytoma cells (1 x 10⁵ cells/ml) (A) were left unstimulated or stimulated 766

with PIA (10 µg/ml) or IL-1 (10 ng/ml) for 24 h with or without prior treatment with IL-

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Figure Legends

1ra (how much?). IL-8 production was quantified in cell supernatants by ELISA. (* vs. untreated cells, or # vs. agonist), or were cotransfected for 24 h with pRLSV40 (a constitutive luciferase reporter gene) plus (B) an empty vector (pCDNA3) or Δ Myd88 expression plasmid or (C) an empty vector (pDC304) or Mal P/H expression plasmid then left untreated or stimulated with PIA (10 µg/ml), IL-1 (10 ng/ml) or LPS (10 µg/ml) as indicated for 24 h. IL-8 levels were corrected for transfection efficiency and expressed in pg/ light unit (L.U.), or (D) PIA (10 µg/ml) or IL-1 (10 ng/ml) with or without prior treatment with CAY10470 (how much? How long?) (* vs. untreated cells, or # vs. agonist).

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Fig. 4. TLR2, not TLR4, mediates the PIA response in U373 MG cells.

- 779 (A) HEKTLR4 cells (1 x 10^5 cells/ml) were stimulated with PIA (10 or 100 μ g/ml) or
- 780 LPS (1 or 10 μ g/ml) fro 24 h and production of IL-8 was quantified in cell supernatants
- by ELISA. (* vs. untreated cells, or # vs. agonist). (B) U373 MG cells (1 x 10⁵ cells/ml)
- were left untreated or stimulated with PIA (10 μ g/ml), LTA (10 μ g/ml) or LPS (10
- $\mu g/ml$) +/- IgG2a control isotype antibody, TLR2.1 or TLR4 neutralising antibodies (25
- $\,$ 784 $\,$ $\,$ µg/ml) for 1 h as indicated. Production of IL-8 was quantified in cell supernatants by
- 785 ELISA. (* vs. untreated cells, or # vs. agonist).

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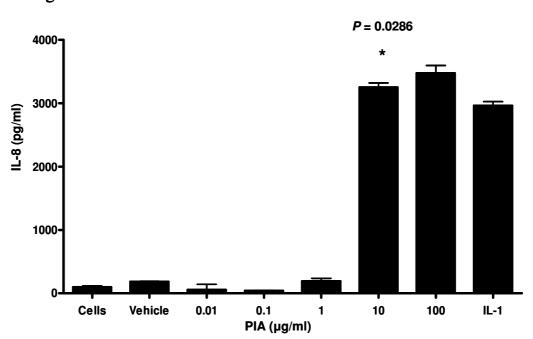
Fig. 5. Primary and transformed human astrocytes express TLR2 and respond to

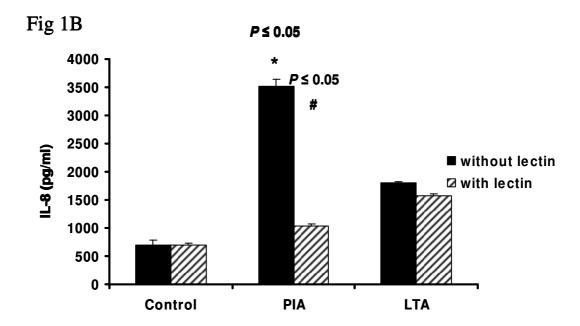
- 788 **PIA.**
- 789 (A) U373 MG or (B) primary human NHA astrocytes (1 x 10⁵/ml) were grown in
- 790 chamber slides, Fc blocked, and labelled with FITC-conjugated anti-TLR2, anti-TLR4

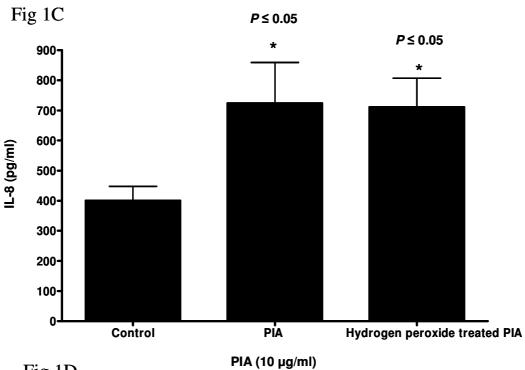
| 791 | or an isotype control Ab. Cells were counterstained with PI and TLR surface expression |
|-----|--|
| 792 | was quantified by laser-scanning microscopy. Graphs show median channel |
| 793 | fluorescence +/-SEM (* vs. isotype, n=6). (B) Inset shows representative histogram of |
| 794 | FITC fluorescence comparing isotype- (clear) and anti-TLR2 Ab (solid)-labelled |
| 795 | samples. (C) NHA cells (1 x 10 ⁵ cells/ml) were left untreated or stimulated with PIA (10 |
| 796 | or 100 $\mu g/ml$) for 48 h. ELISA was used to quantify levels of IL-8 in cell supernatants |
| 797 | (* vs untreated cells). |
| 798 | |
| 799 | Fig. 6. PIA induces the expression of cytokines associated with S. epidermidis |
| 800 | meningitis. |
| 801 | (A) U373 MG astrocytoma cells (1 x 10^5 cells/ml) were left untreated or stimulated with |
| 802 | PIA (10 $\mu g/ml$) for 24 h. Cytokine array analysis was performed on pooled supernatants |
| 803 | from triplicate samples and increases in the production of selected cytokines are |
| 804 | depicted in densitometry units. IL-8, IL-6 and MCP-1 concentrations in (B) the same |
| 805 | samples (* vs untreated cells) or (C) different categories of CSF were measured by |
| 806 | ELISA (* vs normal CSF). |
| 807 | |
| 808 | |
| 809 | |
| 810 | |
| 811 | |
| 812 | |
| 813 | |

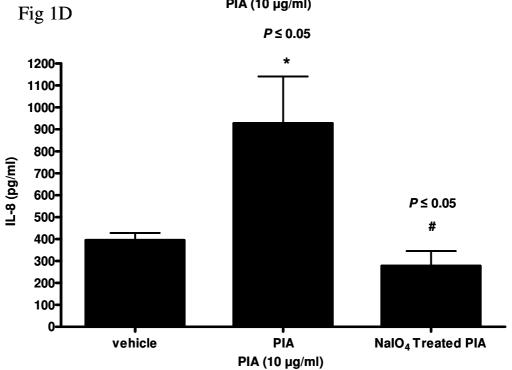
Figures



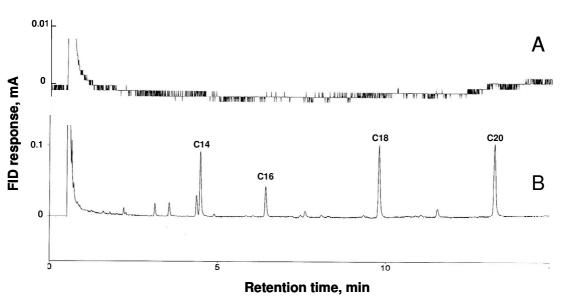


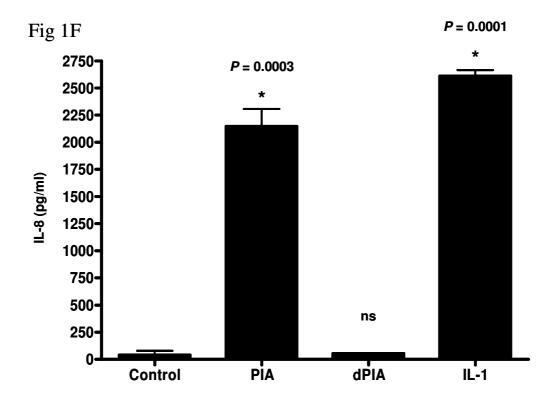




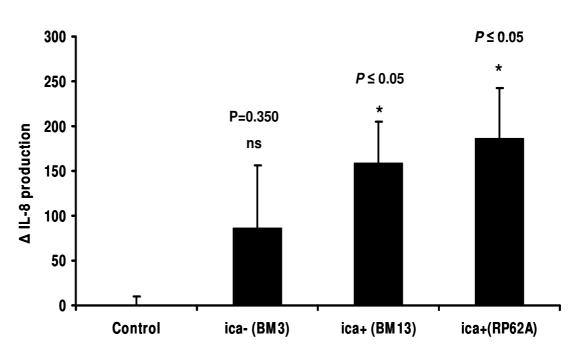




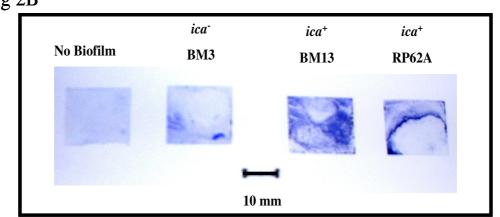


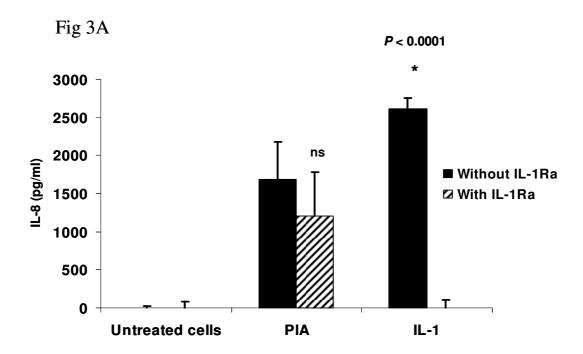






 $Fig\ 2B \hspace{3cm} \text{Isolate/strain}$





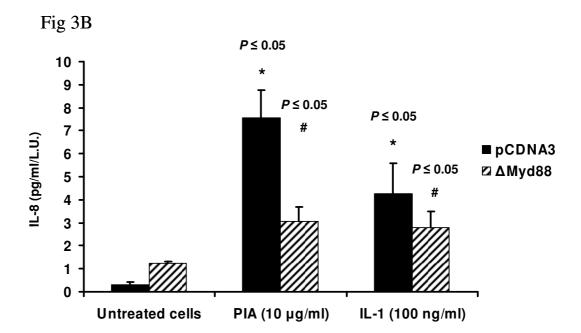


Fig 3C

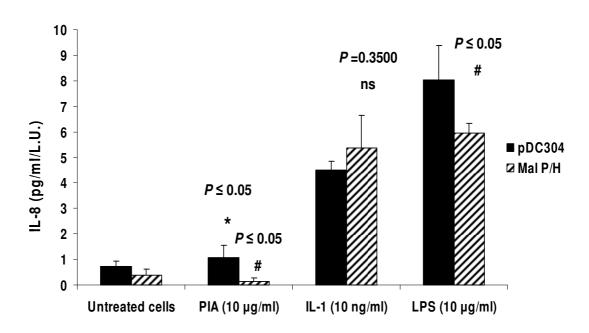


Fig 3D

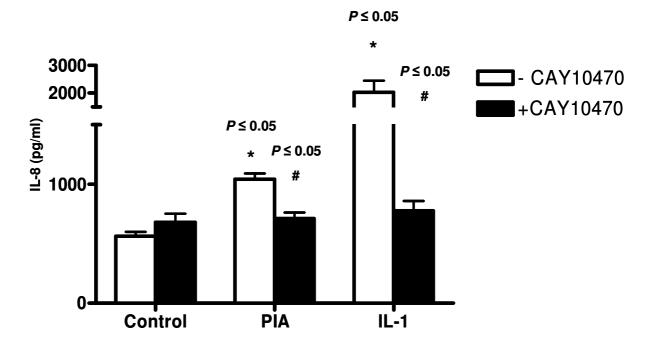


Fig 4A

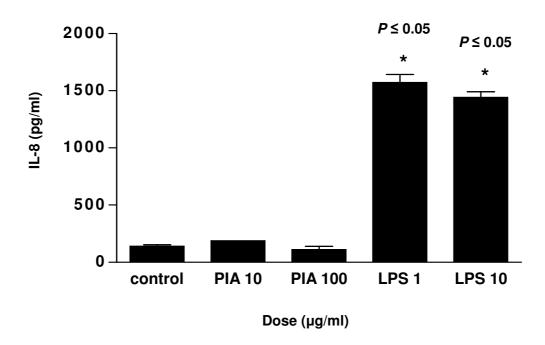
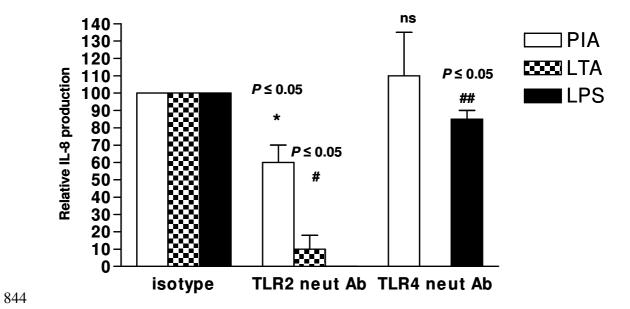


Fig 4B



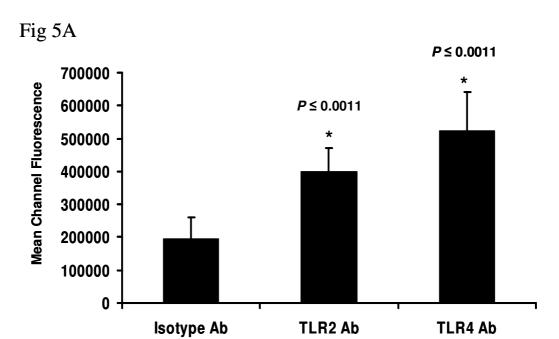
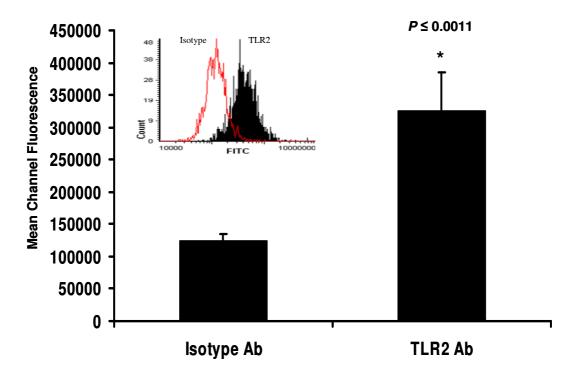


Fig 5B



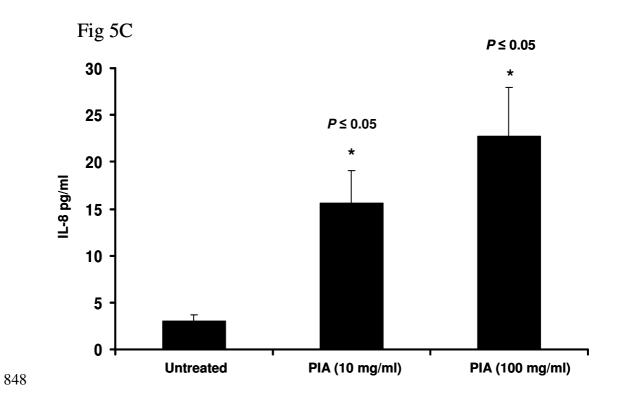


Fig 6 A

