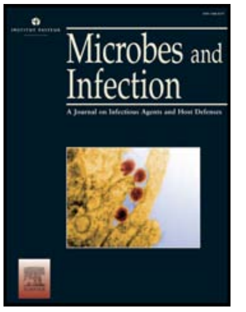
Accepted Manuscript



Surface Layer Proteins Isolated from *Clostridium difficile* Induce Clearance Responses in Macrophages

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PII: S1286-4579(14)00015-X

DOI: 10.1016/j.micinf.2014.02.001

Reference: MICINF 4118

To appear in: *Microbes and Infection*

Received Date: 17 September 2013

Revised Date: 20 December 2013

Accepted Date: 9 February 2014

Surface Layer Proteins Isolated from *Clostridium difficile* Induce Clearance Responses in Macrophages

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Abstract

Clostridium difficile is the leading cause of hospital-acquired diarrhoea worldwide, and if the bacterium is not cleared effectively it can pose a risk of recurrent infections and complications such as colitis, sepsis and death. In this study we demonstrate that surface layer proteins from the one of the most frequently acquired strains of *C. difficile,* activate mechanisms in murine macrophage *in vitro* that are associated with clearance of bacterial infection. Surface layer proteins (SLPs) isolated from *C. difficile* induced the production of pro-inflammatory cytokines and chemokines and increased macrophage migration and phagocytotic activity *in vitro*. Furthermore, we also observed up-regulation of a number of cell surface markers on the macrophage, which are important in pathogen recognition and antigen presentation. The effects of SLPs on macrophages were reversed in the presence of a p38 inhibitor, indicating the potential importance of this signalling protein in how SLP activates the immune system. In conclusion this study shows that surface layer proteins from a common strain of *C. difficile* can activate a clearance response in macrophage and suggests that these proteins are important in clearance of *C. difficile* infection. Understanding how the immune system clears *C. difficile* infection could offer important insights for new treatment strategies.

Keywords

Clostridium difficile; surface layer proteins; macrophage; cytokine; phagocytosis; p38

1. Introduction

Clostridium difficile is a spore-forming, anaerobic Gram-positive bacterium and the leading cause of nosocomial diarrhoea worldwide [1]. Infection usually occurs in hospitalised patients undergoing broad-spectrum antibiotic treatment, where a disruption of the protective microbiota of the gut [2] renders the large intestine more susceptible to colonisation by pathogens. Ingested *C. difficile* spores can then germinate, and cause disease. With the aid of flagella, proteases and other surface proteins, they can penetrate deep into the mucous layer of the intestine [3]. In recent years the emergence of hypervirulent strains has awakened a renewed interest in understanding this pathogen.

The main focus of study in *C. difficile* pathogenesis has long been toxin production. Two main cytotoxic proteins, TcdA and TcdB, are secreted from the bacteria and endocytosed by host cells [4,5], resulting in damage to the epithelial layer of the gut. This induces the production of pro-inflammatory cytokines and migration of immune cells to the site of infection [6]. While the production of these cytotoxic proteins is important in the pathogenesis of *C. difficile* infection, other virulence factors have been shown to play a role. In addition, while much has been investigated about the pathogenesis of *C. difficile*, little is known about the clearance of this pathogen. Investigating mechanisms of clearance is of crucial importance in the understanding of *C. difficile* infection, because many strains of the bacteria are not effectively cleared from the gut. When this occurs, patients may be susceptible to recurrent infection or may develop colitis, which in severe cases can lead to sepsis and death [7].

The Surface Layer Proteins (SLPs) of *C. difficile*, which are considered to act as potential virulence factors, have been generating interest recently. These SLPs coat the entire outer layer of the bacterial cell, are likely a key point of contact between the pathogen and the host immune system and exhibit high variability between strains [8, 9]. Understanding the ability of the host immune system to recognise these proteins is of importance because pathogen recognition is essential for activating the immune system to clear the infection. For example, lipopolysaccharide (LPS), which is present in Gram-negative bacteria, is recognised by toll-like receptor 4 (TLR4) and has been shown to induce the production of chemokines and pro-inflammatory cytokines, to up-regulate phagocytosis and to enhance migration of immune cells to the site of infection [10]. We have recently shown that TLR4 recognises the surface layer proteins of *C. difficile* and that these proteins play a key role in the pathogenesis of *C. difficile* infection, as well as their previously described immuno-regulatory roles [6, 7, 11, 12, 13]. This suggests that SLPs may be capable of activating clearance mechanisms in immune cells, so we wanted to investigate further.

Clearance of pathogens during infection involves immune cells such as macrophage and neutrophils migrating to the site of infection and using specific mechanisms, such as phagocytosis, to effectively clear the pathogen [14]. The role of neutrophils in clearance of *C. difficile* has recently been described [15], but less is known about the role of macrophage in *C. difficile* infection and clearance. The importance of macrophage in clearing infection has been demonstrated previously [16, 17]. Macrophages arrive early to the site of infection and begin production of pro-inflammatory cytokines and chemokines. Secreted chemokines recruit other cells types to the site of the infection, in some cases initiating an adaptive immune response. Macrophages also phagocytose pathogens, which reduces bacterial load and contributes to overall clearance. Indeed it has been shown that Raw 264.7 macrophages can bind and phagocytose *C. difficile* spores. The macrophage prevents these spores from germinating by isolating them from important co-germinants [18]. The recruitment of regulatory T cells, along with the expression of anti-inflammatory cytokines can also help to reduce inflammation and fully resolve infection.

The aim of this study was to examine the effects of SLPs from *C. difficile* on a murine macrophage cell line. Assessing the macrophage responses, including the upregulation of cytokines and chemokines, phagocytosis and migration, may provide important insights in the context of recurring *C. difficile* infections.

2. **Materials and Methods**

2.1 Cell Culture

The J774A.1 murine macrophage cell line was purchased from ECACC and maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) and 2% (v/v) Penicillin-Streptomycin.

2.2 Culture of *C. difficile* and preparation of purified SLPs

C. difficile spores (PCR Ribotype 001; toxin A and B positive; clindamycin resistant; HPA UK reference R13537, Anaerobe Reference Unit, Public Health Laboratory, University Hospital of Wales) isolated from a patient with *C. difficile*-associated disease was used for preparation of SLPs as previously described [11]. Spores were incubated anaerobically for 48 hours on blood agar plates to allow germination and growth of *C. difficile* colonies. A single colony was transferred to BHI/0.05% thioglycolate broth. Cultures were incubated overnight and the S-Layer was removed using 8M Urea/50mM Tris:HCl. This crude extract was dialysed and applied to an anion exchange column attached to an AKTA FPLC system (MonoQ HR 10/10 column, GE Healthcare). Purified SLPs proteins were eluted with a linear gradient of 0–0.3 mol/L NaCl at a flow rate of 4 mL/min. Fractions containing purified SLPs were identified with SDS–PAGE gels stained with Coomassie blue. Pure fractions were combined and assessed for LPS contamination using a *Limulus* amoebocyte lysate (LAL) assay.

2.3 Western Blotting

Protein samples and pre-stained molecular weight markers (Fermentas) were resolved on 12% sodium doecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and quantitatively transferred onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) Bovine Serum Albumin (BSA) in TBST and incubated overnight at 4^{o}C with either antibodies to phospho-p38 (Cell Signalling) or β-actin (Sigma). Membranes were washed and incubated for 2h at room temperature with peroxidase-conjugated anti-mouse or ant-anti rabbit IgG (Sigma) before being developed by chemiluminescence (Millipore). Densitometric analysis was carried out on immunoblots and phospho-p38 is expressed as arbitary units (±SEM) normalised to β-actin. Protein bands were quantified using the GeneSnap acquisition and GeneTools analysis software (GeneGenius Gel Documentation and Analysis System; Syngene).

2.4 Flow Cytometry

J774 macrophage were treated with a JNK inhibitor 420116, (L)-Form, (5μM) (Calbiochem) or p38 inhibitor S8307 (50μM) (Sigma) for 1hour and subsequently stimulated with SLPs (20μg/ml) or positive control LPS (100ng/mL) for 24hours. Macrophage were then washed and stained with specific antibodies for CD14 (eBiosciences) and CD80 (BD) and isotype matched controls. Post 30-minute incubation at 4^{o}C cells were washed and immunofluorescence analysis performed on a FACSAria. Data was analysed using FlowJo Software (Treestar, San Carlos, CA).

2.5 ELISA

J774 macrophage were stimulated with SLPs (20µg/mL) or positive control LPS (100ng/mL), for 24hours. Cells were treated with inhibitors to JNK and p38 for 1 hour. Culture supernatants were removed and stored at -80°C until further analysis. IL-1β, IL-6, IL-12p40, TNFα, MIP-1α, MIP-2 and MCP concentrations in cell culture were analysed by DuoSet ELISA Kits (R&D Systems) according to manufacturer's instructions.

2.6 Migration Assay

J774 macrophage cells were treated for 24 hours with LPS 100ng/mL or SLPs 20µg/mL, counted and plated on Transwell® insert. In the bottom well 600µL of media with/or without chemoattractant, IL-2 and GM-CSF, was placed and cells were left for 4 hours. The Transwell insert was then removed and cells were scrapped and transferred to Eppendorf tubes, followed by centrifugation at 1200 rpm for 5 minutes. The supernatant was removed and pellet was resuspended in 200µL 4% (v/v) formaldehyde/PBS, transferred to FACs tubes and read for 60 seconds on the FACS Aria.

2.7 Phagocytosis Assay

J774 macrophage cells were treated with a JNK inhibitor 420116, (L)-Form, (5µM) (Calbiochem) or p38 inhibitor S8307 (50µM) for 1hour. Cells were then stimulated with SLPs 20µg/mL for 12 hours. Cells were stimulated with LPS 100ng/mL as a positive control. Subsequently 0.5 x 10^6 FITC-labelled latex fluorescent beads (Sigma Aldrich, L4655) were added to all cells. At 0hr and 12hr post-incubation, all cells were washed in FACS buffer, centrifuged, resuspended and analysed by FACS Aria.

2.8 Statistics

One-way analysis of variance (ANOVA) was used to determine significant differences between conditions. When this indicated significance ($p<0.05$), post-hoc Student-Newmann-Keul test analysis was used to determine which conditions were significantly different from each other.

3. Results

3.1 SLPs induce the production of inflammatory cytokines and chemokines by macrophage

Cytokines play an important role in bacterial clearance and chemokines are involved in the recruitment of other cells of the immune system. Given that macrophage represent an important source of both of these immune mediators, we examined their secretion by macrophage in response to SLPs and compared to LPS as a control. Activation of macrophage with either SLPs or LPS induced significant secretion of the cytokines IL-1β, IL-6, TNF-α and IL-12p40 as well as increasing production of the chemokines MIP-1α, MIP-2 and MCP (Figure 1). Indeed the levels of IL-6 and MIP-2 were higher in response to SLPs than to LPS.

3.2 SLPs up-regulate cell surface marker expression on macrophage

Following activation of macrophage there is an increased expression of a number of cell surface markers. These markers are important for interaction with other cells of the immune system, recognition of pathogens and presentation of antigen. Therefore we assessed whether SLPs could induce the expression of TLR2, TLR4, CD40, CD80, CD14 and MHCII and compared this to LPS. Figure 2a demonstrates that the expression of the pathogen recognition receptors TLR2 and TLR4 and their associated co-receptor CD14 were up-regulated following activation of macrophage by SLPs. Furthermore, SLPs also induced an increase in expression of the co-stimulation markers CD40 and CD80 and upregulated MHCII expression. The effect of SLPs on these surface markers was comparable to that of LPS on the same cells.

3.3 Macrophage migration is induced in the presence of SLPs

An important element of bacterial clearance is migration of immune cells, such as macrophage, to the site of infection. Therefore we assessed the effect of SLPs on macrophage migration to chemotactic factors. Macrophage plated in a Transwell plate migrated from the higher chamber to the lower chambers which contained the chemotactic cytokines IL-2 and GM-CSF. This migration was enhanced following exposure of the macrophages to SLPs (Figure 2b). This increase in migration was similar to that of macrophage exposed to LPS, which is known to induce macrophage migration.

3.4 Cytokine and chemokine secretion in response to SLPs is reversed in the presence of a p38 inhibitor but not a JNK inhibitor

We have previously reported that SLPs mediate their effects through TLR4 and have similar immunostimulatory effects to that of LPS. Activation of TLR4 results in subsequent activation of NFκB and the MAPK pathways JNK and p38. In order to assess the potential involvement of these pathways in the effect of SLPs on macrophage activation, we included a p38 and JNK inhibitor in our experiments.

Figure 3 demonstrates that in the presence of a p38 inhibitor, secretion of the cytokines IL-6, IL-12p40 and TNFα was significantly reversed. Furthermore, the presence of the p38 inhibitor significantly suppressed the ability of SLPs to induce macrophage to secrete the chemokines MIP-2 and MCP (Figure 3). These effects were not evident in the presence of a JNK inhibitor (Figure 3).

3.5 SLPs induce phagocytosis in macrophage via a p38-dependant mechanism

Activated macrophages phagocytose particles, such as bacteria, which are then destroyed internally. This is an essential mechanism for clearance of bacterial infections. We examined the effect of SLPs on the ability of macrophage to phagocytose fluorescent latex particles and whether p38 played a role in this. Figure 4 demonstrates that macrophage stimulated with SLPs phagocytose at a higher rate than un-stimulated macrophages. Furthermore, we demonstrate that this effect was significantly reduced in the presence of a p38 inhibitor, but not a JNK inhibitor.

3.6 SLP-induced expression of CD14 and CD80 on macrophage is mediated by p38

As previously shown in Figure 2, SLPs up-regulate important surface markers in a similar manner to LPS. Figure 5 again shows that exposure of macrophages to SLPs increases the expression of CD80 and CD14. The addition of a p38 inhibitor blocked the SLP-induced increase in both CD80 and CD14, an effect that was not seen in the presence of a JNK inhibitor (Figure 5).

3.7 SLPs induce the phosphorylation of p38 in macrophage

Given our data, which demonstrates a role for p38 in SLP-induced macrophage activation, we examined the expression of phosphorlated p38 in macrophage in response to SLP exposure. Figure 6 demonstrates that treatment of macrophage with

SLPs resulted in increased phosphorylation of p38 after 10mins, 30mins and 60mins. LPS was used as a positive control given that it is known to induce p38 phosphorylation.

4. **Discussion**

In this study we demonstrate that SLPs isolated from *C. difficile* ribotype PR 001 activate key mechanisms in macrophage that are important in bacterial clearance. Exposing a murine macrophage cell line to *C. difficile* SLPs induced the macrophage to produce pro-inflammatory cytokines and chemokines, to increase expression of pathogen recognition receptors and other cell surface markers and to increase phagocytosis and migration activity. We further showed that the effects of SLPs on macrophage are mediated by the MAP kinase p38, which is activated downstream of TLR4.

The production of pro-inflammatory cytokines is crucial for bacterial clearance. We show that SLPs from *C. difficile* induced comparable levels of cytokine production by macrophage to that induced by LPS. SLPs stimulated macrophage to produce high levels of IL-12p40, TNFα, MCP and MIP-1 in a similar manner to LPS. Indeed, SLPs induced IL-1ß, IL-6 and MIP-2 more potently than LPS. These cytokines are of importance in fighting bacterial infection. It has been previously shown that IL-1ß is a key cytokine in inducing fever and it can lead to local tissue destruction. IL-1ß also plays a role in adaptive immunity, enhancing differentiation and expansion of $CD4^+$ T cells [19]. Specifically, it is involved in differentiation of $CD4^+$ T cells into T_H17 cells, which are involved in bacterial clearance [20]. Indeed, we have previously shown SLP-activated dendritic cells are strong drivers of both T_H1 and T_H17 responses [11]. IL-1ß has also been shown to be critical in bacterial infection and clearance *in vivo* [21]. IL-1ß-deficient mice infected with *Staphylococcus aureus* developed large skin lesions, high bacterial counts and impaired neutrophil recruitment. This same deficiency was not observed in IL-1α-knockout or wild-type mice. In addition, recombinant IL-1ß injected into IL-1ß knockout mice reversed the detrimental effects [21]. IL-1ß also promotes the production of IL-6 and MCP-1 [22], both of which we found to be up-regulated in this study.

We also demonstrate that *C. difficile* SLPs up-regulate another important inflammatory mediator, TNFα. This pro-inflammatory cytokine can act on blood vessels, increasing their permeability and adhesiveness to leukocytes. This allows for an influx of fluid and immune cells to the site of infection, and it is this increased permeability and fluid accumulation that contributes to the severe diarrhoea observed in *C. difficile*-infected patients. TNFα also acts on dendritic cells, promoting migration to the lymph nodes where an adaptive immune response can be initiated. The role of TNFα in bacterial clearance has been previously shown *in vivo* [23]. TNFα-knockout mice infected with mycobacteria exhibited high bacterial load and succumbed to infection within 40 days. Furthermore, knockout mice injected with moderate levels of TNFα-secreting recombinant *Mycobacterium bovis* BCG survived, displaying controlled bacterial growth and no enlargement of the spleen [23].

The induction of IL-12p40 by SLPs further suggests a role for them in clearance of *C. difficile*. IL-12p40 drives differentiation of naïve $CD4^+$ T cells into a T_H1 subset. These T_H1 cells can then augment the activity of macrophage in a positive feedback loop, secreting IFNγ and IL-2, which act on macrophage to increase phagocytosis and cytokine production. T_H1 cells can also induce IgG production, which contributes to clearance of infection [24]. IL-12p40-knockout mice have previously been shown to be unable to clear *Francisella tularensis* [25]. While the animals survived, they exhibited chronic infection. T cells from wild type mice produced large amounts of

IFNγ, and this was impaired in p40-knockout animals, indicating a role for the p40 subunit in clearance of infection.

We have previously observed up-regulation of important cell surface molecules, such as MHC II, CD40 and TLR4, in DCs in response to SLPs [11]. In this study we see that macrophages respond to SLPs in a similar manner. TLR2, CD40, CD14 and MHCII were expressed at levels comparable to LPS stimulation. This is not surprising, because both SLPs and LPS signal through TLR4. Indeed we have previously shown that TLR4-knockout mice do not effectively clear *C. difficile* infection [11]. Up-regulation of MHCII is important because the molecule is necessary for displaying antigen to $CD4^+$ T cells. Up-regulation of the co-stimulatory molecule CD40 again indicates a role for SLPs in the clearance of *C. difficile* because CD40L on T helper cells can bind to CD40 on macrophage, further activating them to clear pathogens [26]. This suggests that good recognition of SLPs through TLR4 and a subsequent strong immune response is important for clearance. Interestingly there is evidence that the sequence coding for SLPs is different between strains of *C. difficile*, therefore the host response to SLPs from these strains may be weak in some cases, or indeed individual that are immunocompromised may not mount a strong immune response via TLR4 which also may impact on clearance, so these factors should be considered along the ones assessed in this study.

Phagocytosis and cell migration are crucial components of bacterial clearance in the gut. Bacterial load can be reduced by macrophage engulfing bacterial cells into phagosomes, where they are destroyed [27]. We show that stimulation of macrophage with SLPs increased the rate of phagocytosis relative to control cells, indicating that they may be involved in the initiation of clearance mechanisms during *C. difficile* infection. Impaired phagocytosis has been observed in monocyte-derived macrophage in patients with chronic obstructive pulmonary disease [28] in response to common airway pathogens. Reduced phagocytosis of *Staphylococcus pneumoniae* and *Haemophilus influenzae* was observed in diseased patients relative to healthy individuals, implying an important role for phagocytosis in bacterial clearance.

Many studies have also shown the interaction of macrophage and *Clostridium perfringes*, a similar anaerobic pathogen to *C. difficile* that infects the gut [29, 30, 31]. Pathogens can inhibit or modulate the responses of macrophage in the gut to avoid clearance. *Yersinia enterocolitica*, a gram-negative bacteria, interacts with gut macrophage through Yersinia outer proteins (Yops). These outer proteins can act on the actin cytoskeleton of the macrophage, and dephosphorylate proteins involved in focal adhesion, thereby inhibiting phagocytosis [32]. YopP can inhibit phosphorylation of MAPK and NF-κB pathways, inducing apoptosis in macrophage, and contributing to systemic infection [33]. This suppression of macrophage activity in the gut is crucial for successful infection, indicating the necessity of an appropriate macrophage response in clearing gastrointestinal pathogens. Recruitment of neutrophils to the site of infection has been previously shown to be of importance in the clearance of *C. difficile* [15]. Following epithelial damage by *C. difficile* toxins, neutrophils migrating to the lamina propria can provide a barrier against *C. difficile* invading deeper [15]. Interestingly, neutrophil recruitment does not reduce *C. difficile* load in the gut, but appears to prevent systemic dissemination of the intestinal bacteria. The authors did not assess the role of macrophage in their study and our findings suggest that macrophage may have a role in clearance of the pathogen.

We show that SLP-activated macrophage migrated towards the chemotactic cytokines IL-2 and GM-CSF. Migration of immune cells to the site of infection is a key process in the immune response and helps to facilitate bacterial clearance [34]. We show that cells stimulated with SLPs migrate at a greater rate than control cells, suggesting that contact with *C. difficile* enhances the ability of macrophage to migrate to the site of infection. We also show that SLPs induce the production of MIP-1α, MIP-2 and MCP chemokines, signifying a strong role for SLPs in recruiting immune cells to the site of infection. This influx of cells may then increase their rate of phagocytosis, and boost bacterial clearance. These chemokines play a significant role in clearance, because a knockout model of MCP chemokine receptor CCR2 resulted in loss of macrophage recruitment, and inability to clear *Listeria monocytogenes* [35].

P38 is a downstream component of the TLR4 signalling pathway, with TLR4 activation resulting in its phosphorylation and activation [36]. We have previously shown that activation of p38 is important in SLP-induced maturation of DCs [11]. We now report that inhibition of p38 reversed the effects of SLPs on macrophage. We show that IL-6, IL-12p40, TNFα, MIP-2 and MCP production is inhibited in the presence of a p38 inhibitor. We see similar results for cell surface molecules CD14 and CD80, and phagocytosis is also down-regulated in the presence of a p38 inhibitor. We also show that direct phosphorylation of p38 is induced following SLPs-stimulation, with levels of phosopho-p38 increasing over time. This further indicates that SLPs activates macrophage through TLR4. Interestingly, inhibition of JNK, a MAP kinase downstream of TLR4, had no effect on SLP-induced cytokine production. This suggests further specificity in SLP activation of macrophage. Interestingly, previous studies have shown inhibition of p38 can result in an increase in pro-inflammatory cytokine production, yet reduces bacterial clearance [37]. Mice infected with *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* were unable to clear infection following p38 inhibition.

To conclude, this study demonstrates that SLPs from *C. difficile* stimulate key mechanisms in macrophage, which are important for bacterial clearance. The isolated SLPs activated a murine macrophage cell line to produce pro-inflammatory cytokines and chemokines, to increase surface marker expression and to enhance phagocytosis and migration. This strongly suggests a role for macrophage in *C. difficile* clearance during infection and supports our previously published findings that SLPs are important in the recognition of *C. difficile* and in induction of the immune response to the bacterium. Given the continuing challenge that this pathogen poses, understanding how *C. difficile* activates the immune response for efficient bacterial clearance may ultimately provide insights for novel therapies to improve clearance in patients and prevent reoccurrence of infection.

Acknowledgements

The authors acknowledge funding provided under Science Foundation Ireland Research Frontiers Programme and under the Programme for Research in Third Level Institutions (PRTLI) Cycle 4. The PRTLI is co-funded through the European Regional Development Fund (ERDF), part of the European Union Structural Funds Programme 2007–2013. We thank Dr Claire O'Connell for help with manuscript preparation.

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Figure Legends

Figure 1. Pro-inflammatory cytokines and chemokines are secreted by J774 macrophage in response to SLPs. J774 macrophage were stimulated with 20µg/mL SLPs (ribotype 001) and positive control 100ng/ml LPS for 24 hours. Supernatants were then harvested and assessed for levels of IL-1β, IL-6, IL-12p40, TNF-α, MIP-1α, MIP-2 and MCP using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 and *p<0.05 comparing CTL/LPS and CTL/SLP groups as determined by one way ANOVA test.

Figure 2. (A) J774 macrophage up-regulate expression of surface markers in response to SLPs. Cells were stimulated with 20µg/mL SLPs and positive control 100ng/mL LPS for 24 hours. Cells were then stained with specific antibodies for TLR2, TLR4, CD40, CD80, CD14 and MHCII and analysed using flow cytometry. Control macrophage (grey filled histogram) vs LPS 100ng/mL (pink line) and SLPs 20µg/mL (blue dashed line). ***(B) Migration of J774 macrophage is induced in the***

presence of SLPs. J774 macrophage were stimulated with 20µg/mL SLPs and positive control 100ng/mL LPS for 12 hours. Cells were plated at $1x10^5$ in the top chamber of a Transwell® plate with 0.5µg/mL GM-CSF and two units of IL-2 in the lower chamber. After 4 hours the cells that had migrated into the lower chamber were counted by flow cytometry for 60 seconds.

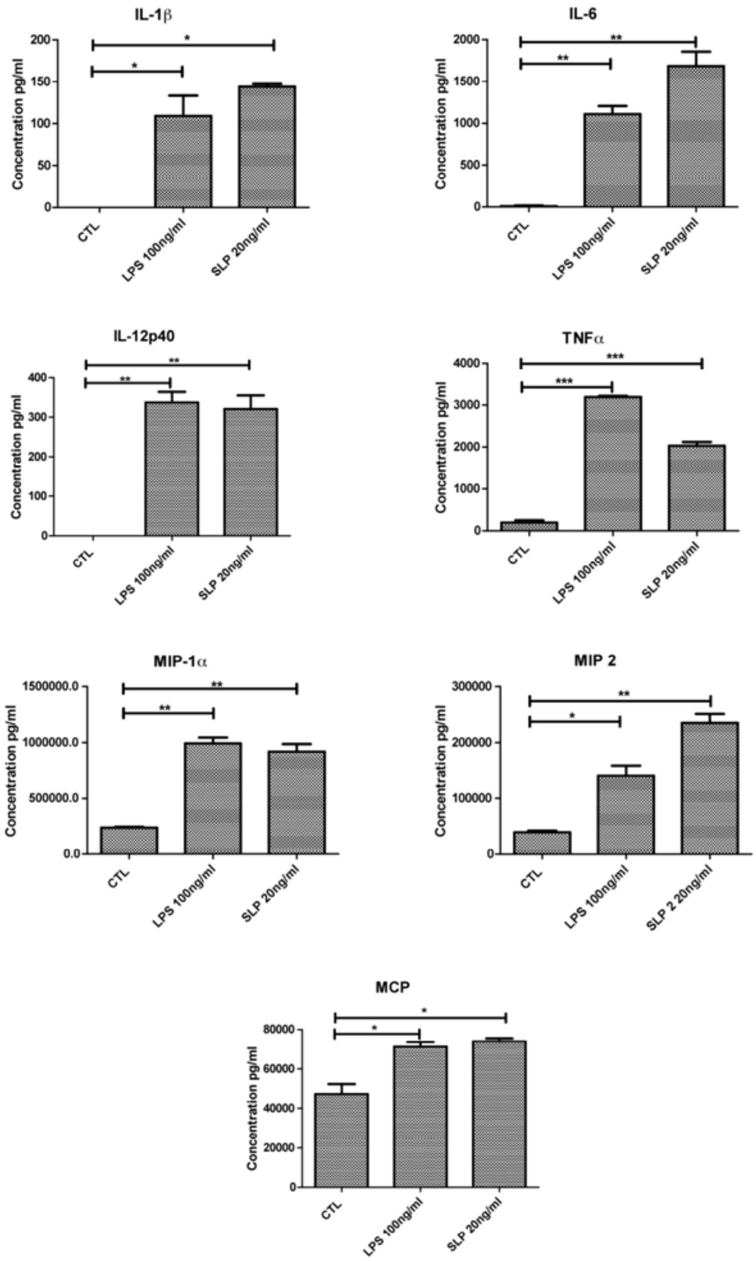
Figure 3. Cytokine and chemokine production in response to SLPs is reversed in the presence of a p38 inhibitor. 50µM p38 inhibitor and 5µM JNK inhibitor were added to J774 macrophage 1 hour prior to 24-hour stimulation with 20µg/mL SLPs (ribotype 001). Supernatants were then harvested and assessed for levels of IL-6, IL-12p40, TNF-α, MIP-2 and MCP using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 and *p<0.05 comparing CTL/SLP vs SLP/SLP/SLP+p38 groups as determined by one way ANOVA test.

Figure 4. SLPs induce phagocytosis in macrophage via a p38-dependant mechanism. 50µM p38 inhibitor and 5µM JNK inhibitor were added to J774 macrophage 1 hour prior to 20µg/mL SLPs (ribotype 001) for 12 hours. Cells were then exposed to fluorescent latex beads for 12 hours, harvested and analysed using FACS Aria.

Figure 5: SLP-induced expression of CD14 and CD80 on J774 macrophage is reversed in the presence of a

***p38 inhibitor*.** 50µM p38 inhibitor and 5µM JNK inhibitor were added to J774 macrophage 1 hour prior to 20µg/mL SLPs (ribotype 001) for 24 hours. Cells were then stained with specific antibodies for CD14 and CD80 using flow cytometry. Control macrophage (grey filled histogram) vs. SLP+/- inhibitors 20µg/mL (blue line).

Figure 6: Phospho-p38 is induced following SLP activation.(A) J774 macrophage were stimulated with 20µg/mL SLPs (ribotype 001) and positive control 100ng/mL LPS for 10-60 mins. Cells were then lysed and immunoblotted for phospho-p38. ß-actin was used as a loading control. (B) Representative immunoblots of 3 experiments are shown and densitometric analysis was carried out on immunoblots and phospho-p38 is expressed as arbitary units (±SEM) normalised to β-actin.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IL-6
IL-12p40
TNFα
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MCP
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CTL
LPS 100ng/ml
SLP 20ng/ml
SLP 2 20ng/ml
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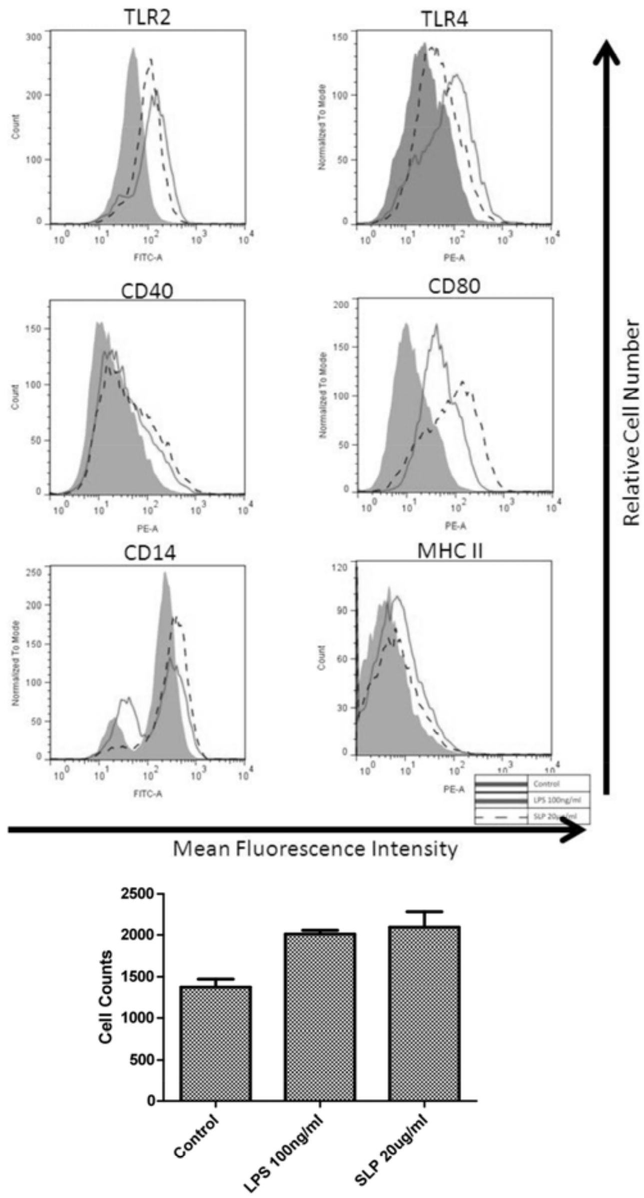
A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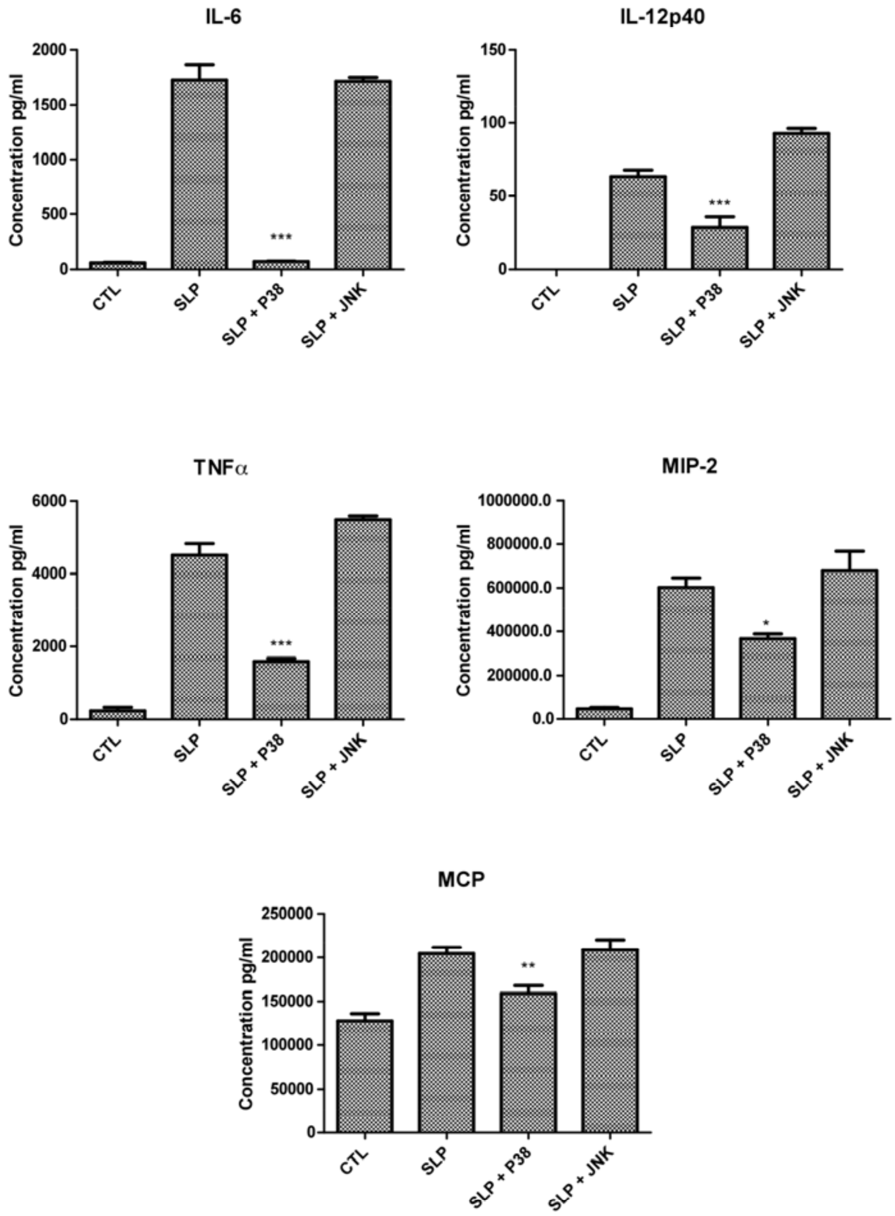
TLR4
CD40
CD80
CD14
MHC II
Count
Normalized To Mode
FITC-A
PE-A
Control
LPS 100ng/ml
Relative Cell Number
Mean Fluorescence Intensity
Cell Counts
Control
LPS 100ng/ml
SLP 20ug/ml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1500
1000
500
0
***
CTL
SLP
SLP + P38
SLP + JNK
IL-12p40
Concentration pg/ml
150
100
50
0
***
CTL
SLP
SLP + P38
SLP + JNK
TNFα
Concentration pg/ml
6000
4000
2000
0
***
CTL
SLP
SLP + P38
SLP + JNK
MIP-2
Concentration pg/ml
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800000.0
600000.0
400000.0
200000.0
0.0
*
CTL
SLP
SLP + P38
SLP + JNK
MCP
Concentration pg/ml
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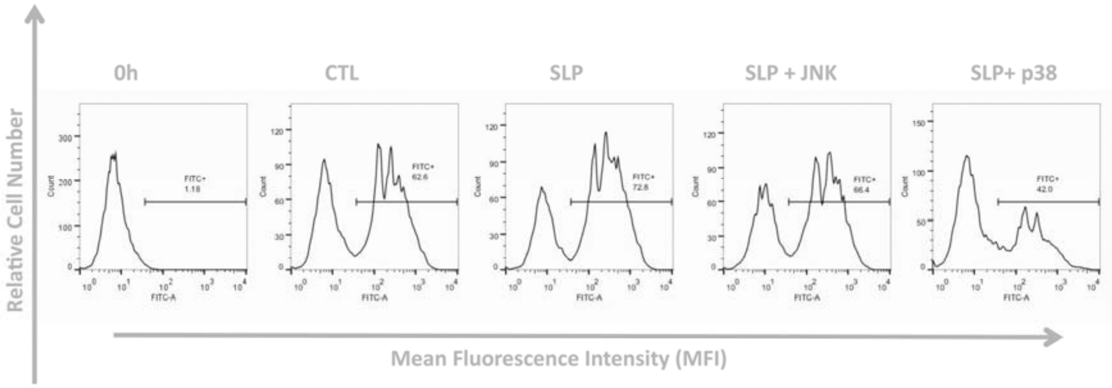
50000
0
**
CTL
SLP
SLP + P38
SLP + JNK


0h
CTL
SLP
SLP + JNK
SLP+ p38
Relative Cell Number
Mean Fluorescence Intensity (MFI)
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FITC+
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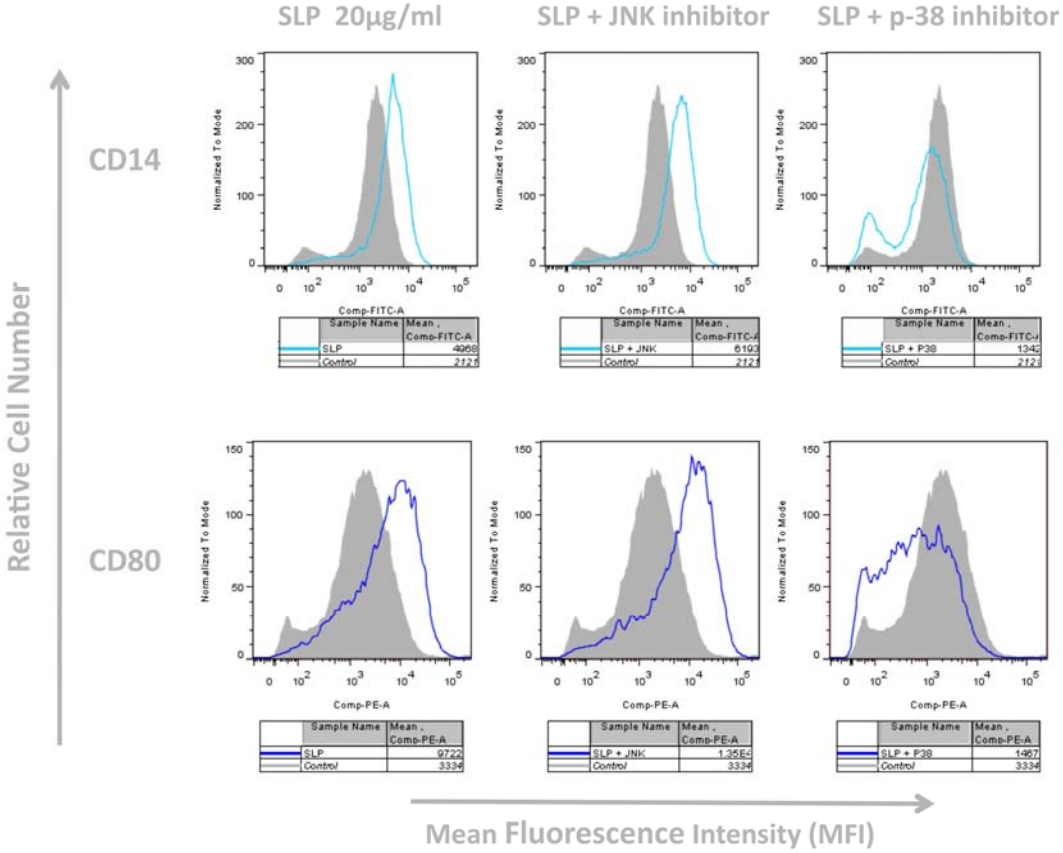
FITC+
72.8
FITC+
66.4
FITC+
42.0
Count
FITC-A


SLP 20µg/ml
SLP + JNK inhibitor
SLP + p-38 inhibitor
CD14
CD80
Relative Cell Number
Mean Fluorescence Intensity (MFI)
Normalized To Mode
Comp-FITC-A
Comp-PE-A
Sample Name
SLP
SLP + JNK
SLP + P38
Control
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1342
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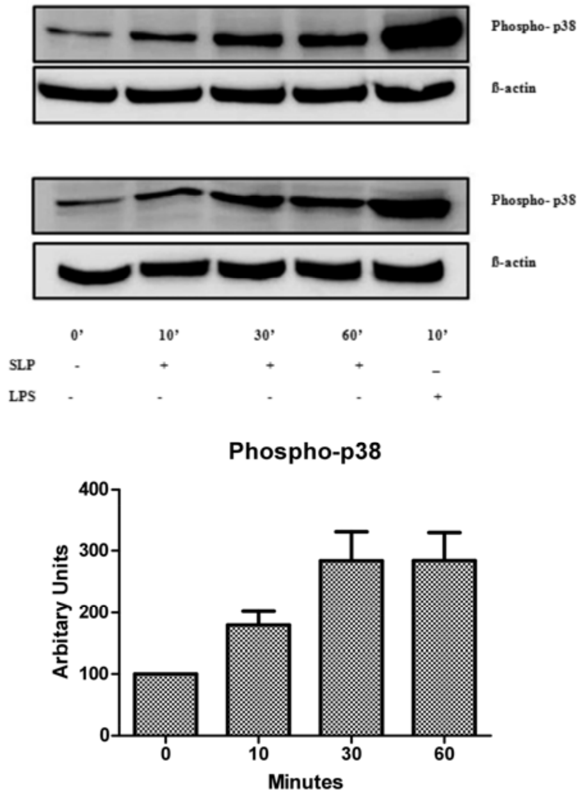
1.35E4
1467
3334


Phospho- p38
ß-actin
Phospho- p38
ß-actin
0' 10' 30' 60' 10'
SLP - + + + _
LPS - - - - +
Phospho-p38
Arbitary Units
400
300
200
100
0
0 10 30 60
Minutes