The effect of Phorbol 12-Myristate 13-Acetate on CD11b and CD62-L cell surface expression of neutrophils and monocytes

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ABSTRACT

Background: Neutrophils and monocytes are phagocytic leukocytes that represent an important component of the non-specific immune system, by which invading micro-organisms and tumour cells are rapidly internalized and destroyed, via a number of biological mechanisms. Additionally, these phagocytic leukocytes play a key role in facilitating leukocyte-endothelial interactions in response to pro-inflammatory agents, during an inflammation. The expression of specific adhesion molecules such as CD62-L and CD11b, on the cell surface of neutrophils and monocytes, play an integral role with regards to cell migration, rolling, firm adhesion and subsequent cellular activation (leukocyte adhesion cascade). Leukocytes can be stimulated by chemotactic agents such as Phorbol 12-myristate 13-acetate (PMA). PMA is a direct stimulant of protein kinase C signaling pathway, which promotes cellular activation.

Methods: The aim of this pilot study was to determine the effect of PMA on CD62-L and CD11b cell surface expression of neutrophils and monocytes. Venous blood samples were collected from the ante-cubital fossa from healthy individuals, following written informed consent (n=4). Neutrophils and monocytes were isolated via a density gradient centrifugation method. Subsequently, neutrophil and monocyte cell surface expression of CD62-L and CD11b were measured by labelling with fluorescent anti-CD62-L and anti-CD11b monoclonal antibodies via flow cytometry.

Results: Following stimulation with PMA, monocytes displayed a decrease in CD62-L and increase in CD11b cell surface expression (p=0.103 and p=0.026 respectively). With respect to neutrophils, PMA resulted in a similar cellular response, where the cell surface expression of CD62-L decreased whilst CD11b increased (p=0.098 and p=0.025 respectively).

Discussion and Conclusion: In summary, this pilot study confirms the potent effect that PMA has on leukocyte function. Although studies involving PMA have previously been documented, this study provides a sound platform to continue work in this field of leukocyte biology. However, the validation and reliability of these findings would require to be assessed through the assessment of larger cohorts.

Keywords: neutrophils, monocytes, CD11b, CD62-L, PMA, leukocyte adhesion cascade.

1. INTRODUCTION

Neutrophils and monocytes are phagocytic leukocytes that represent an important component of the non-specific immune system, by which invading micro-organisms and

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tumour cells are rapidly internalized and destroyed via a number of biological mechanisms. Additionally, these phagocytic leukocytes play a key role in facilitating leukocyte-endothelial interactions in response to pro-inflammatory agents, during an inflammation. Leukocyte migration is an essential mechanism for the recruitment of leukocytes into the tissue from the blood vessel, following the stages known as: capture, rolling, slow rolling, firm adhesion and transmigration [1]. It requires a network of adhesion molecules primarily mediated by L, P and E selectins to allow naïve leukocytes to be captured and begin rolling along the endothelium of the blood vessel [2]. Synergetic interactions between adhesion molecules regulate the migration of naïve, antigen-activated effector, and memory leukocytes. L-selectin (CD62-L) is exclusively expressed on leukocytes and initiates the capture and the subsequent rolling, by binding to glycosylation-dependent cell adhesion molecule-1, sialylayted glycoprotein of 200 kD and podocalyxin-like proteins, collectively known as peripheral node addressins.

Integrins, which are large complex transmembrane glycoproteins, have been identified to play a key role in both adhesion and mediation of cell-to-cell interactions such as: cellular trafficking, migration and phagocytosis [3]. CD11b/CD18 is a non-covalently linked heterodimer of α_M and β2 subunits respectively, on the surface of leukocytes including monocytes and neutrophils. α_M subunits mediate adhesion and spreading on a series of α_Mβ2 ligands including, ICAM-1, whilst the β2 subunits supported migration and attachment to receptors such as C3bi mediating complement-coated particle uptake [4]. CD62-L (or L-selectin) is constitutively expressed on the surface of monocytes but is rapidly down regulated once the monocytes have been activated. This is due to proteomic cleavage of CD62-L from the surface leaving a portion of the protein, which is soluble and functional [5,6]. Similarly, CD11b is constitutively expressed on the surface of leukocytes, and is stored in the specific granules and transported to the cell surface upon activation. Upon upregulation, the affinity of CD11b receptors are increased where this promotes firm attachment to the endothelium to allow trans-endothelial migration to the site of inflammation or injury [7].

The magnitude of cell surface CD11b can be up-regulated rapidly from an intracellular pool, which translocate to the surface in response to cell activation. Once activated, CD11b binds to actin and associated signalling proteins, as well as acting as a signalling partner for N-Formylmethionine-leucyl-phenylalanine (fMLP), and lipopolysaccharide (LPS) receptors [8]. Studies have shown that the expression of CD11b is essential for firm adhesion, which is a process that precedes the transmigration of cells to tissue at the site of inflammation [9]. Interestingly, further studies have also demonstrated that the CD11b-CD18 complex can recognise double stranded RNA that are only present in viral infections [10]. Findings from this study demonstrated that the CD11b-CD18 complex can selectively bind to double stranded RNA, speculating that when monocytes have differentiated into macrophages the CD11b-CD18 complex may mediate extracellular immune responses as well as migration.

CD62-L is expressed on most leukocytes including monocytes, granulocytes, lymphocytes, NK cells, bone marrow myeloid progenitor cells and on a subset of thymocytes [11]. CD62-L has an important role in leukocyte capture on endothelial surfaces, homing of naïve lymphocytes to lymph nodes and Peyers patches via high endothelial venules (HEV) [12]. For leukocytes to migrate into peripheral tissues and adhere to sites of inflammation, CD62-L is rapidly shed from the cell surface [11]. Investigations into monocyte migration through HEVs reported that CD62-L is a key mediator during leukocyte migration [13]. Recent research has shown, via real-time fluorescence time-lapse microscopy that CD62-L plays a key role during the leukocyte adhesion cascade, especially during leukocyte recruitment and migration [14].

Changes to the expression of CD62-L and CD11b on phagocytic leukocytes have also been reported in a clinical setting, involving orthopaedic patients and those with cardiovascular disease. This in turn, highlights the significance of leukocytes and their interactions with the endothelium in a clinical setting [6, 15, 16]. Phorbol Myristate Acetate (PMA) is a potent tumour promoter, a stimulator of 'respiratory burst' and a membrane-active stimulator of oxidative metabolism, which can be used to show the cytotoxicity of human neutrophils and monocytes [17]. Respiratory or oxidative burst is the release of potent reactive oxygen species (ROS) that are dedicated to aiding the elimination invading microorganisms. Upon cellular activation, cytosolic proteins within the leukocytes translocate to the membrane forming a multi-component electron-transfer system, where this catalyses the reaction forming superoxide anion and hydrogen peroxide, which both have bactericidal properties [18].

Leukocytes can be stimulated by chemotactic factors such as PMA. PMA is known to be a direct stimulant of protein kinase C and so triggers the diacylglycerol signalling pathway which promotes cellular activation [19-21]. Reports have demonstrated that cell stimulation with PMA leads to activation, marked by prolonged cellular aggregation, CD18 phosphorylation, exocytosis and phagocytosis [22-26].

The main aim of this pilot study was to investigate the effect of PMA on the cell surface expression of CD11b and CD62-L on neutrophils and monocytes.

2. METHODOLOGY

2.1 Subject Volunteers

Ethical Approval was granted by the local ethics committee of the Faculty of Science and Engineering at the University of Chester. Four healthy male volunteers were recruited after written informed consent (n=4). The test subjects were aged between 20 and 48 years old (mean 34 ± 14 years), and all were non-smokers. None had any history of cardiovascular disease, with initial haematological analysis via a full blood count confirming that test results were within the normal clinical reference ranges (Table 1). During this investigation, 10 ml of venous blood samples subject volunteers were collected and analysed within a 4-hour time period, to ensure live cell viability.

Parameter	Mean	SD (±)	Normal Reference Values*
WBC	5.58	0.48	4.0-11.0 $x10^9$/L
LY	41.15	5.13	15-55%
MO	5.08	1.38	2-8%
GR	53.78	5.61	25-75%
RBC	4.89	0.21	4.50-6.50 $x10^{12}$/L
Hb	14.90	0.77	13.5-17.5 g/dL
Hct	45.38	2.71	40-52%

MCV	92.73	1.53	80-95 fL
MCH	30.43	0.47	27-34 pg
MCHC	32.80	0.55	20-35 g/dL
RDW	13.25	0.99	11-15%
Plts	320	60	150-400 x10^9/L
MPV	7.60	0.44	7.8-11.0 fL
Pct	0.24	0.05	0.190-0.360%

***(Adapted from [27])**

Table 1. Full blood count analysis. Data presented as mean ± standard deviation.

2.2 Blood Samples

Venous blood was collected from healthy volunteers by venepuncture of the ante-cubital vein, and collected into vacutainers containing sufficient EDTA-K_2 to give a final concentration of 1.5mg/ml (Becton & Dickinson U.K., Ltd., between Towns Road, Cowley, Oxford, OX4-3LY). All blood samples were stored at room temperature as a standard practice.

2.3 Blood Counts

Full blood counts were performed using a Coulter® MicoDiff[18] automated cell counter (Beckman Coulter, UK). Isolated leukocytes (polymorphonuclear and mononuclear) were also counted in order to assess yield and purity, and to optimally adjust leukocyte concentrations.

2.4 Isolation of Leukocytes

Suspensions of polymorphonuclear (granulocytes such as neutrophils) and mononuclear (lymphocytes and monocytes) leukocytes were prepared by density gradient sedimentation on ficoll hypaque solutions [28]. Following isolation, cells were re-suspended in phosphate buffered saline (PBS) supplemented with dipotassium EDTA (1.5 mg/ml) to yield a final cell count of 2×10^6 cells/ml. All chemicals were supplied by Sigma-Aldrich, UK.

2.5 Flow Cytometry

All flow cytometric analyses were performed using a Becton and Dickenson FACScan flow cytometer. During these studies 5000 gated events were recorded.

2.6 Measurement of Cell Surface Expression of CD62L

The monoclonal antibodies used were mouse anti-human CD62L (MCA1076F) and isotype-matched control IgG2b (MCA691F) and were purified immunoglobulin/fluororescein isothiocyanate (Ig/FITC) conjugates (AbD Serotec Ltd., U.K.). Following isolation of leukocyte subpopulations and adjustment of concentration (2×10^6 cells/ml), 10 µl of the

monoclonal antibody (0.1 mg/ml) was added to 100 µl of the appropriate cell suspension. These were incubated at room temperature for 30 minutes, prior to assay analysis using flow cytometry of gated monocytes and neutrophils.

For cells treated with PMA, 10µl of PMA working solution (3.14nM as defined by Sigma-Aldrich data sheets) was then added to the appropriate tubes (PMA positive), and tubes were incubated with untreated preparations (PMA negative) at 37°C for a further 15 minutes, prior to assay analysis using flow cytometry. Negative controls (PBS) represented auto-fluorescence. This method was repeated for CD11b analysis.

2.7 Measurement of Cell Surface Expression of CD11b

The monoclonal antibodies used were mouse anti-human CD11b (MCA551F) and isotype-matched control IgG1 (MCA928F) and were purified immunoglobulin/fluoro-rescein isothiocyanate (Ig/FITC) conjugates (AbD Serotec Ltd., U.K.). Following isolation of leukocyte subpopulations and adjustment of concentration (2×10^6 cells/ml), 10 µl of the monoclonal antibody (0.1 mg/ml) was added to 100 µl of the appropriate cell suspension. These were incubated at room temperature for 30 minutes, prior to assay analysis using flow cytometry of gated monocytes and neutrophils.

2.7 Statistical Analysis

During this study, all results were presented as mean ± standard deviation (SD). Initially, all the data were tested to determine if the results were normally distributed. The Shapiro-Wilk statistical test was employed with data being considered as normally distributed if P = .05. Where data were normally distributed, t-tests were employed adopting a 5% level of significance.

3. RESULTS

3.1. Full Blood Counts

Following venepuncture, red cell, platelet and partial differential white cell counts (full blood counts) were measured using the Coulter® MicroDiff[18], confirming normal haematological results (Table 1).

3.2 Efficacy of Leukocyte Isolation Methods

The efficacy of the density gradient centrifugation to separate PMNs and monocytes from whole blood, as described by Lennie *et al*. for the isolation of leukocyte subpopulations is shown in Figure 1. The isolation of polymorphonuclear (PMN) cells (77% ± 3.33) was shown to provide a higher mean percentage yield following cell isolation methods, in comparison to mononuclear (MN) cells (65% ± 5.01) (Figure 1). A possible explanation for this could be due to the vast numbers of PMN cells in comparison to MN cells being present in the normal peripheral blood circulation. The effectiveness of the isolation method was also complimented by the erythrocyte contamination value, which was shown to be 0.04% (± 0.03). Following isolation procedures, PMN (neutrophils) and MN cells (monocytes only) were then used for analysis via flow cytometry, where isolated cells were selected by assignment of gates normally associated with neutrophils and monocytes.

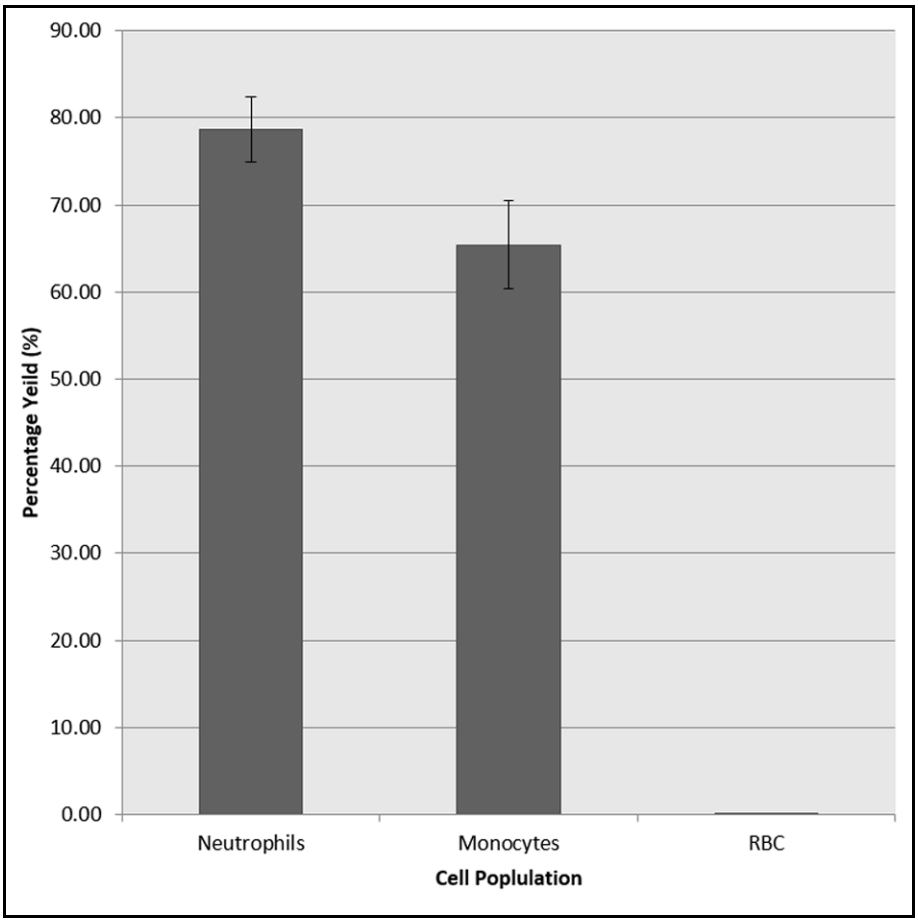

Figure 1. Efficacy of the density gradient centrifugation method for the isolation of leukocyte subpopulations was measured using the Coulter® MicroDiff[18] apparatus. The data represent mean % yields (± standard deviation) of granulocytes (neutrophils) and mononuclear cells (lymphocytes and monocytes), by comparison of erythrocyte contamination.

3.3 Assignment of Flow Cytometric Gates for Isolated Leukocyte Subpopulations

During flow cytometry, the gating of phagocytic leukocyte sub-populations (neutrophils and monocytes) were adjusted so that the percentage of cells analysed were identical to those identified using a Coulter® MicroDiff[18] apparatus. Lymphocytes, red blood cells and debris were excluded from defined gates. All flow cytometric analyses were performed using a Becton and Dickenson FACScan flow cytometer (Figure 2).

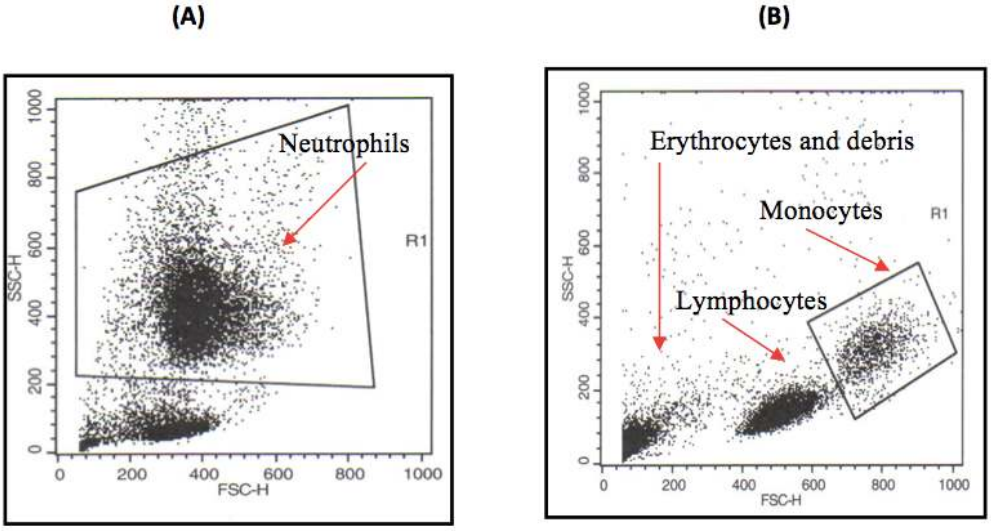

Figure 2. Gating of leukocyte subpopulations during flow cytometric analysis. Gates were adjusted so that the percentage of cells analysed were identical to those identified using Coulter MicroDiff. Lymphocytes, red blood cells and debris were excluded from defined gates. Leukocyte subpopulations were selected by assignment of gates normally associated with (A) neutrophils and (B) monocytes.

3.4 The Effect of PMA on CD62L Cell Surface Expression of Neutrophils and Monocytes

As can be seen from figures 3 and 4, neutrophils and monocytes displayed decreasing expression of CD62L following treatment with PMA (3.14nM), (un-stimulated neutrophils: 20.86 ± 6.86; PMA treated neutrophils: 17.28 ± 8.88; un-stimulated monocytes: 28.59 ± 8.57; PMA treated monocytes: 21.12 ± 2.49). Negative controls of PBS and EDTA were used to show background noise. Although trends of decreasing neutrophil and monocyte CD62L cell surface expression was observed following treatment with PMA, these did not show any statistical differences (p=0.098 and p=0.103 respectively). Interestingly, monocytes appeared to express slightly more CD62L on their cell surfaces in comparison to neutrophils, prior to treatment of cells with PMA. A possible explanation for this could be that monocytes are morphologically bigger than neutrophils.

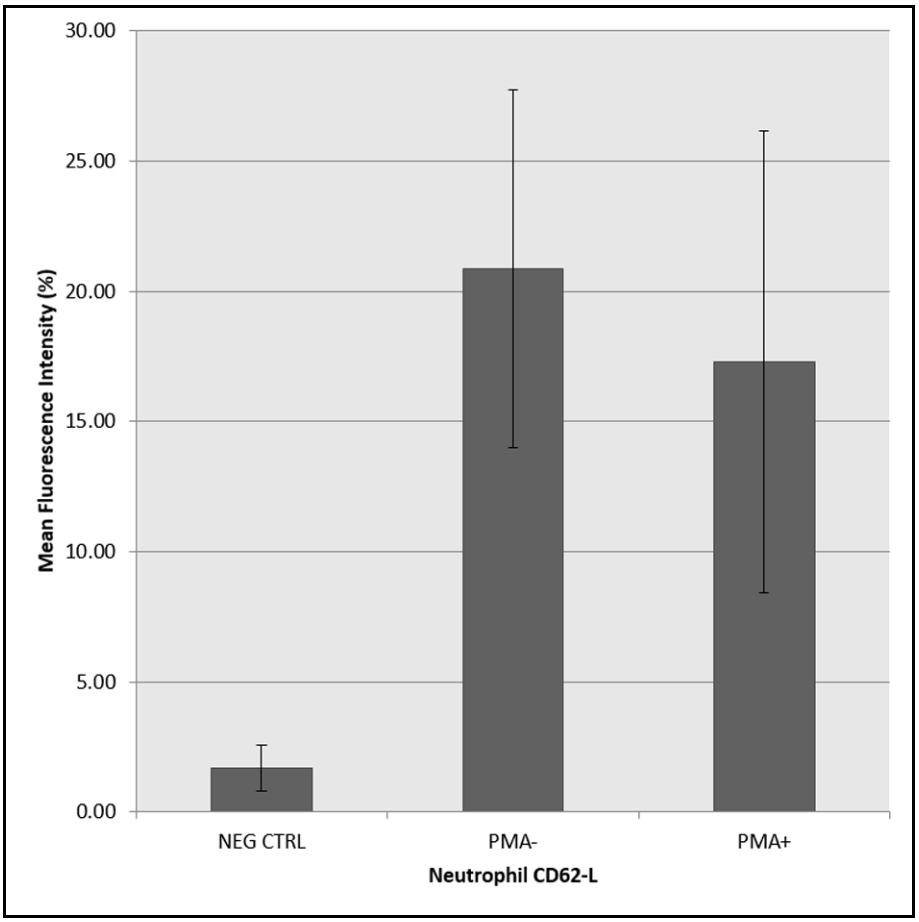

Figure 3. The effect of PMA on CD62-L cell surface expression on neutrophils (± standard deviation), peak MFI: 1.78, 28.70 and 27.35 respectively. p=0.098 shown as determined by t-test.

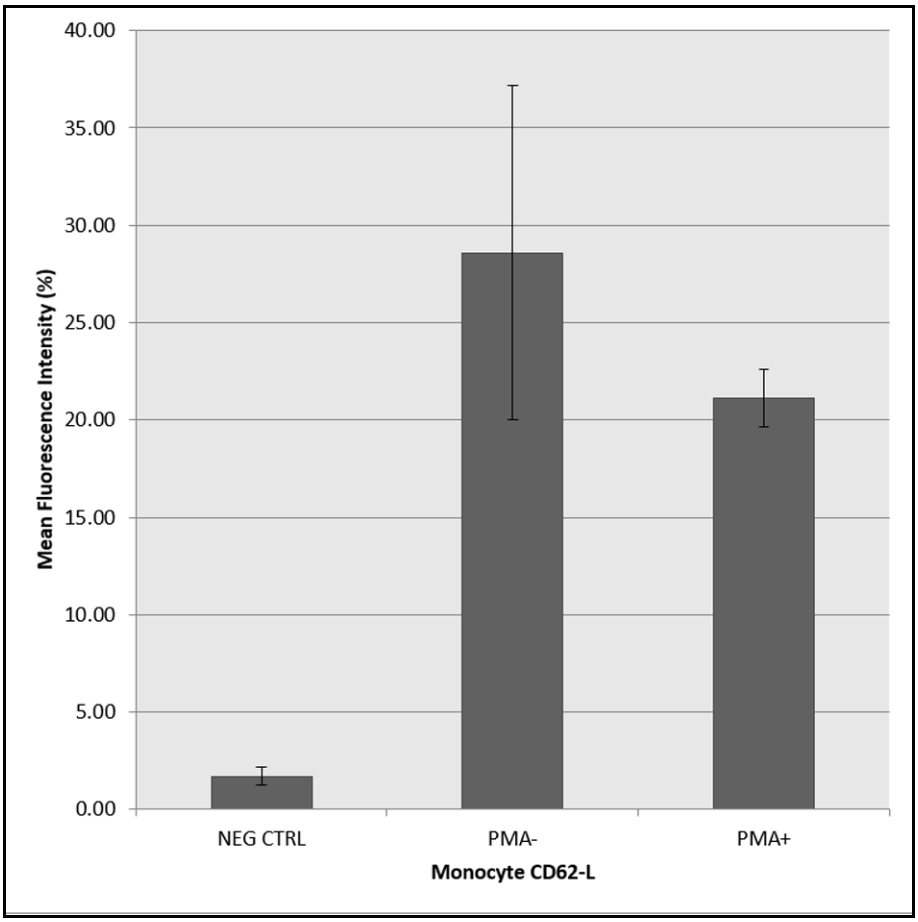

Figure 4. The effect of PMA on CD62-L cell surface expression on monocytes (± standard deviation), peak MFI: 1.96, 39.62, 23.33 respectively. p=0.103 as determined by t-test.

3.5 The Effect of PMA on CD11b Cell Surface Expression of Neutrophils and Monocytes

As can be seen from figures 5 and 6, neutrophils and monocytes displayed increasing CD11b expression following treatment with PMA (3.14nM), (un-stimulated neutrophils: 19.56 ± 10.15; PMA treated neutrophils: 38.42 ± 4.82; un-stimulated monocytes: 29.15 ± 4.91; PMA treated monocytes: 51.71 ± 12.23). Significant increases in both neutrophil and monocyte CD11b cell surface expression was observed following treatment with PMA (p=0.025 and p=0.026 respectively, as determined by t-test) Interestingly, monocytes appeared to express a 1.5-fold higher expression of CD11b on their cell surfaces in comparison to neutrophils, prior to treatment of cells with PMA. A possible explanation for this could be that monocytes are bigger than neutrophils and express a larger surface area that can express more adhesion molecules.

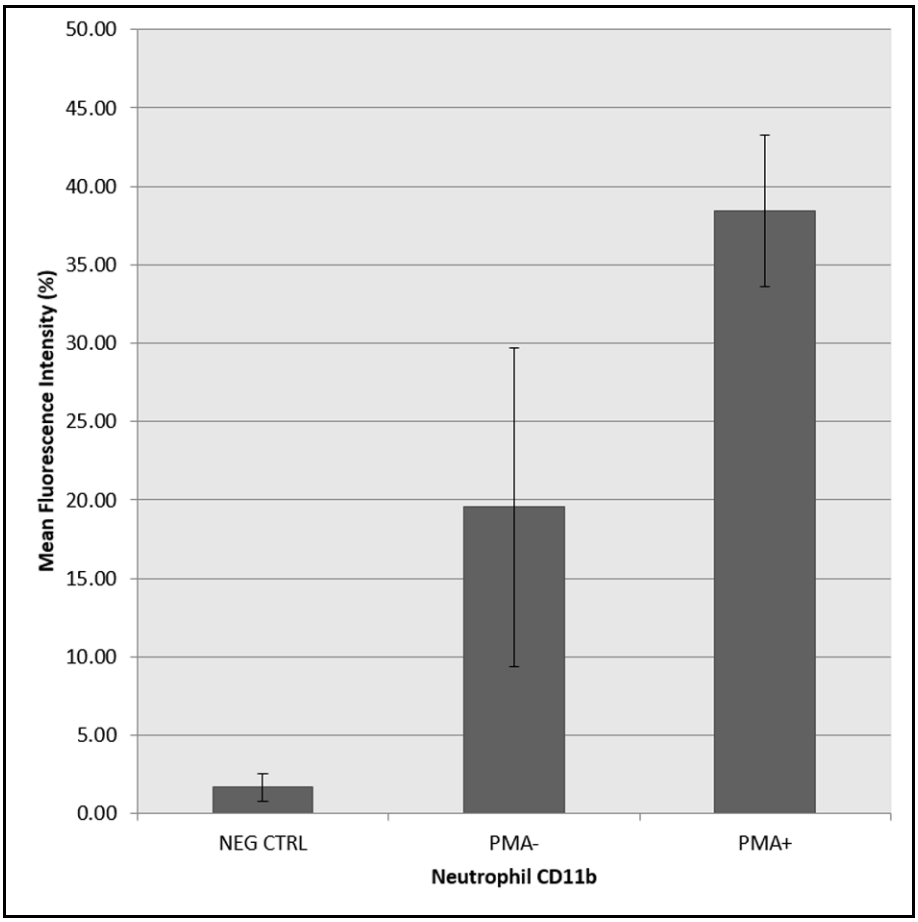

Figure 5. The effect of PMA on CD11b cell surface expression on neutrophils (± standard deviation), peak MFI: 2.50, 25.73, 43.77 respectively. p=0.025 as determined by t-test.

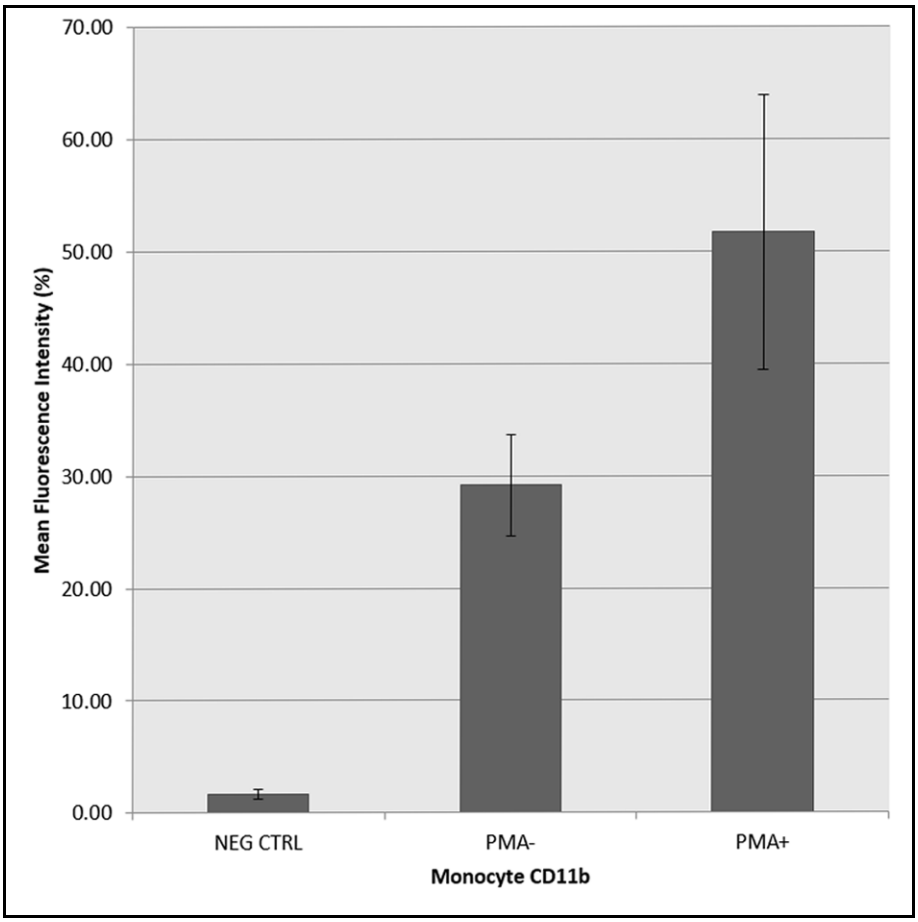

Figure 6. The effect of PMA on CD11b cell surface expression on monocytes (± standard deviation), peak MFI: 1.96, 35.87, 69.95 respectively. p=0.026 as determined by t-test.

4. DISCUSSION

A preliminary part of this research was to verify methods for the isolation of leukocytes, prior to undertaking further studies. Isolating leukocytes employing a Ficoll-Hypaque density gradient technique was of importance during these investigations. Optimisation of this technique allowed for the isolation of concentrated fractionated leukocyte sub-populations of high purity for use during investigative analysis. Data collected from the isolation of leukocyte studies demonstrated high percentage yields for both PMN and MN cells. With regards to PMNs an average yield of 77% (± 3.33) was achieved, with MN cells providing an average yield of 65% (± 5.01). The efficacy of this leukocyte isolation protocol was also complemented by the very low erythrocyte contamination, which was found to be 0.04% (± 0.003) (Figure 1). All blood cell counts performed during this study were analysed using a highly reliable Coulter® MicroDiff[18] analyser. This preliminary study demonstrated the high degree of reproducibility of this method for the isolation of PMNs and MN cells, prior to further investigations using gated neutrophil and monocyte cell preparations for flow cytometric analysis on leukocyte function.

During the investigations to compare the effect of PMA on leukocyte cell surface adhesion molecules, isolated cells were treated individually with PMA (3.14nM) to induce cell activation. Previous studies have demonstrated that PMA promotes cellular activation in terms of increased CD11b production, and in agreement with others, the present study demonstrated that treatment of isolated leukocytes with PMA, resulted in altered levels of specific adhesion molecules, namely CD62-L and CD11b [9, 19-21, 29].

CD62-L has been demonstrated to play an important and integral part during the leukocyte adhesion cascade in inflammation, especially during the initial capture of leukocytes to the endothelium and during the rolling process [6, 30-33]. FITC anti-human CD62-L was used during these investigations to determine the cell surface expression on isolated leukocytes using flow cytometry. Studies presented gating of isolated blood cells labelled with FITC anti-human CD62-L. The CD62-L cell surface expression of monocytes was consistently higher than that seen in neutrophils for un-stimulated cells. Following exposure to PMA, CD62-L cell surface expression on both neutrophils and monocytes ($p>0.05$) decreased to values lower than un-activated cells, suggesting that PMA plays an integral role with regards to promoting the shedding of CD62-L from the cells surface. Although the investigations performed during this study were carried out *in vitro*, it suggests that leukocyte activation is followed by shedding of CD62-L from the cell surface, allowing for normal leukocyte-endothelial interactions to transpire [34].

CD11b has been demonstrated to play a key role during the leukocyte adhesion cascade and inflammation, specifically during the slow rolling and firm adhesion stages of the cascade [31, 35]. FITC anti-human CD11b was used during these investigations, to determine integrin cell surface expression by isolated leukocytes using flow cytometric evaluation. The studies presented gating of isolated blood cells labelled with FITC anti-human CD11b. The expression of CD11b on monocytes was consistently higher than that seen in neutrophils. The higher CD11b expressed by monocytes could be due to the larger cell size, and hence surface area of monocytes compared to neutrophils. Following exposure to PMA, CD11b expression by both neutrophils and monocytes significantly increased ($p<0.05$). Although the experiments performed during this study were carried out *in vitro*, isolated leukocytes did however up-regulate their CD11b cell surface expression upon exposure to PMA. Interpretation of the results from this study suggests that following activation this adhesion molecule may play a key role in facilitating leukocyte-endothelial interactions in response to pro-inflammatory agents, during an inflammatory response.

Although no power calculations were performed as part of the statistical analysis pertaining to the study, it is acknowledged that a major limiting factor of this study was the relatively small number of subjects recruited. In order to fully appreciate the effects of PMA on the parameters measured, more participants could have been recruited. This in-turn would have been beneficial to some of the statistical trends that were observed, that otherwise may have resulted in significant differences. It is also important to consider that patients which participated in the study were within normal ranges for haematological values and therefore defined as ‘healthy’. However, at the time of the investigation it was not possible to compare findings with possible patient implications (e.g. diabetes) that may influence leukocyte activation. Due to time and financial restrictions, it would have also been interesting to undertake similar investigations involving other chemotactic agents such as fMLP, which is a bacterial peptide that interacts with its specific plasma membrane receptor, formyl peptide receptor, and is considered a potent stimulant of phagocytic leukocytes. Other leukocyte parameters such as elastase and the production of reactive oxygen species, such as hydrogen peroxide or superoxide, could have also been considered for the study. Despite these limitations, the work undertaken in the present study provides a sound platform to undertake further work in the field of leukocyte biology.

4. CONCLUSION

This pilot study confirms the potent effect that PMA has on leukocyte function as indicated in changes to CD62L and CD11b cell surface adhesion molecules. Although studies involving PMA and leukocytes have previously been documented, this study provides a sound platform to continue work in the field of leukocyte biology. However, the validation and reliability of these findings would require to be assessed through the assessment of larger cohorts.

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COMPETING INTERESTS

The author(s) declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

The design of the study and subject recruitment was carried out by JAM and SFH, whilst both SFH and RL were involved in the blood sampling procedures. JAM performed all of the analytical procedures, with help from SFH during blood separation procedures and FC analysis. SFH provided supervisory support during the study and JAM drafted the manuscript. All authors read and approved the final manuscript.

ETHICAL APPROVAL

Ethical Approval was granted by the local ethics committee of the Faculty of Science and Engineering at the University of Chester

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ABBREVIATIONS

PMA – Phorbol 12-myristate 13-acetate

fMLP – N-Formylmethionine-leucyl-phenylalanine

LPS – lipopolysaccharide

HEV – high endothelial venules

ROS – reactive oxygen species

PBS – phosphate buffered saline

SD – standard deviation

MFI – mean fluorescence intensity

PMN – polymorphonuclear

MN – mononuclear