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CITATION

Stevens, Niall; Sadovskaya, Irina; Jabbouri, Said; Sattar, Tafiq; O'Gara, James P.; Humphreys, Hilary; et al. (2009): Staphylococcus epidermidis polysaccharide intercellular adhesin induces IL-8 expression in human astrocytes via a mechanism involving TLR2.. Royal College of Surgeons in Ireland. Journal contribution. https://hdl.handle.net/10779/rcsi.10785737.v2

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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] Funding for this project is gratefully acknowledged from the Health Research Board.

Keywords: Human, Bacterial, Cytokines

Running Title: PIA activation of TLR2

Abbreviations: blood brain barrier, BBB; coagulase-negative staphylococci, CoNS; central nervous system, CNS; cerebrospinal fluid, CSF; cerebrovascular endothelial cell, CVE; cell wall teichoic acid, CWTA; deacetylated PIA, dPIA; external ventricular drain/device, EVD; intercellular adhesion, *ica*; IL-1 receptor antagonist, IL-1ra; lipopolysaccharide, LPS, lipoteichoic acid, LTA; mean channel fluorescence, MCF;

normal human astrocytes, NHA; propidium iodide, PI; polysaccharide intercellular adhesin, PIA.

Summary

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.

Introduction

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→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*-succinylation 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 pro-inflammatory 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 NFκB activation, and the production of pro-inflammatory 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

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 ($P \leq 0.05$ 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 ($P \leq 0.05$) 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 H_2O_2 (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 μg 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 cells

184 We next assessed the role of acetylation in the ability of PIA to induce IL-8
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 *ΔMyd88 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 ΔMyD88 on the PIA effect. Figure 3B shows that expression of a ΔMyD88 transgene significantly inhibits PIA-induced IL-8 expression. IL-1 was used as a positive control as ΔMyD88 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 μg, 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 μg 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% ($P \leq 0.05$). 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.

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 E_2 (PGE_2), 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

294 protozoan structures (Kopp *et al*., 2003). Kielan *et al*. (2005) have shown that TLR2
295 recognises *S. aureus* peptidoglycan in a brain abscess model. Furthermore, it was
296 reported that TLR2 expression on astrocytes is necessary for the recognition of Gram-
297 positive peptidoglycan, and stimulation of these cells by cell wall material of *S. aureus*
298 has been reported to result in the production of pro-inflammatory mediators (Esen *et al*.,
299 2004). Recently Zahringer *et al*. (2008) proposed that TLR2 is activated only by
300 lipoproteins or lipopeptides, and that the activity of all other putative compounds so far
301 reported as TLR2 agonists is due to contaminating lipopeptides/proteins. Having
302 thoroughly determined the purity of our PIA preparations via NMR, fatty acid analysis
303 and lectin, NaOH, H_2O_2 and sodium *meta* periodate inhibition studies we are confident
304 that our observed effects represent a true phenomenon.

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

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

317 be important in PIA-mediated biofilm formation and immune evasion (Heilmann *et al*.,
318 1996, Vuong *et al*., 2004). Using a mouse model of device-related infection, it was
319 shown that a *S. epidermidis* Δ*icaB* mutant was significantly impaired in its ability to
320 establish infection compared to a wild type strain (Vuong *et al*., 2004). Kalka-Moll and
321 co-workers (Kalka-Moll *et al*., 2002) reported that bacterial polysaccharides from *B.*
322 *fragilis*, *S. aureus*, and *S. pneumoniae* with zwitterionic charge motifs represent an
323 unusual group of carbohydrates with possibly unique immunogenic properties. Since
324 PIA can be considered as a zwitterionic polymer the immunogenic properties of this
325 polysaccharide may also be charge dependent. Our observation that deacetylation of PIA
326 with NaOH abolishes its IL-8 activating activity support this.

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

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

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

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

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

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

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

Experimental procedures

Cell culture and treatments. U373 MG human astrocytoma cells were obtained from the European Collection of Cell Cultures (ECACC), primary normal human astrocytes (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 10% FCS, 1% L-glutamine, 1% sodium-pyruvate, 1% non-essential amino acid (GIBCO®), and 1% penicillin/streptomycin (Invitrogen Life Sciences). NHA cells were cultured in the supplied astrocyte basal medium (Clonetics®, Lonza) supplemented with 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 (Invitrogen Life Sciences). HEKTLR4 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO®) supplemented with 10% FCS, 1% penicillin/streptomycin (Invitrogen Life Sciences), and 1% blastocidin (10 mg/ml) (Invivogen). Twenty-four hours prior to agonist treatment, cells were placed under serum-free conditions or in media containing 1% FCS for LPS stimulations. Cells were maintained at 37 ^{o}C in a humidified atmosphere of 5% CO_2.

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

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^{o}C for 24 h. Bacterial biofilms on the bottom

405 of the flasks were washed twice with NaCl (0.9% w/v) and collected in 0.9% NaCl using
406 6 mm diameter glass beads. The suspension was then sonicated twice, 30 s each, on ice
407 using an Ikasonic sonicator (IKA Labortechnik) set to 50% amplitude and 50% duty
408 cycle. The sonicate was clarified twice by centrifugation (2,000 x g, 4 ^{o}C, 15 min, then
409 8,700 x g, 4 ^{o}C, 10 min). Following this, the supernatant was concentrated using an
410 Amicon 10 kDa cutoff ultrafiltration cell. TCA was added to a concentration of 5% and
411 the precipitate was removed by centrifugation (8,700 x g, 4 ^{o}C, 10 min). The now
412 protein-free extract was fractionated on a Sephacryl S-300 column (1 x 80 cm), eluted
413 with water. Amino sugar-rich fractions corresponding to HMW PIA were pooled and
414 lyophilized.
415 The purity of the PIA was also examined by ^{1}H-NMR (Varian UNITY INOVA
416 500 instrument). CWTA was obtained from the SDS-treated *S. epidermidis* RP62A as
417 described previously (Sadovskaya *et al*., 2004).
418
419 *Purity of PIA assay.* The purity of the PIA was checked by ^{1}H-NMR (Varian UNITY
420 INOVA 500 instrument) and monosaccharide analysis, as described previously
421 (Sadovskaya *et al*, 2005). Glucosamine was the only monosaccharide present in the PIA
422 preparation. In order to exclude the possible contamination with LTA, *S. epidermidis*
423 LTA and the PIA preparation were analysed for the presence of fatty acids as fatty acids
424 methyl ester (FAME) by gas liquid chromatography (GLC). LTA of *S. epidermidis*
425 RP62A was prepared from defatted cells as described in (Iwasaki *et al*., 1986). The
426 purified *S. epidermidis* LTA (0.5 mg) was heated in 2 M HCl in absolute methanol at 85
427 °C for 6 h. The reaction mixture was evaporated to dryness under a stream of nitrogen,

the residue dissolved in 250 µl 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 µl 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 µg/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 ^{o}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.

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

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

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

465

466 *Collection of CSF specimens*. CSF was collected from neurosurgical patients with an
467 EVD *in situ* at Beaumont Hospital, Dublin, by healthcare professionals following
468 informed consent and using the appropriate clinical criteria as approved by the hospital
469 ethics committee, over a three year period as part of routine care. CSF specimens were
470 examined for sterility or for the presence of *S. epidermidis*, following growth on
471 Columbia blood agar. *S. epidermidis* was identified using the API®20 Staph
472 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^{o}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-$_{\kappa}$B$_5$-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 ^{o}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 Victor^2, 1420 multilabel counter) using the Promega luciferase assay system, according to the manufacturer's instructions.

Laser-scanning cytometry. U373 MG astrocytoma cells and NHA cells were seeded (1 x 10^5 cells/well) in four well chamber slides (Nunc, UK), in duplicate. Slides were fixed in methanol (AnalaR) for 5 min, and then labeled indirectly with goat-anti TLR 2 (Santa Cruz Biotechnology, USA) and anti-goat FITC, then counter-stained with propidium iodide (Molecular probes, USA), washed twice in PBS + 1% Tween and allowed to dry. Fluorescence excitation was provided by a 488 nm laser line. Nuclear (PI) and TLR2 (FITC) fluorescence were measured at 588 +/- 10 nm or 530 +/- 20 nm, respectively. Antibody-staining cells were then identified and quantified using CompuCyte software on the basis of integrated green fluorescence (Kamentsky *et al*., 1997).

Data and Statistical analysis. Data was analyzed using GraphPad Prism 4.0 software package (GraphPad). Data obtained from cytokine array analysis was analyzed using the Ray®Bio Analysis Tool (Ray®Biotech Inc.) Results are expressed as mean ± SD, and were compared by One-tailed student *t*-test. Differences were considered significant when the *P* value was ≤ 0.05.

Acknowledgements

We thank Dr. E. Vinogradov, IBS, NRC Canada for the ^{1}H-NMR analysis of PIA and dPIA.

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745 **Figure Legends**

746 **Fig. 1. PIA induces IL-8 production from U373 MG astrocytoma cells.**

747 U373 MG astrocytoma cells (1 x 10^5/ml) were left unstimulated or stimulated with (A)
748 vehicle, IL-1 (10 ng/ml) or PIA as indicated for 24 h, (B) PIA or LTA (10 µg/ml, 24 h)
749 with or without prior treatment with lectin, (C) PIA or H_2O_2-treated PIA (10 µg/ml, 24
750 h), (D) PIA or $NaIO_4$-treated PIA (10 µg/ml, 24 h) and production of IL-8 was
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).
753 Assignments of FAME was made tentatively using different standards (Supelco^{TM} 37
754 component FAME mix; olive oil FAME). (F) IL-8 ELISA of supernatants from PIA (10
755 µg/ml), dPIA (10 µg/ml), or IL-1 (10 ng/ml) treated cells (24h). (* vs untreated cells).
756

757 **Fig. 2. IL-8 induction from U373 MG cells by *S. epidermidis* biofilm.**

758 (**A**) ΔIL-8 expression (pg/ml) from U373 MG astrocytoma cells (1 x 10^6 cells/ml)
759 following stimulation fro 24 h with an adhered biofilm on a glass surface, formed by ica$^-$
760 (BM3) or ica$^+$ (BM13) *S. epidermidis* isolates. The known *ica*$^+$ biofilm-positive RP62A
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 NFκB, but not IL-1, are involved in PIA-induced IL-8**
765 **expression.**

766 U373 MG astrocytoma cells (1 x 10^5 cells/ml) (A) were left unstimulated or stimulated
767 with PIA (10 µg/ml) or IL-1 (10 ng/ml) for 24 h with or without prior treatment with IL-

768 1ra (how much?). IL-8 production was quantified in cell supernatants by ELISA. (* vs.
769 untreated cells, or # vs. agonist), or were cotransfected for 24 h with pRLSV40 (a
770 constitutive luciferase reporter gene) plus (B) an empty vector (pCDNA3) or ΔMyd88
771 expression plasmid or (C) an empty vector (pDC304) or Mal P/H expression plasmid
772 then left untreated or stimulated with PIA (10 μg/ml), IL-1 (10 ng/ml) or LPS (10
773 μg/ml) as indicated for 24 h. IL-8 levels were corrected for transfection efficiency and
774 expressed in pg/ light unit (L.U.), or (D) PIA (10 μg/ml) or IL-1 (10 ng/ml) with or
775 without prior treatment with CAY10470 (how much? How long?) (* vs. untreated cells,
776 or # vs. agonist).
777
778 **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 μg/ml) or
780 LPS (1 or 10 μg/ml) fro 24 h and production of IL-8 was quantified in cell supernatants
781 by ELISA. (* vs. untreated cells, or # vs. agonist). (B) U373 MG cells (1 x 10^5 cells/ml)
782 were left untreated or stimulated with PIA (10 μg/ml), LTA (10 μg/ml) or LPS (10
783 μg/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).
786
787 **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 10^5/ml) were grown in
790 chamber slides, Fc blocked, and labelled with FITC-conjugated anti-TLR2, anti-TLR4

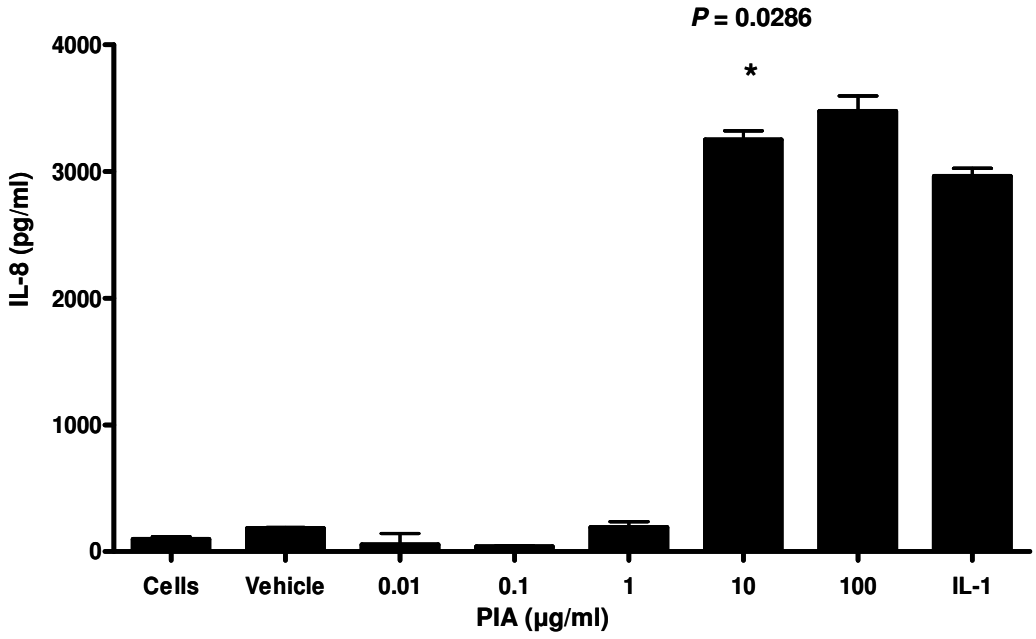
or an isotype control Ab. Cells were counterstained with PI and TLR surface expression was quantified by laser-scanning microscopy. Graphs show median channel fluorescence +/-SEM (* vs. isotype, n=6). (B) Inset shows representative histogram of FITC fluorescence comparing isotype- (clear) and anti-TLR2 Ab (solid)-labelled samples. (C) NHA cells (1 x 10^5 cells/ml) were left untreated or stimulated with PIA (10 or 100 μg/ml) for 48 h. ELISA was used to quantify levels of IL-8 in cell supernatants (* vs untreated cells).

Fig. 6. PIA induces the expression of cytokines associated with *S. epidermidis* meningitis.

(A) U373 MG astrocytoma cells (1 x 10^5 cells/ml) were left untreated or stimulated with PIA (10 μg/ml) for 24 h. Cytokine array analysis was performed on pooled supernatants from triplicate samples and increases in the production of selected cytokines are depicted in densitometry units. IL-8, IL-6 and MCP-1 concentrations in (B) the same samples (* vs untreated cells) or (C) different categories of CSF were measured by ELISA (* vs normal CSF).

814 **Figures**

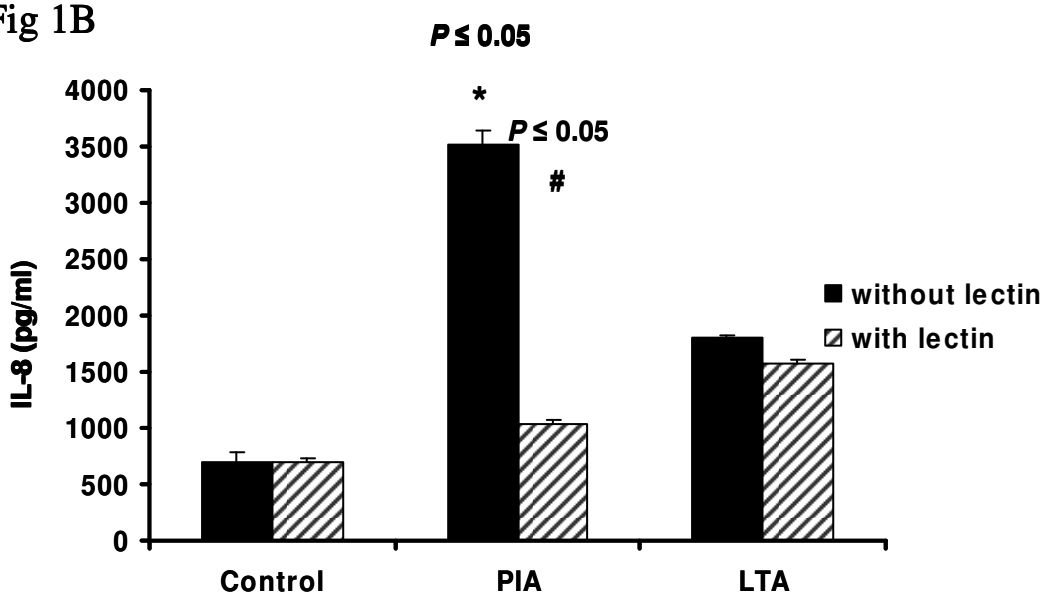
Fig 1A



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Fig 1B



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Fig 1C

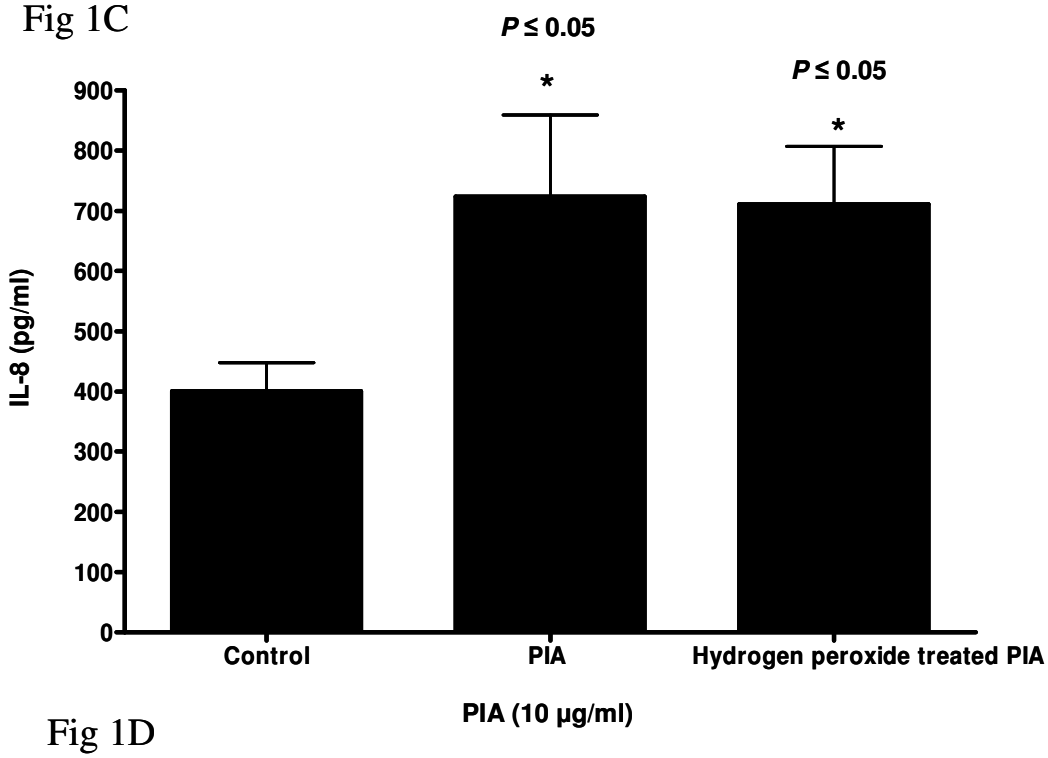
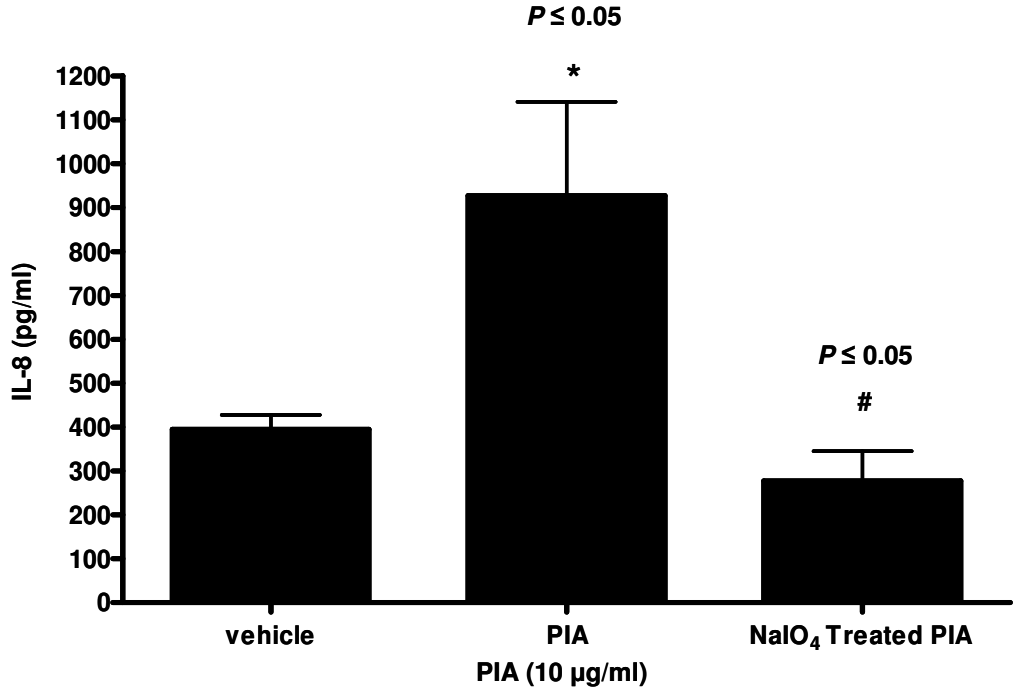


Fig 1D

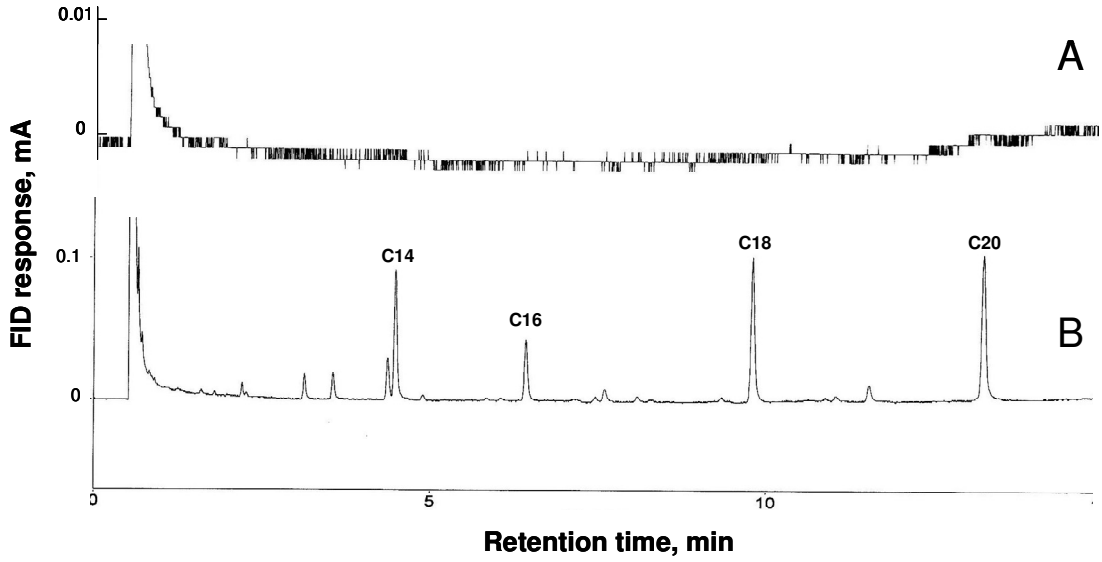


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820

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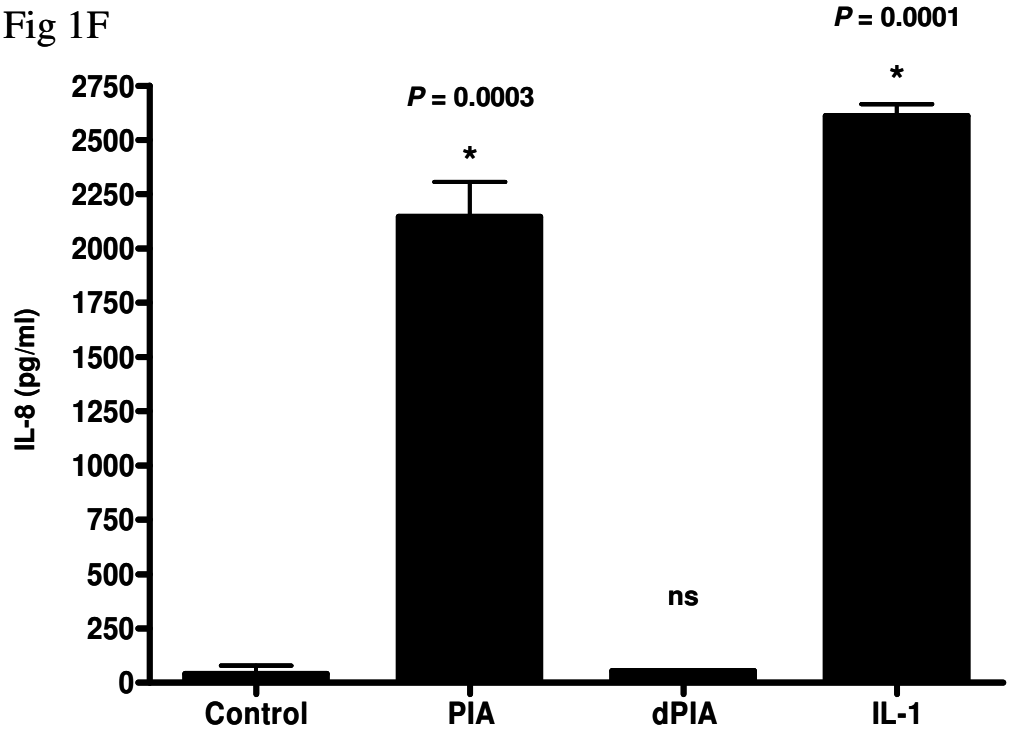
Fig 1E



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Fig 1F



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Fig 2A

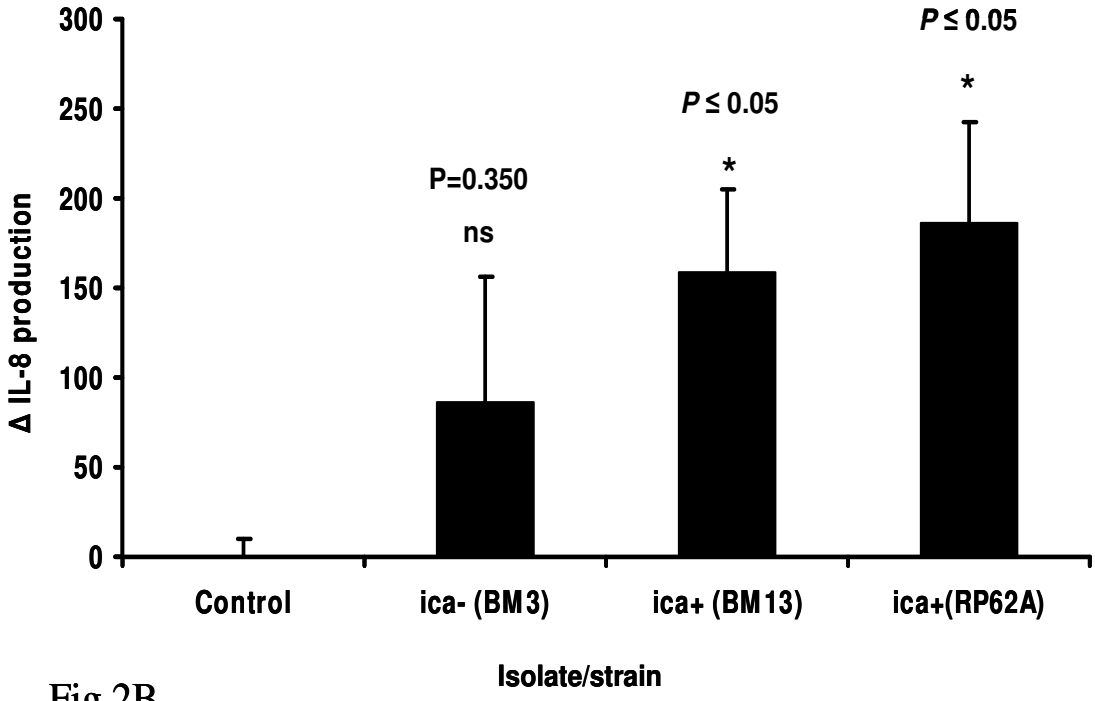
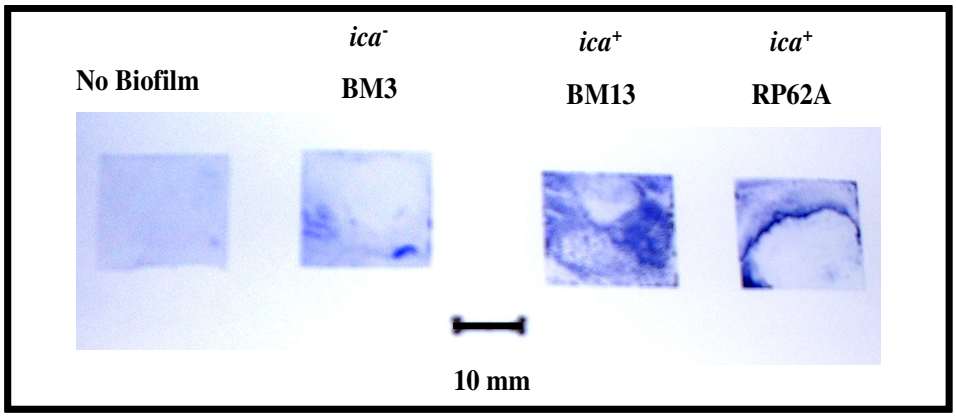


Fig 2B



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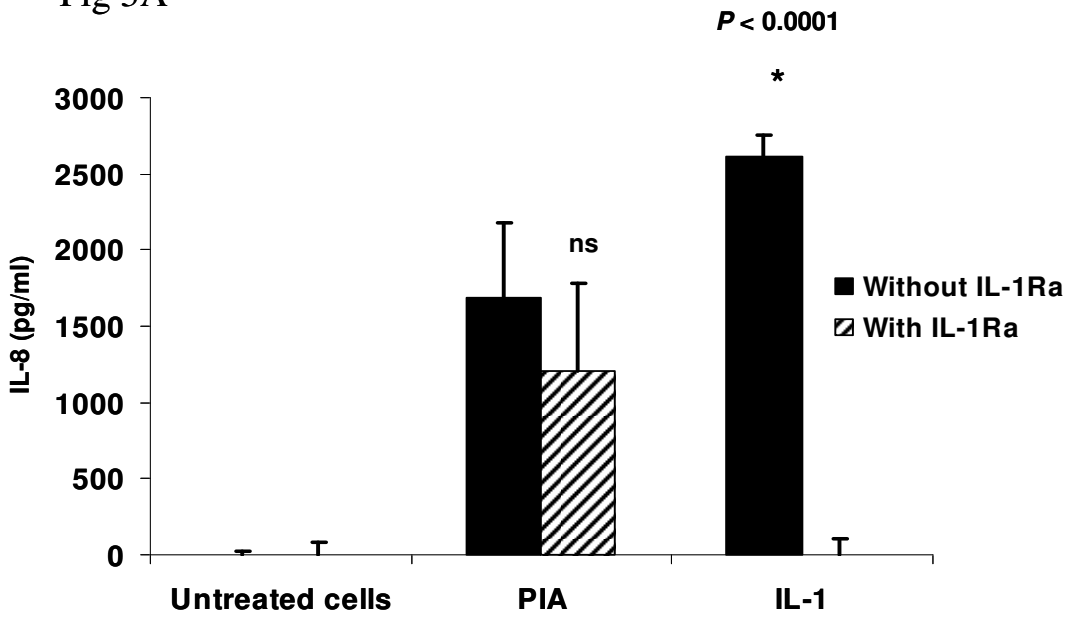
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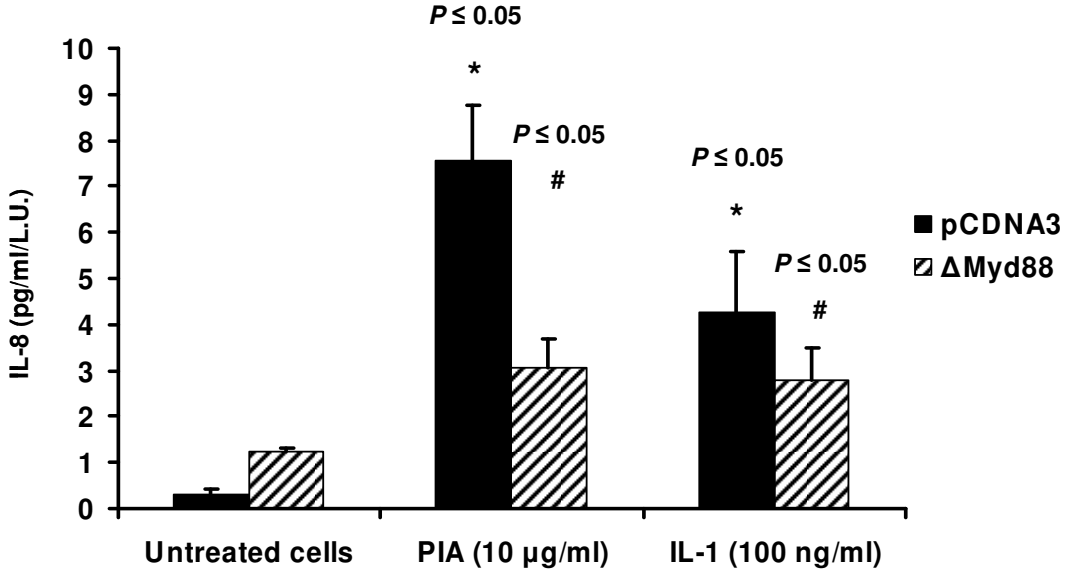
Fig 3A



833

834

Fig 3B

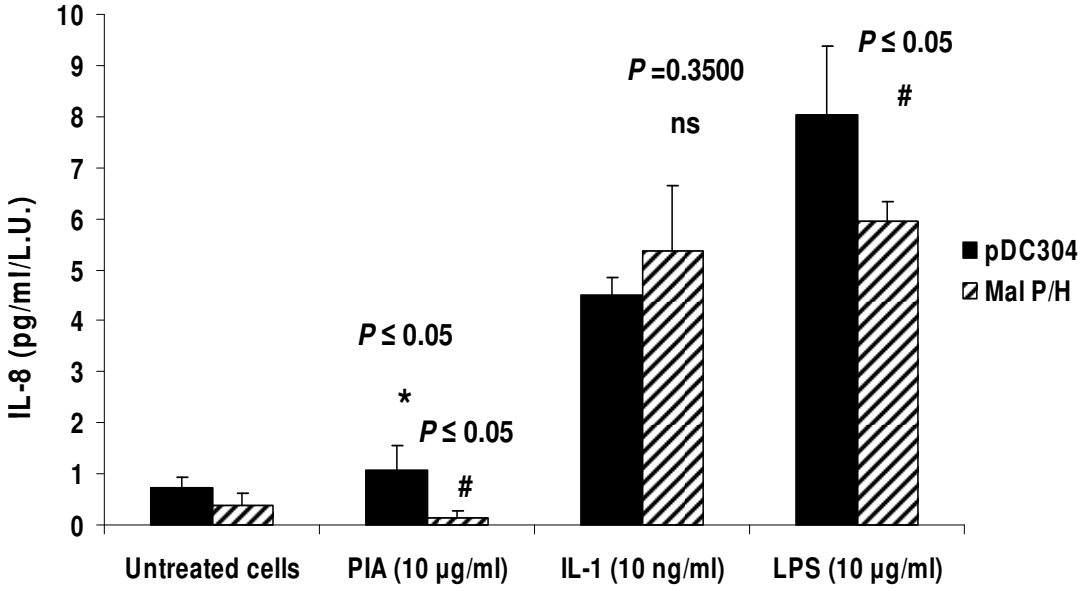


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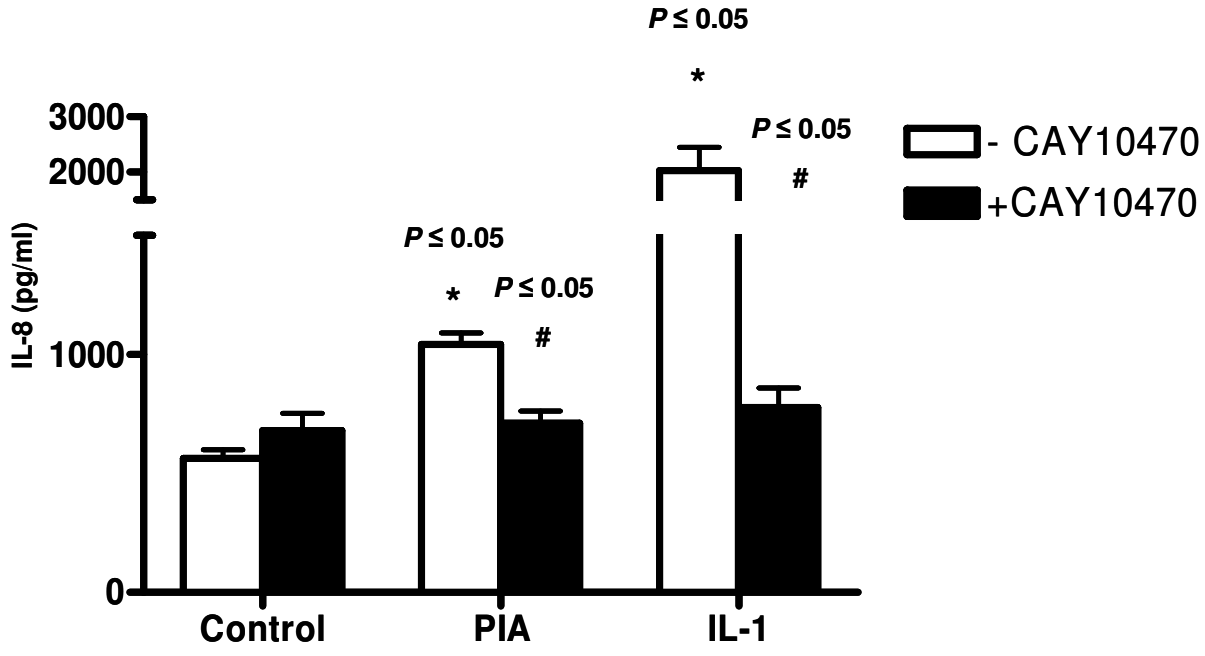
Fig 3C



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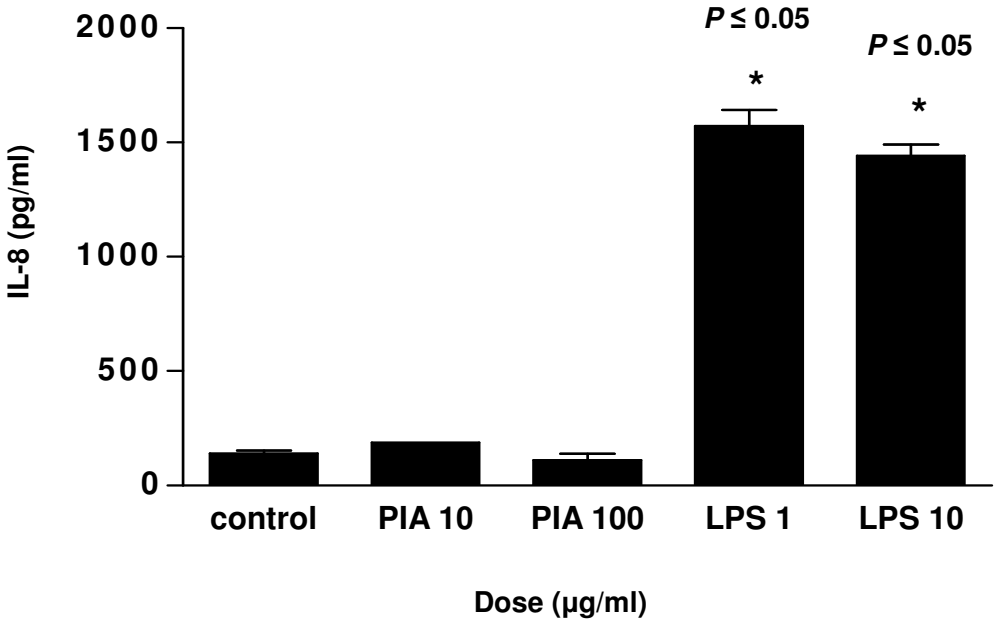
Fig 3D



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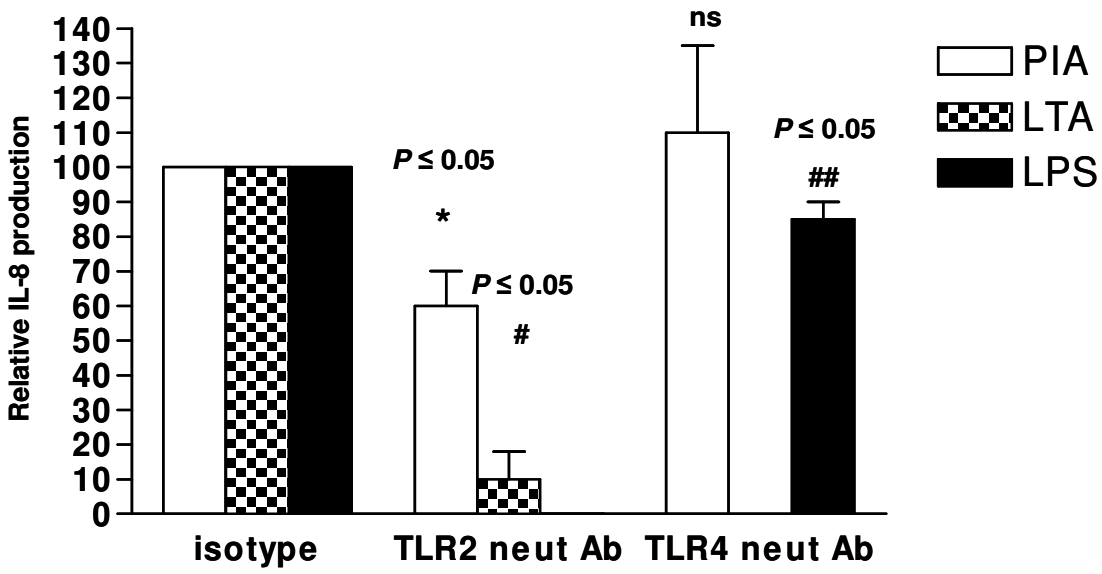
Fig 4A



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843

Fig 4B


Relative IL-8 production
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
P ≤ 0.05
*
P ≤ 0.05
#
ns
P ≤ 0.05
##
isotype
TLR2 neut Ab
TLR4 neut Ab
PIA
LTA
LPS

844

Fig 5A

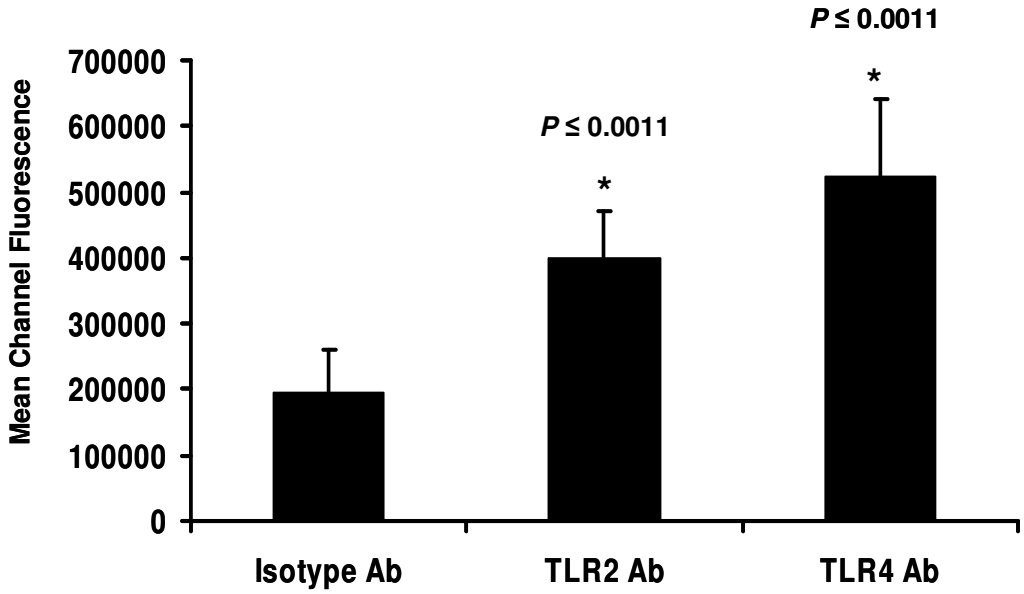
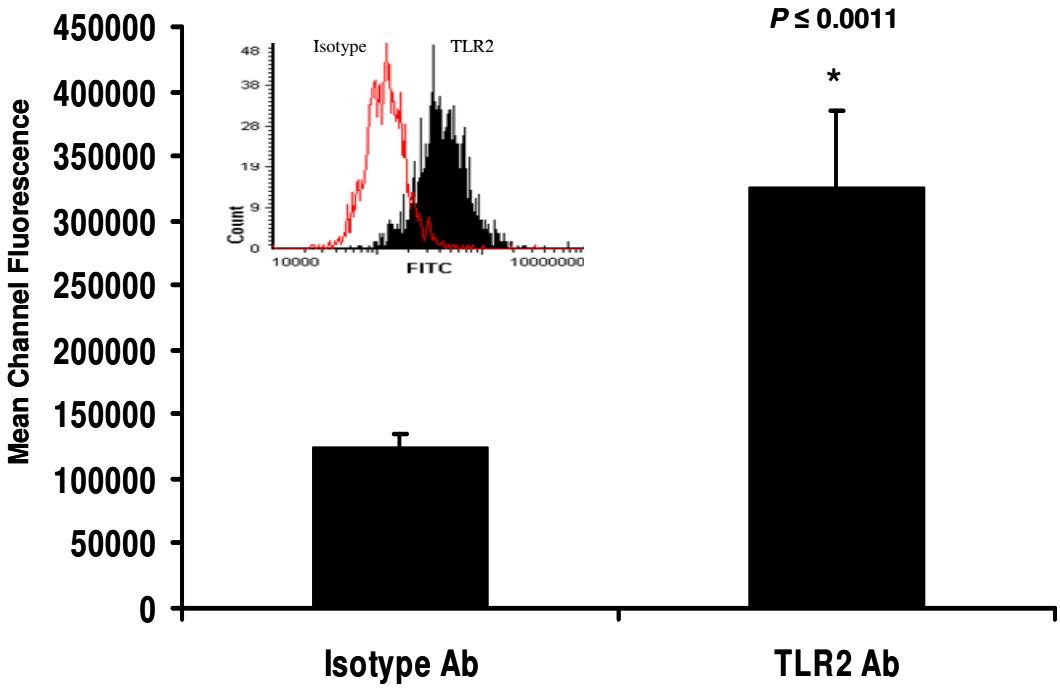


Fig 5B



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846

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Fig 5C

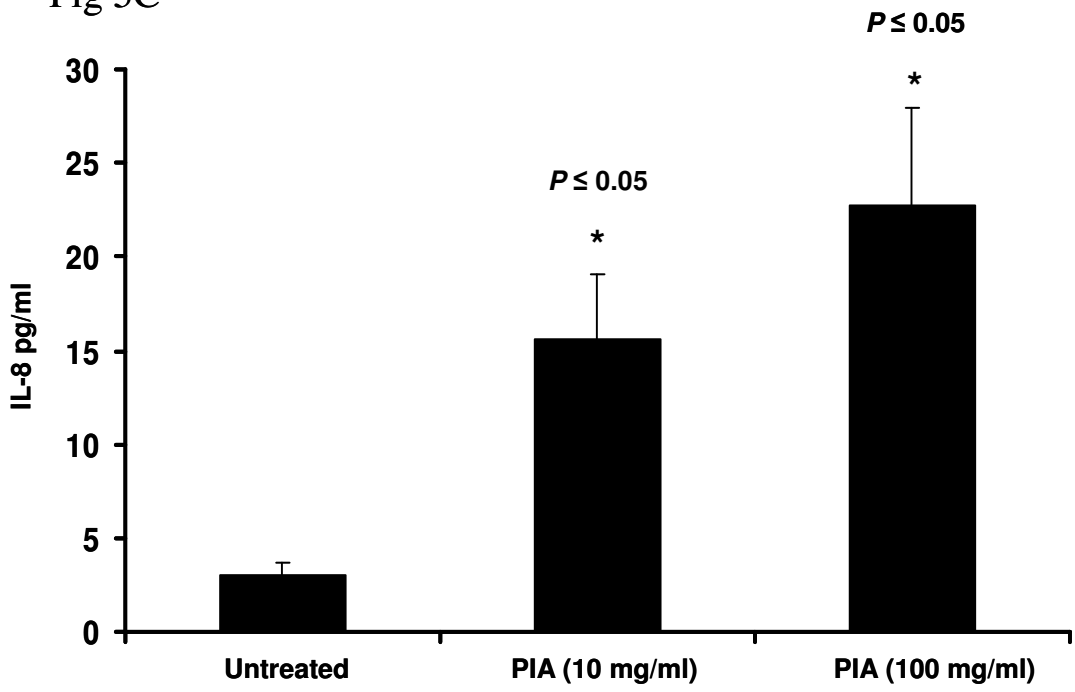

P ≤ 0.05
*
P ≤ 0.05
*
IL-8 pg/ml
30
25
20
15
10
5
0
Untreated
PIA (10 mg/ml)
PIA (100 mg/ml)

Fig 6 A

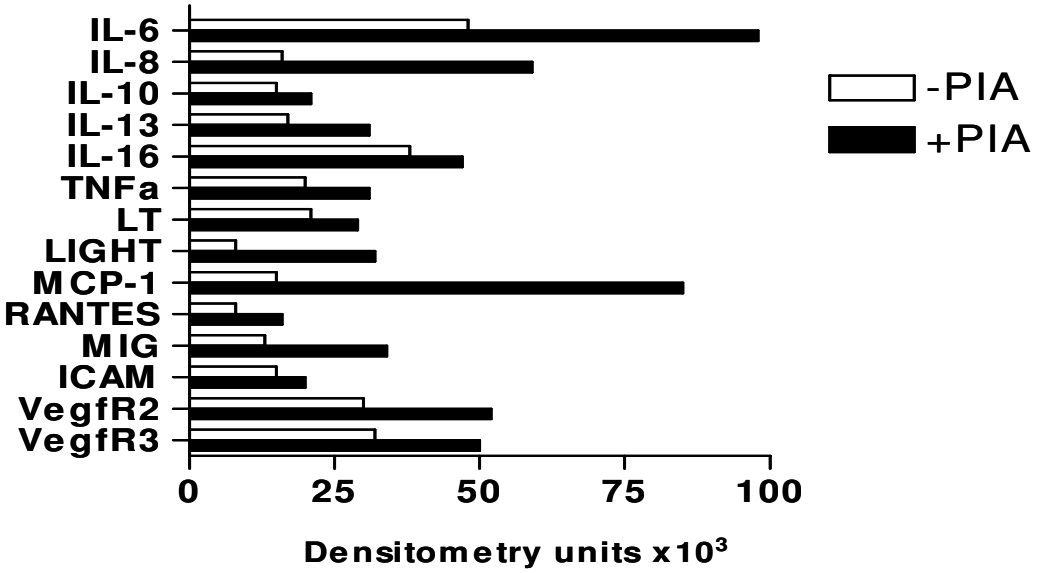
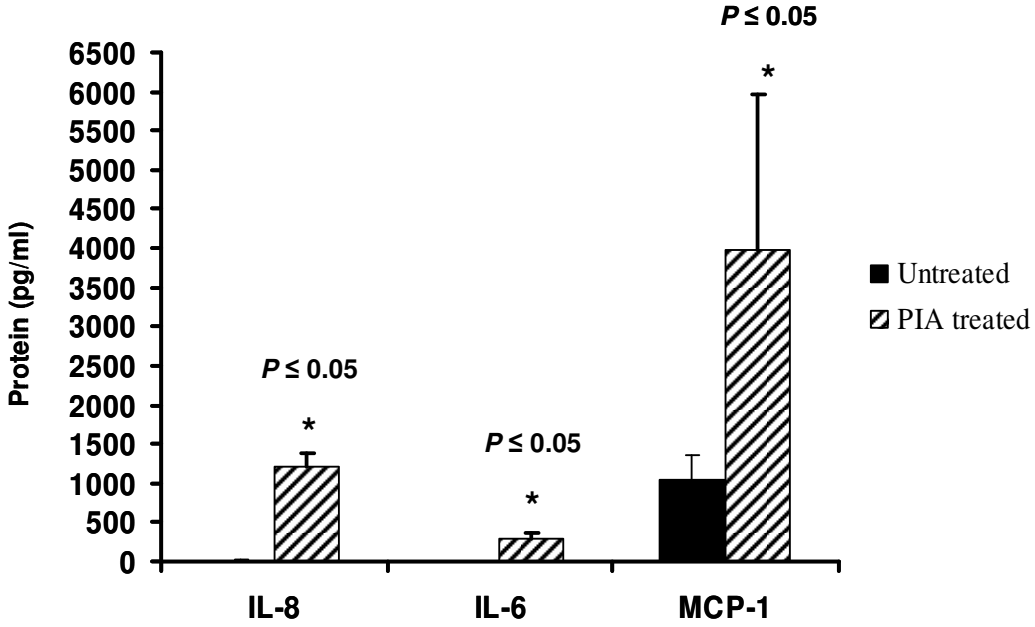


Fig 6B



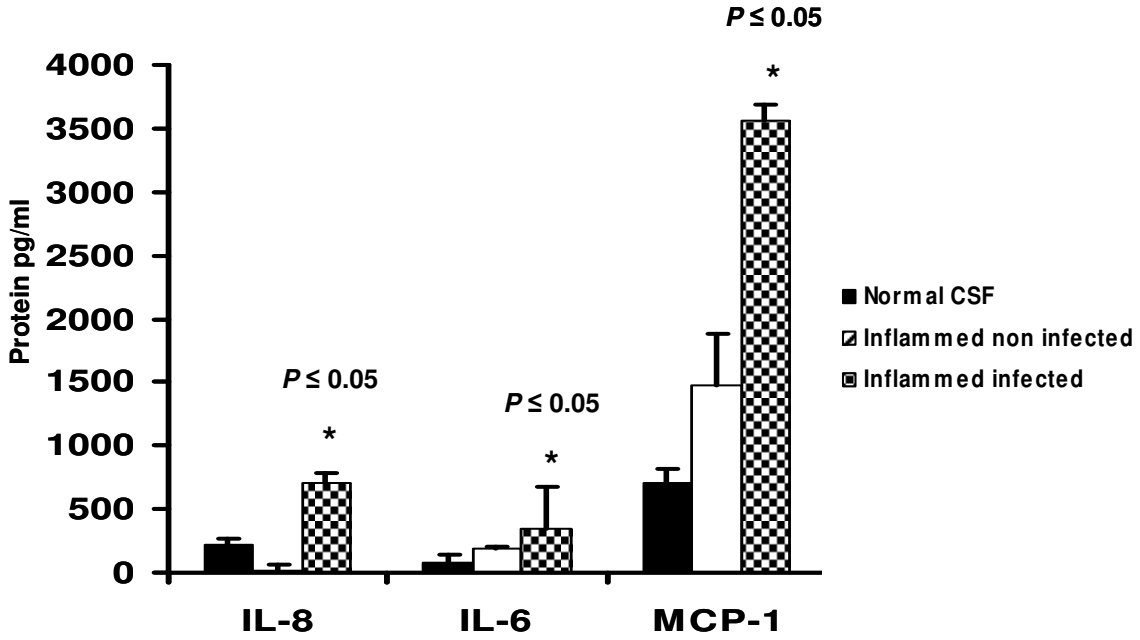
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Fig 6C



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