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N-formyl peptides drive mitochondrial damage associated molecular pattern induced neutrophil activation through ERK1/2 and P38 MAP Kinase signalling pathways.

Jon Hazeldine[1*], Peter Hampson[1,2*], Francis Adusei Opoku[3], Mark Foster[3] and Janet. M. Lord[1].

[1]NIHR Surgical Reconstruction and Microbiology Research Centre, School of Immunity and Infection, Birmingham University Medical School, Birmingham, B15 2TT.
[2]Healing Foundation Centre for Burns Research, Queen Elizabeth Hospital, Birmingham, B15 2WB, UK.
[3]Royal Centre for Defence Medicine, Birmingham Research Park, Birmingham, B15 2SQ, UK.

* These authors contributed equally to this manuscript.

Running title: MtDAMP-induced neutrophil activation requires MAPK signalling

Key Words: Neutrophils, Mitochondrial damage associated molecular patterns, trauma, Mitogen activated protein kinases.

Summary

Traumatic injury results in a systemic inflammatory response syndrome (SIRS), a phenomenon characterised by the release of pro-inflammatory cytokines into the circulation and immune cell activation. Released from necrotic cells as a result of tissue damage, damage associated molecular patterns (DAMPs) are thought to initiate the SIRS response by activating circulating immune cells through surface expressed pathogen recognition receptors. Neutrophils, the most abundant leukocyte in human circulation, are heavily implicated in the initial immune response to traumatic injury and have been shown to elicit a robust functional response to DAMP stimulation.

Here, we confirm that mitochondrial DAMPs (mtDAMPs) are potent activators of human neutrophils and show for the first time that signalling through the mitogen-activated-protein-kinases p38 and Extracellular-signal-related-kinase 1/2 (ERK1/2) is essential for this response. At 40 and/or 100 μg/ml, mtDAMPs activated human neutrophils, indicated by a significant reduction in the surface expression of L-selectin, and triggered a number of functional responses from both resting and Tumour necrosis factor-α primed neutrophils, which included reactive oxygen species (ROS) generation, degranulation, secretion of interleukin-8 and activation of p38 and ERK1/2 MAPKs. Pre-treatment of neutrophils with Cyclosporin H, a selective inhibitor of Formyl Peptide Receptor-1 (FPR-1), significantly inhibited mtDAMP-induced L-selectin shedding as well as p38 and ERK1/2 activation, suggesting that *N*-formyl peptides are the main constituents driving mtDAMP-induced neutrophil activation. Indeed, no evidence of L-selectin shedding or p38 and ERK1/2 activation was observed in neutrophils challenged with mitochondrial DNA alone. Interestingly, pharmacological inhibition of p38 or ERK1/2 either alone or in combination significantly inhibited L-selectin shedding and IL-8 secretion by mtDAMP-challenged

neutrophils, revealing for the first time that MAPK activation is required for mtDAMP-induced neutrophil activation and function.

Our findings demonstrate that signalling through FPR-1 and activation of p38 and ERK1/2 MAPKs are key events in mtDAMP-induced neutrophil activation. Gaining an understanding of the signalling pathways involved in mtDAMP-induced neutrophil activation may assist in the development of future therapeutic strategies aimed at targeting the SIRS response to improve the outcome of the hospitalised trauma patient. Reducing the severity of the inflammatory response may realise substantial benefits for the severely injured trauma patient.

Introduction

Ranking in the top ten causes of death worldwide, traumatic injury accounts for nearly six million fatalities per year [1]. Whilst recent advancements in medical care, notably those targeting blood loss and coagulopathy, have markedly reduced mortality rates that are due to trauma, the development of secondary complications, such as acute respiratory distress syndrome (ARDS), sepsis and multiple organ failure (MOF) remains a significant cause of morbidity and mortality in the hospitalised trauma patient. Common to the development of these and other post-traumatic complications is an overt systemic inflammatory response. Termed the systemic inflammatory response syndrome (SIRS), this phenomenon is characterised by a robust pro-inflammatory cytokine and chemokine response, activation of the complement system and immune dysregulation [2-4].

In a seminal paper published in 2010, Zhang and colleagues described a potential mechanistic explanation for how tissue damage arising from traumatic injury initiates a SIRS response [5]. The group showed for the first time that severe injury in humans leads to the release of endogenous damage-associated molecular patterns (DAMPs) from ruptured mitochondria [5], an observation that has since been confirmed by numerous independent groups [6-8]. *In vitro*, these so called mitochondrial DAMPs (mtDAMPs), which include mitochondrial DNA (mtDNA) and formyl peptides, trigger robust functional responses from neutrophils, such as degranulation, chemotaxis and secretion of the chemokine interleukin (IL)-8, all of which were accompanied by activation of the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 [5]. Furthermore, the study showed that injecting mtDAMPS into mice triggered an *in vivo* SIRS response, characterised by amongst other things, “priming” of circulating neutrophils and neutrophil-mediated organ damage [5]. Thus, these results along with those of other studies [9-11], suggest that the

systemic inflammation observed in severely injured patients may be attributable in part to mtDAMP-induced activation of circulating neutrophils [10].

MtDAMPs are a collection of molecules, which include mtDNA, cytochrome C, formyl peptides and ATP [12]. Pre-treating neutrophils with either chloroquine, an inhibitor of toll-like receptor 9, which recognises mtDNA, or cyclopsorin H (CsH), a potent and selective formyl peptide receptor (FPR) antagonist has been shown to significantly reduce mtDAMP-induced MAPK activation, calcium signalling and degranulation [9;11], suggesting that mtDNA and formyl peptides are the main instigators driving mtDAMP-induced activation of neutrophils. ATP on the other hand has been shown in an *in vivo* model of sterile injury to assist in neutrophil recruitment to the site of tissue damage by activating signalling pathways in endothelium that initiate neutrophil adhesion [13].

Although it is well established that exposing neutrophils to mtDAMPS induces a multitude of functional responses and activates the MAPK signalling cascade [5;9-11], these aspects have only ever been studied in isolation. Thus, the primary aim of this study was to determine whether activation of the MAPKs ERK 1/2 and/or P38 is required for the functional responses elicited by neutrophils following mtDAMP stimulation. In addition, we investigated which of the two prominent constituents of mtDAMPS, namely mtDNA and *N*-formyl peptides, are primarily responsible for mtDAMP-induced neutrophil activation.

Results

MtDAMPs are potent activators of human neutrophils and stimulate robust functional responses

Shedding of L-selectin, a receptor that facilitates the initial interaction of circulating neutrophils with the endothelium, from the surface of neutrophils is considered a marker of neutrophil activation [14]. When compared to untreated controls, we found that freshly isolated human neutrophils exposed to 40 or 100 μg/ml mtDAMPs expressed significantly less L-selectin on their surface, with this reduction occurring as early as five minutes post treatment (Figure 1A). In addition to surface density, a significant decline in the percentage of neutrophils that expressed L-selectin was observed following a ten minute exposure to mtDAMPs (Figure 1A). Thus, mtDAMPs are potent activators of human neutrophils.

In addition to activating resting neutrophils, treatment with mtDAMPs induced robust functional responses. In agreement with observations made in previous studies [5;9-11], we found that exposure to 40 and/or 100 μg/ml of mtDAMPs resulted in the rapid generation of ROS (Figure 1B), secretion of the chemokine IL-8 (Figure 1C) and degranulation (Figure 1D).

MtDAMP stimulation activates ERK1/2 and p38 signalling in human neutrophils

To investigate the effect of mtDAMP treatment on the activation kinetics of the MAPKs p38 and ERK1/2, we treated neutrophils with 40 or 100 μg/ml mtDAMPs over a 10-minute period, after which, activation was measured by assessing phosphorylation using phosphospecific antibodies and Western blotting. At each concentration, mtDAMPs led to a rapid activation of ERK1/2 (Figure 2A) and p38 (Figure 2B) MAPK, with a significant increase in phosphorylation detected for both MAPK within two minutes of stimulation. This activation was more prolonged in response to 100 μg/ml mtDAMPs with a significant

increase in MAPK phosphorylation still detectable ten minutes after stimulation. In contrast, phosphorylation of both kinases had returned to baseline levels within five minutes of stimulation with 40 µg/ml mtDAMPs.

Formyl peptides but not mtDNA activate and induce functional responses in resting neutrophils

The term mtDAMPs describes a collection of molecules, which includes proteins, lipids and DNA. In the seminal paper that first described an immune stimulatory capacity of mtDAMPs, mtDNA was highlighted as a potent activator of neutrophil function [5]. However, we found no evidence of neutrophil activation or function when freshly isolated human neutrophils were challenged with mtDNA (Figure 3). When compared to untreated controls, neutrophils treated with 1 or 5 µg/ml mtDNA did not differ in their expression of L-selectin, when measured as either mean fluorescent intensity, an indicator of surface density, or as the percentage of L-selectin positive cells (Figure 3A). Furthermore, we observed no activation of either P38 or ERK1/2 MAPK signalling in neutrophils exposed to mtDNA over a ten-minute time course (Figure 3B). However, phosphorylation of both kinases was observed in neutrophils treated with 100 µg/ml mtDAMPs over the same time course (Figure 3A and 3B).

In contrast to mtDNA, we found formyl peptides present within mtDAMPs to be potent activators of human neutrophils. When compared to their untreated counterparts, neutrophils pre-treated with CsH, a selective antagonist of formyl peptide receptor 1, expressed significantly greater amounts of L-selectin on their surface (Figure 4A) and a greater percentage of CsH-treated neutrophils retained L-selectin on their surface at the end of a sixty-minute time course. In terms of a functional response, preventing formyl peptide receptor signalling markedly reduced the activation kinetics of both P38 and ERK1/2 MAPK (Figure 4B).

ERK1/2 and p38 signalling is crucial for mtDAMP-induced neutrophil activation and function

The data presented so far has confirmed the findings of previous studies by demonstrating that exposure to mtDAMPs, in particular formyl peptides, elicits robust functional responses from neutrophils and activates MAPK signalling [5;9]. However, whether the activation of the MAPKs ERK1/2 and/or p38 is required for mtDAMP-induced neutrophil activation and function has not been determined. To address this issue, we treated freshly isolated neutrophils with 10 μM of the ERK inhibitor PD098059 and/or the p38 inhibitor SB202190 prior to mtDAMP stimulation.

Inhibition of ERK1/2 or p38 signalling alone partially reduced mtDAMP-induced neutrophil activation. This is demonstrated in Figure 5A, which shows significantly greater amounts of L-selectin was retained on the surface of SB202190 or PD098059-treated neutrophils following stimulation with 100 μg/ml mtDAMPs when compared to untreated controls (Figure 5A). Similar results were obtained when the percentage of L-selectin positive neutrophils was measured (data not shown). However, when used in combination, pre-treatment with ERK1/2 and P38 inhibitors completely abrogated the reduction in L-selectin expression that we observed from the surface of untreated neutrophils following exposure to 100 μg/ml mtDAMPs (Figure 5A).

In line with the results for neutrophil activation, inhibition of MAPK signalling also reduced the functional response of neutrophils when challenged with mtDAMPs. We found that when compared to their untreated counterparts, neutrophils that had been pre-treated with PD098059 or SB202190 either alone or in combination secreted significantly less IL-8 following stimulation with 100 μg/ml mtDAMPs (Figure 5B). Thus, both p38 and ERK1/2

activation is crucial not only for mtDAMP-induced activation of neutrophils but also their functional response.

Discussion

Traumatic injury leads to a systemic inflammatory response syndrome (SIRS), which is characterised by the release of pro-inflammatory cytokines and immune-dysregulation. Damage-associated molecular patterns released from necrotic tissue are thought to initiate the SIRS response via their binding to pattern recognition receptors (PRRs) on immune cells. Lam and colleagues were the first to demonstrate that mitochondrial derived DAMPs were released into the circulation following trauma, showing that plasma mitochondrial DNA (mtDNA) levels were thousands fold higher in trauma patients when compared to healthy controls [15]. Since then, numerous studies have shown elevated mtDNA levels in trauma patients [5;6;8;16]. In addition, post-trauma plasma levels of mtDNA have been associated with post-traumatic complications including SIRS and sepsis [6;8], as well as mortality [16]. The group of Hauser were the first to demonstrate a link between mtDAMPs and the initiation of the SIRS response by showing that mtDAMPs not only serve as chemoattractants for neutrophils but also stimulate IL-8 production and MMP-8 release by these cells. Moreover, intravenous mtDAMP injection into rats resulted in neutrophil lung infiltration and tissue damage. Following cellular necrosis, mitochondria release a range of mtDAMPs including mtDNA, *N*-formyl peptides, ATP, cytochrome-c and mitochondrial lipids. Despite the association between tissue damage, elevation of mtDAMPs and SIRS, the precise mechanism of mtDAMP-induced neutrophil activation has been under studied.

Here, we have confirmed that mtDAMPs are potent activators of human neutrophils. At concentrations of 40 and 100 µg/ml, mtDAMPs elicited a range of functional responses from resting or TNF-α primed neutrophils, which included L-selectin shedding, ROS production, degranulation and IL-8 release. This activation was abrogated in the presence of CsH, a potent and selective FPR-1 antagonist, suggesting a key role for mitochondrial derived *N*-formyl peptides in mtDAMP-induced neutrophil activation. Indeed, we found no evidence

of neutrophil activation (as measured by shedding of L-selectin) or function (induction of MAPK signalling) when these cells were treated with mtDNA alone. This latter observation is in contrast to the findings of earlier studies that have demonstrated robust MAPK activation in neutrophils following mtDNA stimulation [5;11]. The results are however in line with those of Crouser et al [17] who showed that stimulation of monocytes with mtDAMPs led to a significant increase in IL-8 secretion, a response that was not altered by the removal of mtDNA from the mtDAMP preparation [17]. Moreover, Sun et al [18] demonstrated that mtDAMPs could induce endothelial cell permeability and that this could be abolished by protease treatment of the mtDAMPs, implicating a predominant role for mitochondrial proteins in driving this permeability response [18].

Whilst the contradictory findings outlined above raise questions as to whether mtDNA is directly involved in driving neutrophil activation following sterile injury, evidence is accumulating that suggests circulating levels of mtDNA are an indicator of injury severity [6;15] and can be linked to patient outcome [6-8;16]. Regarding this latter point, significantly higher concentrations of mtDNA have been measured in plasma samples taken from traumatically injured subjects upon hospital admission who subsequently succumb to their injuries when compared to those who survive the insult [7;8]. Besides mortality, elevated levels of plasma mtDNA have been linked to the onset of multiple organ dysfunction syndrome [7] and shown to be an independent predictor for the development of a SIRS response following acute traumatic injury [6].

In addition to *N*-formyl peptides, mitochondria contain a number of other proteins that can act as mtDAMPs when released into the circulation. For example, numerous studies have demonstrated extracellular ATP to have immunomodulatory properties. In particular, the work of Kubes and colleagues, in a murine model of sterile injury, has shown extracellular ATP to have a role in neutrophil recruitment via P2X7R signalling and Nlrp3 inflammasome

activation [13]. Furthermore, a recent study has demonstrated that human neutrophils can be directly activated by ATP via the P2Xl receptor [19]. It has also been demonstrated that serum cytochrome-c levels are elevated in patients with SIRS and that high levels were associated with the development of multi-organ failure [20], thus suggesting a possible link between serum cytochrome-c levels and systemic inflammation. Interestingly, intra-articular injection of cytochrome-c has been shown to induce arthritis in mice, an effect that was abrogated by depletion of neutrophils [21]. Studies such as these raise the possibility of a link between mitochondrial proteins (other than *N*-formyl peptides) and the initiation of the SIRS response via neutrophil activation.

The precise signalling mechanisms by which mtDAMPs induce neutrophil activation have not previously been fully characterised. The group of Hauser showed that mtDAMPs lead to the activation of p38 and ERK1/2 MAPK signalling pathways in neutrophils [5;9]. However, the requirement of these pathways in mtDAMP induced neutrophil activation was not determined. Here, we show that the activation of these signalling pathways is required for mtDAMP induced neutrophil activation. Firstly, we showed that mtDAMP treatment, but not mtDNA treatment, led to the activation of p38 and ERK1/2 MAPK signalling pathways in neutrophils. Importantly, pharmacological inhibition of these pathways prevented mtDAMP induced L-selectin shedding and IL-8 release. Furthermore, blocking of the FPR-1 receptor using CsH, not only prevented mtDAMP induced p38 and ERK1/2 activation, a finding in line with previously published data [9], but also inhibited L-selectin shedding. To the best of our knowledge, these data demonstrate for the first time that p38 and ERK1/2 signalling downstream of the FPR-1 receptor is crucial for mtDAMP-induced neutrophil activation, thereby implicating extracellular *N*-formyl peptides as key initiators in the systemic inflammatory response to sterile injury. This is important as it offers a therapeutic possibility to dampen down an inappropriate SIRS response in trauma patients.

In a recent article, Fernandez-Ruiz and colleagues showed that isolated human monocytes pre-treated with mtDAMPs generated significantly less IL-1β, IL-6, IL-12p70 and TNF-α upon lipopolysaccharide challenge when compared to their untreated counterparts [22]. This observation of mtDAMP-induced endotoxin tolerance led the group to speculate that the refractory state of monocytes observed in many "sterile" pathologies may result in part from their *in vivo* exposure to mtDAMPs [22]. Whether this phenomenon also applies to neutrophils is to our knowledge currently unknown. However, it is of interest that in studies where impaired neutrophil function has been reported post trauma, the presence of exhausted neutrophils in the circulation has been proposed as a possible mechanism by which to explain the functional aberrations reported [23;24]. Thus, given the results presented in this paper combined with those of others [5;9-11], it is plausible to suggest that exposure to mtDAMPs may be responsible in part for the hyperactive state of neutrophils that is observed in the immediate aftermath of traumatic injury as well as the refractory state of neutrophils that has been described in the days following injury. Initial mtDAMP exposure would activate circulating neutrophils, triggering a multitude of functional responses such as ROS generation and degranulation, which would result in a circulating pool of exhausted neutrophils that are refractory to subsequent stimulation. Thus, by influencing the function of circulating neutrophils, the generation of and exposure to mtDAMPs may be one factor underlying the increased susceptibility of the hospitalised trauma patient to nosocomial infection.

Materials and methods

Preparation of mitochondrial DNA (mtDNA) and mitochondrial damage associated molecular patterns (mtDAMPs)

MtDNA and MtDAMPs were obtained from mitochondria that had been isolated from the K562 tumour cell line (ATCC®, Teddington, Middlesex, UK). To isolate mitochondria, K562 cells were treated for 10 minutes on ice with 1X RIPA lysis buffer (Upstate Biotechnology, New York, USA) supplemented with protease inhibitor cocktail (1:100; Sigma-Aldrich, Dorset, UK) prior to being subjected to 120 strokes in a tight fitting dounce homogeniser. Once lysed, cells were centrifuged at 800 x g for 10 minutes at 4^0C to pellet nuclear material. Supernatants were then spun at 3000 x g for 30 minutes at 4^0C to pellet mitochondria.

MtDNA was extracted from mitochondrial pellets using a QIAamp® DNA blood mini kit according to manufacturer's instructions (Qiagen, Manchester, UK). The concentration and purity of isolated MtDNA was determined by spectrophotometry (Nanodrop 2000; Thermo Scientific, MA, USA). For purity measurements, the A260/A280 ratio was routinely 1.8-2.0, indicating no significant contamination by mitochondrial proteins. Once isolated, mtDNA was stored at -80^{o}C prior to use. To prepare mtDAMPs, isolated mitochondrial pellets, re-suspended in 1 ml of Hank's balanced salt solution (Gibco, Life Technologies, Paisley, UK) containing protease inhibitor cocktail (1:100; Sigma-Aldrich) were disrupted in a sonicating water bath for 12 minutes. Post treatment, samples were centrifuged at 15,000 x g for 10 minutes at 4^0C. Supernatants containing mtDAMPS were collected and protein concentration determined by spectrophotometry. Samples were stored at -80^{o}C prior to use.

Neutrophil Isolation and treatment

Peripheral blood neutrophils were isolated by Percoll density gradient centrifugation. Neutrophil purity and viability was assessed by Giemsa staining (Diff-Qik; Gentaur Europe, Brussels, Belgium) and trypan blue (Sigma-Aldrich) staining respectively. Routinely, the purity of neutrophil preparations and cell viability were ≥98%. Following isolation, neutrophils were re-suspended at a concentration of 1 or $5x10^6$ /ml in RPMI-1640 media supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (Sigma-Aldrich; hereafter referred to as complete medium) or Hank's balanced salt solution (HBSS) supplemented with calcium and magnesium (hereafter referred to as $HBSS^{+/+}$; Gibco, Life Technologies).

To assess the importance of mitogen activated protein kinase (MAPK) signalling in the functional response of neutrophils to mtDAMP stimulation, neutrophils were treated for 1 hour (37^oC, 5% CO_2) with 10 µM of the P38 inhibitor SB203580 (Cell Signalling Technology) and/or 10 µM of the ERK 1/2 inhibitor PD98059 (Cell Signalling Technology) or vehicle control prior to treatment with mtDAMPS. To inhibit signalling through formyl peptide receptors, freshly isolated neutrophils were treated with 1 uM cyclosporin H (Abcam, Cambridge, UK) for 1 hour at 37^oC in a humidified 5% CO_2 atmosphere prior to mtDAMP stimulation.

Measurement of L-selectin expression

$2x10^5$ freshly isolated neutrophils and those pre-treated with 10 µM SB203580 and/or 10 µM PD98059 or 1uM cyclosporin H were stimulated for 0, 5, 10, 30 and 60 minutes at 37^oC in a humidified 5% CO_2 atmosphere with either 1 or 5 µg/ml of mtDNA or 40 µg/ml or 100 µg/ml of mtDAMPs. Post treatment, cells were pelleted (250 x g, 5 minutes, 4^0C), supernatants discarded and neutrophils stained with 10 µg/ml of a mouse anti-human L-selectin-FITC conjugated antibody (Clone Dreg56; eBioscience, Hatfield, UK) or its concentration-matched isotype control for 20 minutes on ice. Post incubation, cells were washed (250 x g, 5 minutes, 4^0C) once in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA)), re-suspended in PBS and transferred to polypropylene FACS tubes. Flow cytometry was conducted using an AccuriC6™ bench top cytometer (BD Biosciences, Oxford, UK) with 10,000 neutrophils acquired for analysis, which was performed using CFlow Plus (BD Biosciences). The percentage of antigen positive cells was recorded along with the corresponding mean fluorescence intensity (MFI) values.

Measurement of reactive oxygen species (ROS) generation

ROS generation was assessed by luminol-amplified chemiluminescence, a technique that measures the generation of myeloperoxidase-derived oxygen metabolites [25]. Neutrophils ($1x10^5$ in $HBSS^{+/+}$) were dispensed into wells of a 96-well white-bottomed flat plate (Corning) that contained 1µM luminol (pH 7.3; Sigma-Aldrich). Cells were then stimulated with 100 µg/ml mtDAMPS or vehicle control, after which ROS generation was assessed at 1 minute intervals over a period of 60 minutes using a Berthold Centro LB 960 luminometer (Berthold Technologies, Hertfordshire, UK). Experiments were performed in quadruplicate,

with ROS production measured as relative light units (RLU) and calculated as area under the curve (AUC).

Neutrophil degranulation assay

A myeloperoxidase (MPO) release assay was used to measure neutrophil primary granule release. Freshly isolated neutrophils (4 x 10^5 in $HBSS^{+/+}$ containing 1% BSA) were primed for 30 minutes (37^{o}C, 5% CO_2) with 10 ng/ml tumour necrosis factor-alpha (TNF-α; Sigma-Aldrich). Post treatment, neutrophils were stimulated with 40 or 100 µg/ml mtDAMPS or vehicle control for 1 hour (37^{o}C, 5% CO_2). Untreated neutrophils served as a marker of spontaneous degranulation, whilst neutrophils treated for 5 minutes (37^{o}C, 5% CO_2) with 1% Triton X-100 (Sigma-Aldrich) served as the readout of maximum degranulation. Following treatment, cells were pelleted (250 x g, 5 minutes) and supernatants collected, to which 50 µl of 3,3',5,5'-Tetramethylbenzidine (Life Technologies) was added. Samples were incubated for 30 minutes in the dark at room temperature, before being read at 650 nm (ELx808 absorbance microplate reader, BioTek® UK, Bedfordshire, UK). MPO release was calculated using the following equation: % MPO release = (Experimental degranulation – spontaneous degranulation)/ (Maximum degradation – spontaneous degranulation) X 100.

Measurement of interleukin (IL)-8.

Freshly isolated neutrophils (2x10^5 in complete media) or those pre-treated with 10 µM SB203580 and/or 10 µM PD98059 were stimulated for 6 hours with 40 µg/ml or 100 µg/ml of mtDAMPS or vehicle control at 37^{o}C in a humidified 5% CO_2 atmosphere. Post stimulation, cells were pelleted (3000 rpm, 5 minutes), supernatants collected and stored at -80^{o}C prior to analysis. The concentration of IL-8 in cell free supernatants was determined using singleplex technology according to manufacturer's instructions (Bio-Rad, Hertfordshire, UK).

Western blotting

Freshly isolated neutrophils (1x10^6 in complete medium) and those pre-treated with either 10 µM SB203580, 10 µM PD98059 or 1uM cyclosporin H were incubated with 40 µg/ml or 100 µg/ml mtDAMPS for 2, 5 and 10 minutes at 37^{o}C in a humidified 5% CO_2 atmosphere. Post stimulation, neutrophils were pelleted by centrifugation (1500 x g, 2 minutes, 4^{o}C), supernatants discarded and cell lysates prepared by the addition of hot sodium dodecyl

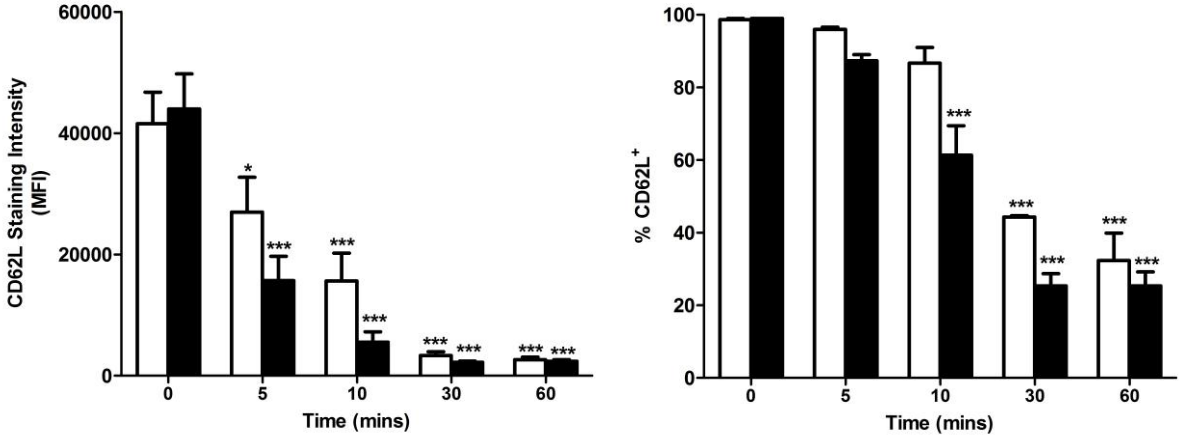
sulphate (SDS) sample buffer (4% SDS (v/v), 0.1 M dithiothreitol, 20% glycerol (v/v), 0.0625 M Tris-HCL and 0.004% bromophenol blue (w/v)). Lysates were separated on 10% SDS-polyacrylamide gels and proteins transferred to polyvinylidene difluoride membranes (Scientific Laboratory Supplies, Yorkshire, UK). To prevent non-specific binding, membranes were incubated with 5% bovine serum albumin (BSA) in Tris buffered saline (TBS; 200 mM Tris (pH 7.5), 1.5 M NaCl) containing 0.1% Tween-20 (TBST) for 1 hour at RT, before being probed overnight at 4^{o}C with either a rabbit anti-human phospho-p38 or a rabbit anti-human phospho-ERK antibody (both diluted 1:1000 in TBST containing 2.5% BSA; Cell Signalling Technology). Post incubation, blots were washed in TBST and incubated for 1 hour at RT with a goat anti-rabbit secondary antibody conjugated to horse radish peroxidase (HRP; diluted 1:4000 in TBST; GE Healthcare, Buckinghamshire, UK). HRP activity was detected using enhanced chemiluminescence (Geneflow, Staffordshire, UK). To confirm equal loading of proteins, blots were stripped with stripping buffer (10% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 0.08% (v/v) ß-mercaptoethanol) for 45 minutes at 50^{0}C, washed for 2 hours in distilled H_20 and blocked for 1 hour at RT in 5% BSA/TBST, before being probed overnight at 4^{0}C with either a total p38 or ERK 1/2 antibody (both diluted 1:2000 in TBST; Cell Signalling Technology). Densitometry was performed using the National Institute of Health ImageJ software package.

Statistical analyses

Statistical analyses were performed using GraphPad Prism® software (GraphPad Software Ltd, California, USA). Data distribution was examined using the Kolmogorov-Smirnov test. For data that followed a normal distribution, paired student T-tests, a repeated measures ANOVA with Dunnett's multiple comparison post hoc test or a two-way repeated measures ANOVA with a Bonferroni post hoc test were performed. For non-normally distributed data, a Friedman test with Dunn's multiple comparison post hoc test or a Wilcoxon matched-pairs signed rank test was used. Data are presented as mean ± standard error of the mean (SEM). The minimum level of confidence at which results were considered statistically significant was $p \leq 0.05$.

Figure 1

(A)



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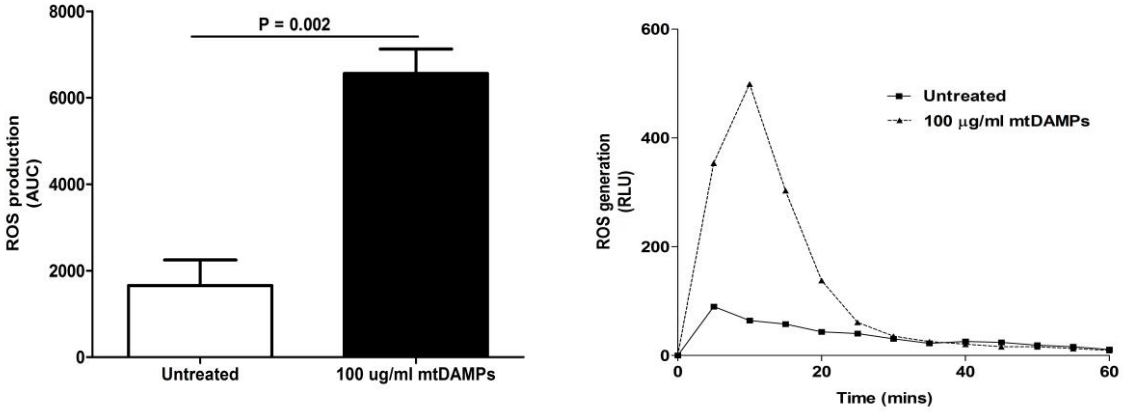

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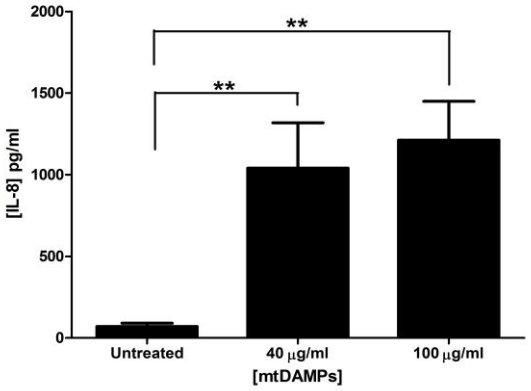

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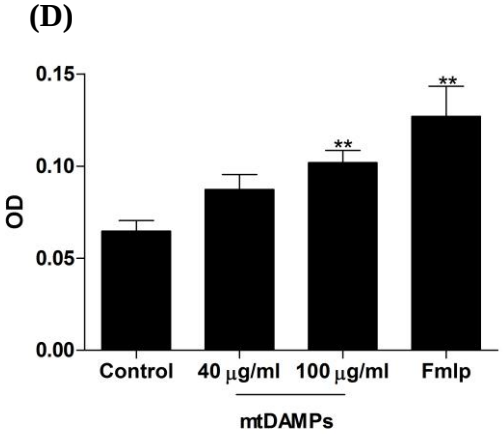
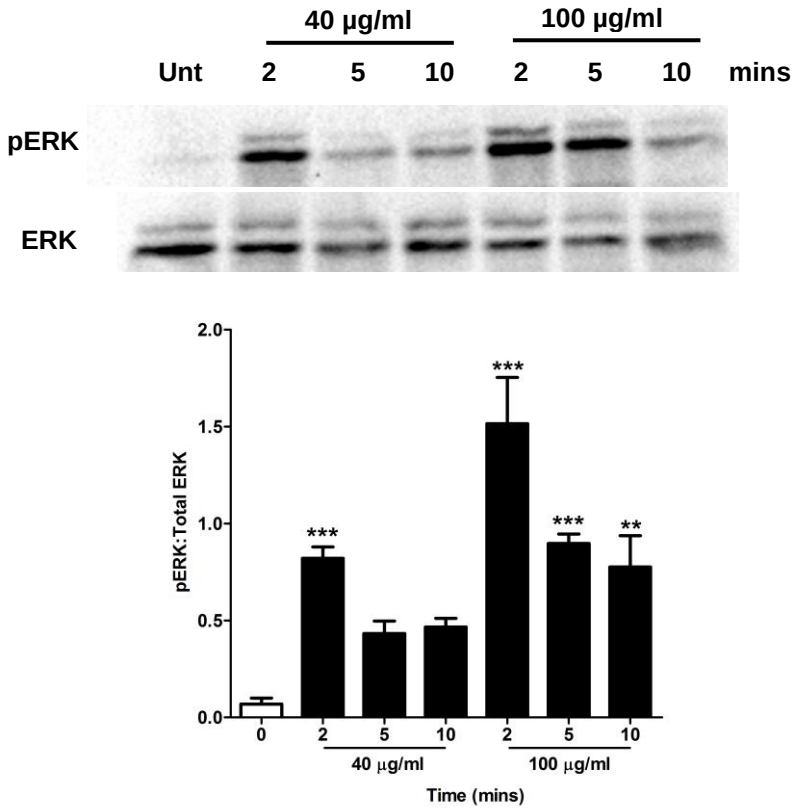


Figure 2

(A)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(B)

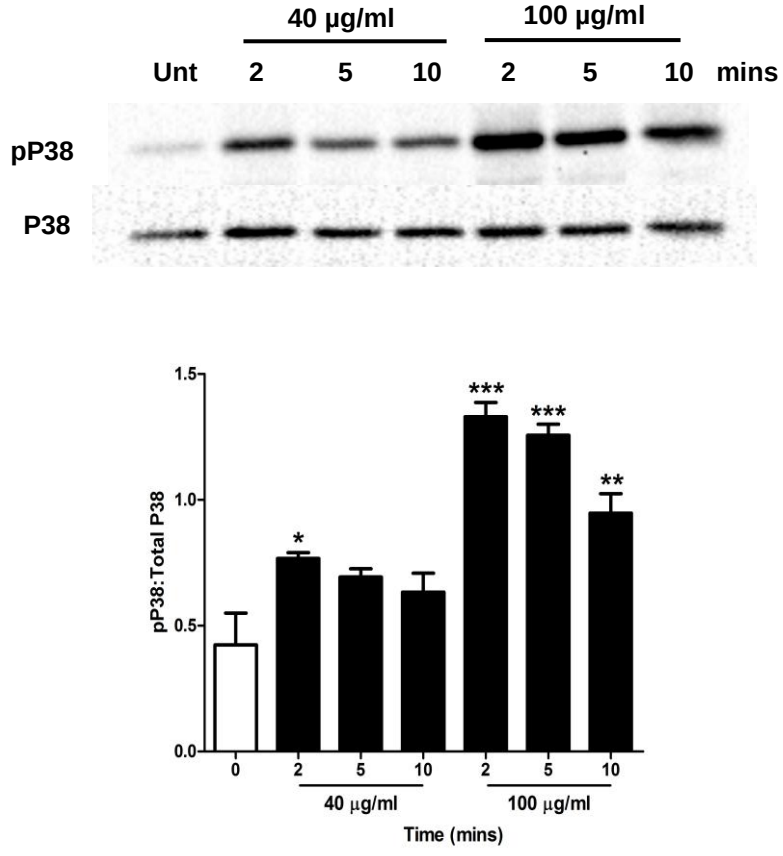
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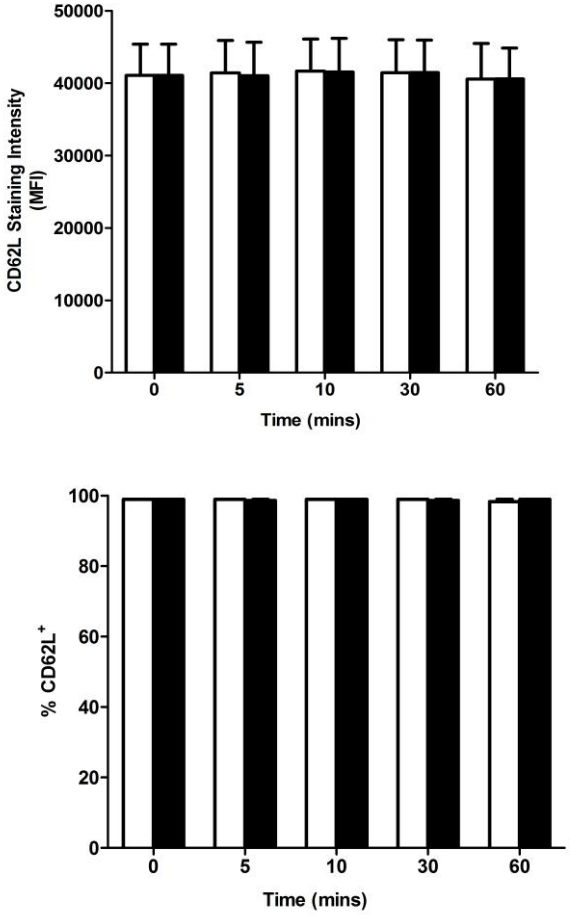
Figure 3

(A)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Time (mins)

(B)

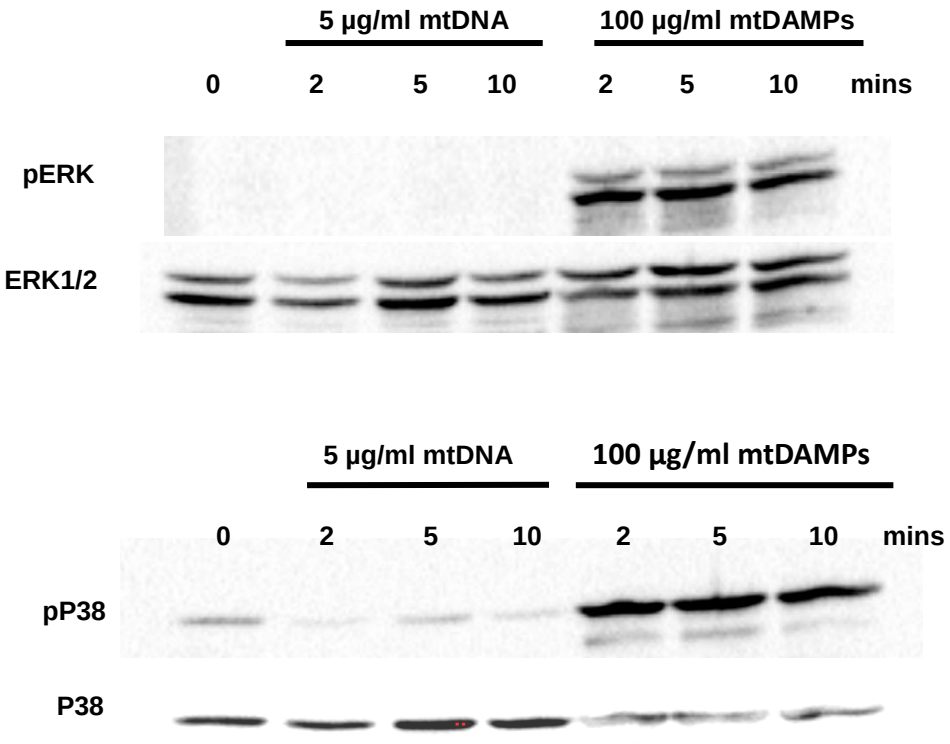
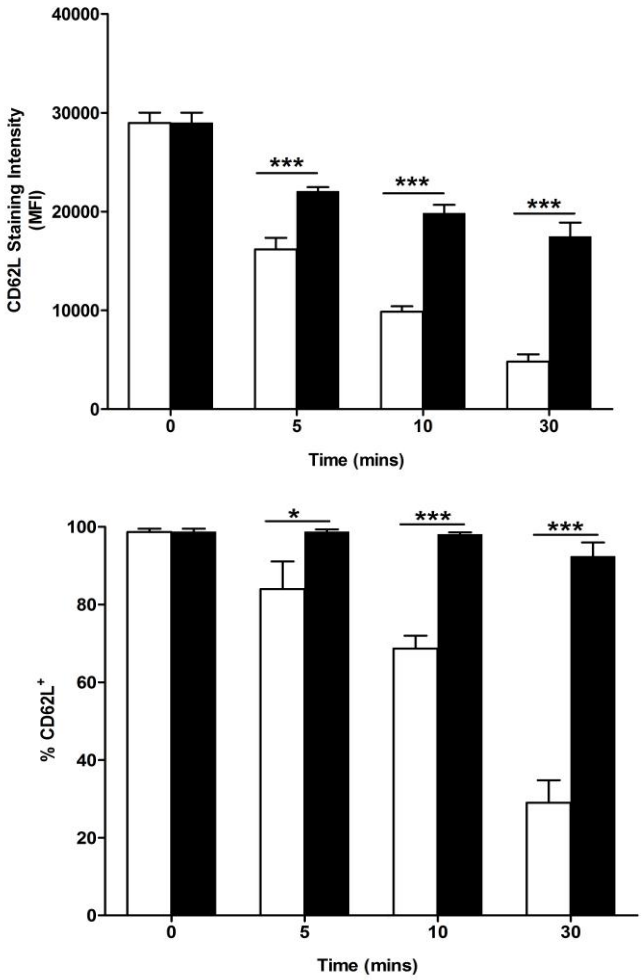
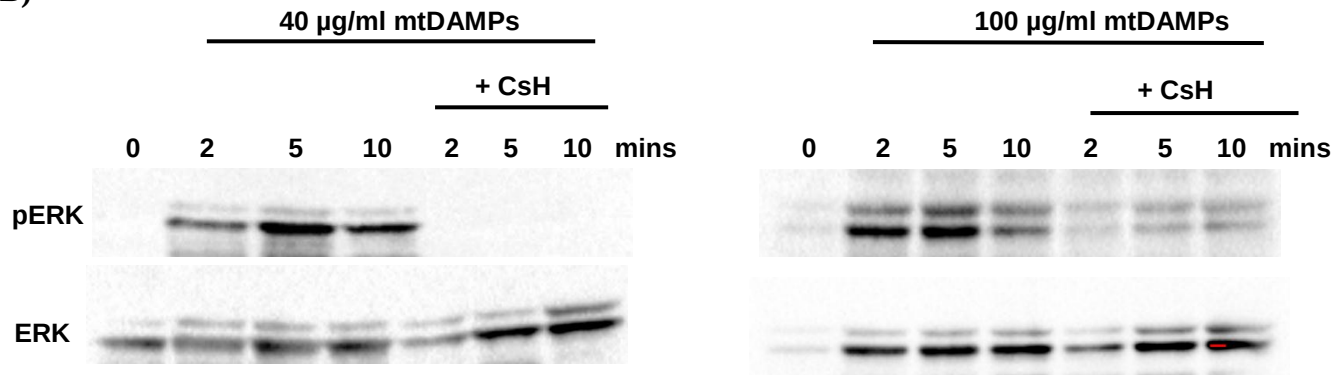

5 µg/ml mtDNA
100 µg/ml mtDAMPs
0
2
5
10
2
5
10
mins
pERK
ERK1/2
5 µg/ml mtDNA
100 µg/ml mtDAMPs
0
2
5
10
2
5
10
mins
pP38
P38

Figure 4

(A)


CD62L Staining Intensity (MFI)
40000
30000
20000
10000
0
***
***
***
0
5
10
30
Time (mins)
% CD62L+
100
80
60
40
20
0
*
***
***
0
5
10
30
Time (mins)

(B)


40 µg/ml mtDAMPs
+ CsH
100 µg/ml mtDAMPs
+ CsH
0 2 5 10 2 5 10 mins
0 2 5 10 2 5 10 mins
pERK
ERK

(C)

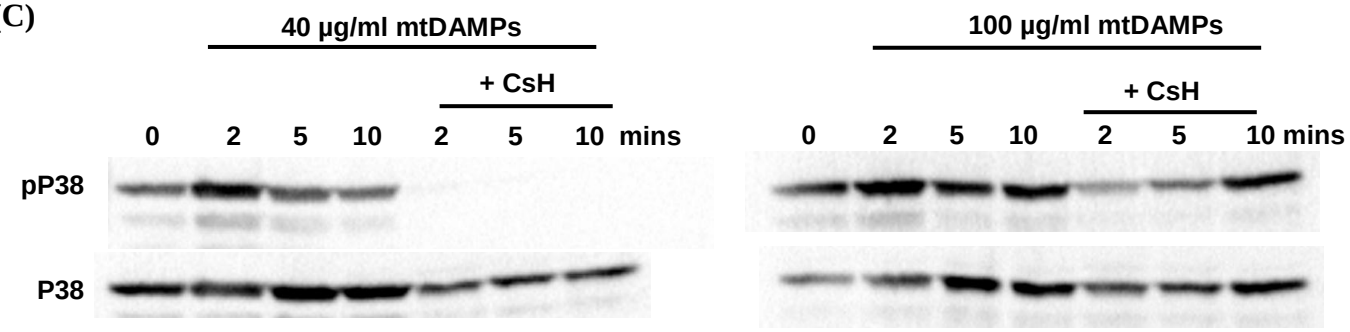
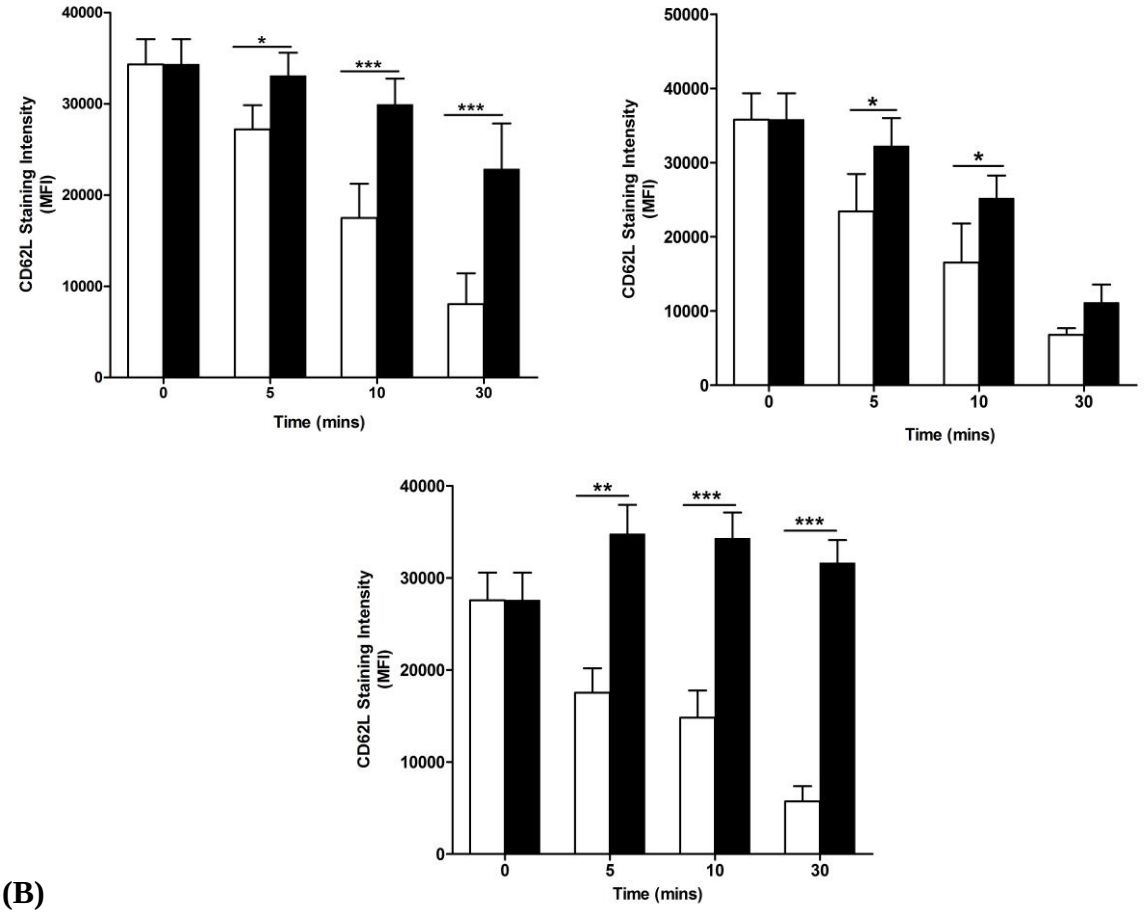

40 µg/ml mtDAMPs
+ CsH
100 µg/ml mtDAMPs
+ CsH
0 2 5 10 2 5 10 mins
0 2 5 10 2 5 10 mins
pP38
P38

Figure 5

(A)


CD62L Staining Intensity (MFI)
Time (mins)
0
5
10
30
40000
30000
20000
10000
50000
*
**
***

(B)

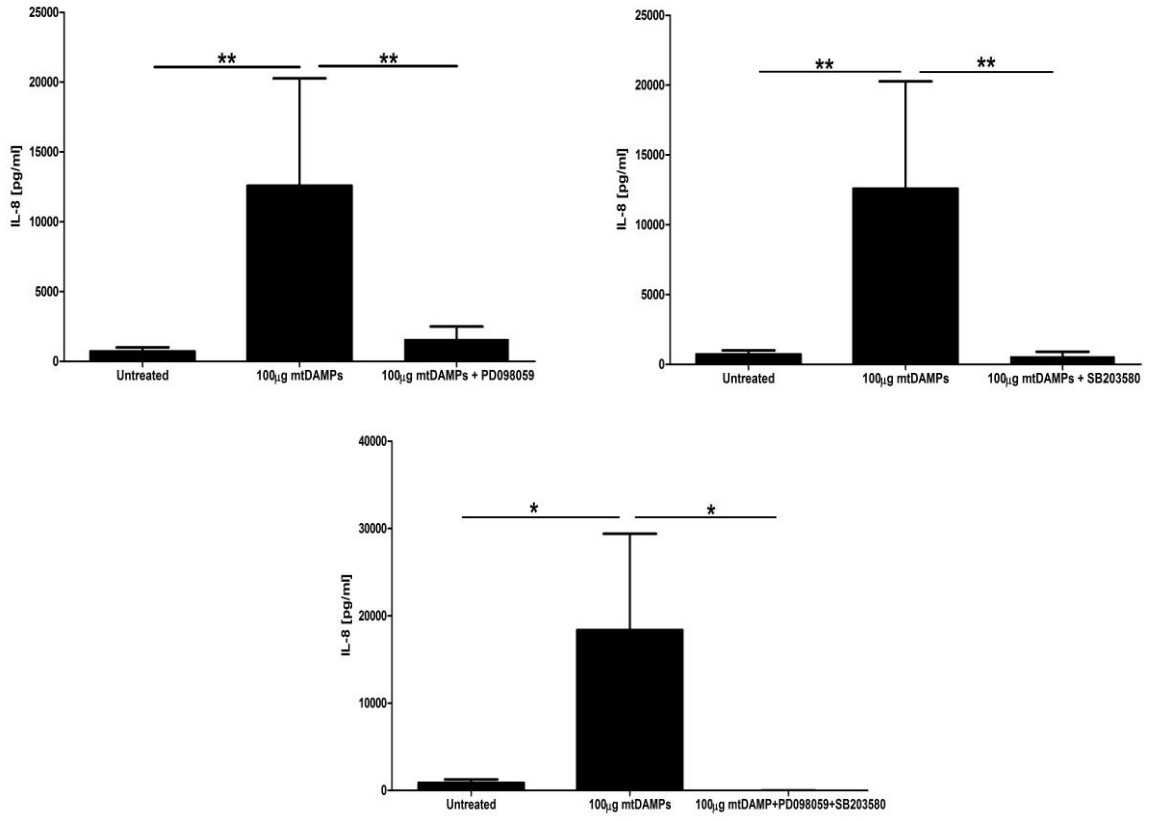

IL-8 [pg/ml]
25000
20000
15000
10000
5000
0
**
*
Untreated
100µg mtDAMPs
100µg mtDAMPs + PD098059
100µg mtDAMPs + SB203580
100µg mtDAMP+PD098059+SB203580

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Figure Legends

Figure 1. Exposure to mtDAMPs activates human neutrophils and triggers a robust functional response. (A) Neutrophil activation upon stimulation with 40 µg/ml (open bars) or 100 µg/ml (filled bars) mtDAMPs was assessed by L-selectin staining over a 60-minute period. Data are presented as L-selectin surface density (left panel) and the percentage of L-selectin positive neutrophils (right panel). Data are mean ± SEM (n=3). * = P<0.05, *** = P<0.0001 test vs. untreated sample. MFI, Mean fluorescent intensity. **(B)** ROS generation by freshly isolated neutrophils stimulated with 100 µg/ml mtDAMPs was measured over a 60-minute period by luminol-based chemiluminescence. In the left hand panel, ROS generation is presented as area under the curve (AUC) and represents the mean ± SEM (n=3). In the right hand panel, a representative plot of ROS generation presented as relative light units (RLU) is shown. **(C)** IL-8 secretion by neutrophils stimulated with 40 or 100 µg/ml mtDAMPs (n = 10). Data are mean ± SEM. ** = P<0.01. **(D)** Degranulation by neutrophils upon stimulation with 40 or 100 µg/ml mtDAMPs or the bacterial peptide fMLP (positive control) assessed by measuring myeloperoxidase activity in culture supernatants. Data are mean ± SEM (n-7). * = P<0.05, ** = P<0.01 test vs. untreated sample.

Figure 2. St**imulation with mtDAMPs activates MAPK signalling in resting human neutrophils**. **(A-B)** Activation of the MAPKs ERK1/2 **(A)** and P38 **(B)** assessed by Western blotting in freshly isolated neutrophils treated with 40 or 100 µg/ml mtDAMPs over a 10-minute time course. Top panels: Representative Western blots depicting MAPK phosphorylation kinetics. Bottom panels: Densitometric analysis of MAPK activation. Data are mean ± SEM (n=3). * = P<0.05, ** = P<0.01, *** = P<0.0001 compared to time 0.

Figure 3. Mitochondrial DNA does not activate human neutrophils or induce MAPK phosphorylation. (A) Activation of neutrophils in response to 1 µg/ml (open bars) or 5 µg/ml (filled bars) mitochondrial DNA assessed by L-selectin staining. Data are presented as percentage of L-selectin positive neutrophils (top panel) and surface density by MFI (bottom panel). Data are mean ± SEM (n-5). Activation of the MAPKs ERK1/2 **(B)** and P38 **(C)** in response to 5 µg/ml mtDNA or 100 g/ml mtDAMP stimulation assessed by Western blotting over a 10-minute time course. Representative Western blots depicting MAPK phosphorylation kinetics are shown. Data are mean ± SEM (n=3).

Figure 4. Formyl peptides within mtDAMPs activate human neutrophils and trigger MAPK signalling. (A) Activation of neutrophils pre-treated with the formyl peptide receptor antagonist cyclosporin H (CsH; filled bars) or vehicle control (open bars) in response to stimulation with 100 µg/ml mtDAMPs assessed by L-selectin staining. Data are presented as percentage of L-selectin positive neutrophils (top panel) and surface density by MFI (bottom panel). Data are mean ± SEM (n=3). *=P<0.05, ***=P<0.0001 compared to samples treated with 100 µg/ml mtDAMPs alone. Activation of the MAPKs ERK1/2 **(B)** and P38 **(C)** in response to 40 and 100 µg/ml mtDAMP stimulation was compared between neutrophils pre-treated with CsH or vehicle control by Western blotting over a 10-minute time course. Representative Western blots depicting MAPK phosphorylation kinetics are shown.

Figure 5. ERK1/2 and p38 signalling is required for mtDAMP-induced neutrophil activation and IL-8 secretion. (A) Activation of neutrophils pre-treated with the ERK1/2 inhibitor PD098059 (left panel; filled bars), the p38 inhibitor SB202190 (right panel; filled bars) or both (bottom panel; filled bars) in response to stimulation with 100 µg/ml mtDAMPs (open bars) assessed by L-selectin staining. Data, presented as surface density by MFI, are mean ± SEM (n=3). *=P<0.05, **=P<0.01 ***=P<0.0001 compared to samples treated with 100 µg/ml mtDAMPs alone. **(B)** IL-8 production by neutrophils treated with PD098059 (left panel, n=9), SB202190 (right panel, n=9) or both (bottom panel, n=6) prior to a 6-hour stimulation with 40 or 100 µg/ml mtDAMPs. Data are mean ± SEM.

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