

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

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

34 Integrins, which are large complex transmembrane glycoproteins, have been identified to
35 play a key role in both adhesion and mediation of cell-to-cell interactions such as: cellular
36 trafficking, migration and phagocytosis [3]. CD11b/CD18 is a non-covalently linked
37 heterodimer of α_M and β_2 subunits respectively, on the surface of leukocytes including
38 monocytes and neutrophils. α_M subunits mediate adhesion and spreading on a series of
39 $\alpha_M\beta_2$ ligands including, ICAM-1, whilst the β_2 subunits supported migration and attachment
40 to receptors such as C3bi mediating complement-coated particle uptake [4]. CD62-L (or L-
41 selectin) is constitutively expressed on the surface of monocytes but is rapidly down
42 regulated once the monocytes have been activated. This is due to proteomic cleavage of
43 CD62-L from the surface leaving a portion of the protein, which is soluble and functional
44 [5,6]. Similarly, CD11b is constitutively expressed on the surface of leukocytes, and is stored
45 in the specific granules and transported to the cell surface upon activation. Upon
46 upregulation, the affinity of CD11b receptors are increased where this promotes firm
47 attachment to the endothelium to allow trans-endothelial migration to the site of inflammation
48 or injury [7].

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

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

70 Changes to the expression of CD62-L and CD11b on phagocytic leukocytes have also been
71 reported in a clinical setting, involving orthopaedic patients and those with cardiovascular
72 disease. This in turn, highlights the significance of leukocytes and their interactions with the
73 endothelium in a clinical setting [6, 15, 16]. Phorbol Myristate Acetate (PMA) is a potent
74 tumour promoter, a stimulator of 'respiratory burst' and a membrane-active stimulator of
75 oxidative metabolism, which can be used to show the cytotoxicity of human neutrophils and
76 monocytes [17]. Respiratory or oxidative burst is the release of potent reactive oxygen
77 species (ROS) that are dedicated to aiding the elimination invading microorganisms. Upon
78 cellular activation, cytosolic proteins within the leukocytes translocate to the membrane
79 forming a multi-component electron-transfer system, where this catalyses the reaction
80 forming superoxide anion and hydrogen peroxide, which both have bactericidal properties
81 [18].

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

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

89 2. METHODOLOGY

90

91 2.1 Subject Volunteers

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

Parameter	Mean	SD ($\pm$)	Normal Values*	Reference
WBC	5.58	0.48	$4.0-11.0 \times 10^9/L$	
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	$4.50-6.50 \times 10^{12}/L$	
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 $\times 10^9$ /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 $\pm$ 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-K₂ 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® MicoDiff¹⁸ 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/fluorescein isothiocyanate (Ig/FITC) conjugates (AbD Serotec Ltd., U.K.). Following isolation of leukocyte subpopulations and adjustment of concentration (2×10^6 cells/ml), 10 μ l of the

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

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

135 2.7 Measurement of Cell Surface Expression of CD11b

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

143 2.7 Statistical Analysis

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

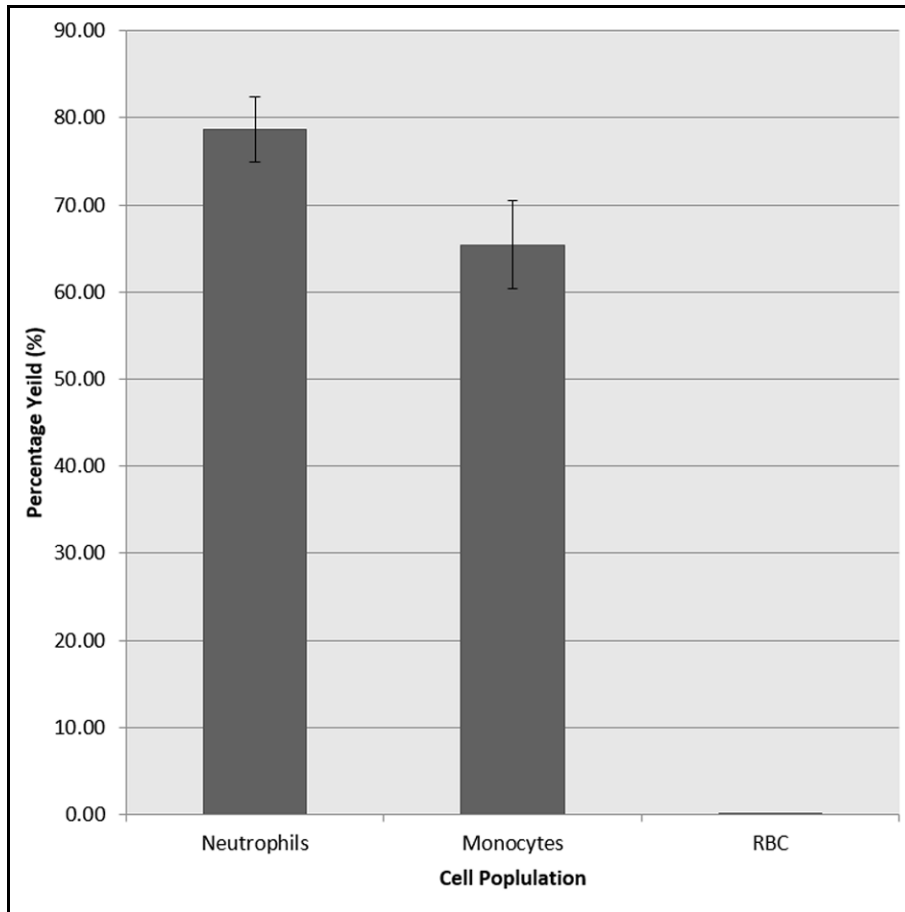
150 3. RESULTS

152 3.1. Full Blood Counts

153 Following venepuncture, red cell, platelet and partial differential white cell counts (full blood
154 counts) were measured using the Coulter[®] MicroDiff¹⁸, confirming normal haematological
155 results (Table 1).

156 3.2 Efficacy of Leukocyte Isolation Methods

157 The efficacy of the density gradient centrifugation to separate PMNs and monocytes from
158 whole blood, as described by Lennie *et al.* for the isolation of leukocyte subpopulations is
159 shown in Figure 1. The isolation of polymorphonuclear (PMN) cells ($77\% \pm 3.33$) was shown
160 to provide a higher mean percentage yield following cell isolation methods, in comparison to
161 mononuclear (MN) cells ($65\% \pm 5.01$) (Figure 1). A possible explanation for this could be due
162 to the vast numbers of PMN cells in comparison to MN cells being present in the normal
163 peripheral blood circulation. The effectiveness of the isolation method was also
164 complimented by the erythrocyte contamination value, which was shown to be 0.04% ($\pm$
165 0.03). Following isolation procedures, PMN (neutrophils) and MN cells (monocytes only)
166 were then used for analysis via flow cytometry, where isolated cells were selected by
167 assignment of gates normally associated with neutrophils and monocytes.

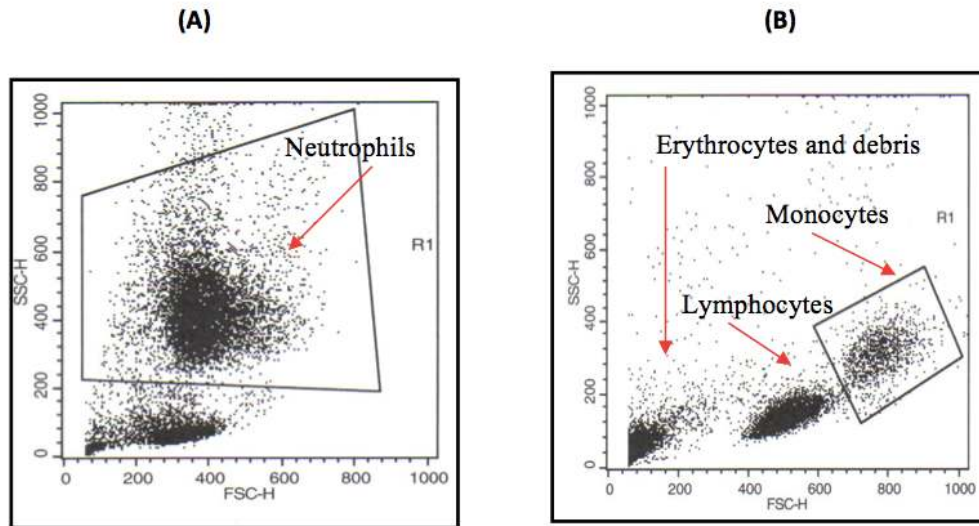


168

169 **Figure 1. Efficacy of the density gradient centrifugation method for the isolation of**
 170 **leukocyte subpopulations was measured using the Coulter® MicroDiff¹⁸ apparatus.**
 171 The data represent mean % yields (± standard deviation) of granulocytes (neutrophils) and
 172 mononuclear cells (lymphocytes and monocytes), by comparison of erythrocyte
 173 contamination.

174 **3.3 Assignment of Flow Cytometric Gates for Isolated Leukocyte** 175 **Subpopulations**

176 During flow cytometry, the gating of phagocytic leukocyte sub-populations (neutrophils and
 177 monocytes) were adjusted so that the percentage of cells analysed were identical to those
 178 identified using a Coulter® MicroDiff¹⁸ apparatus. Lymphocytes, red blood cells and debris
 179 were excluded from defined gates. All flow cytometric analyses were performed using a
 180 Becton and Dickinson FACScan flow cytometer (Figure 2).

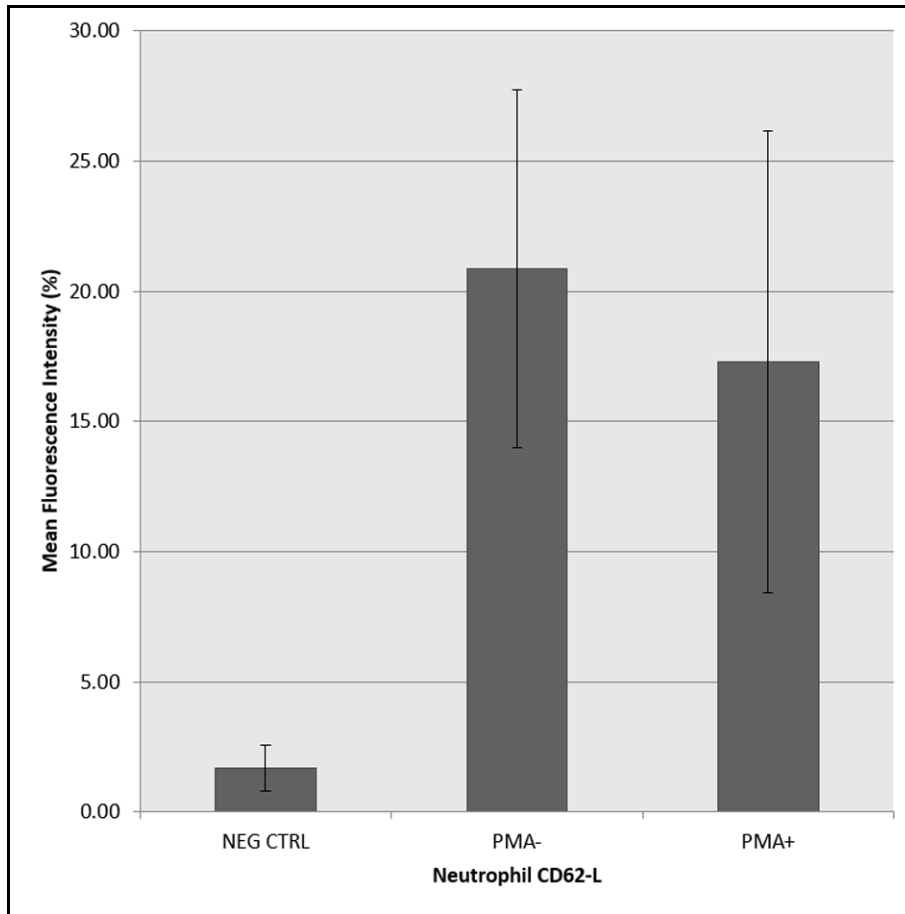


181

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

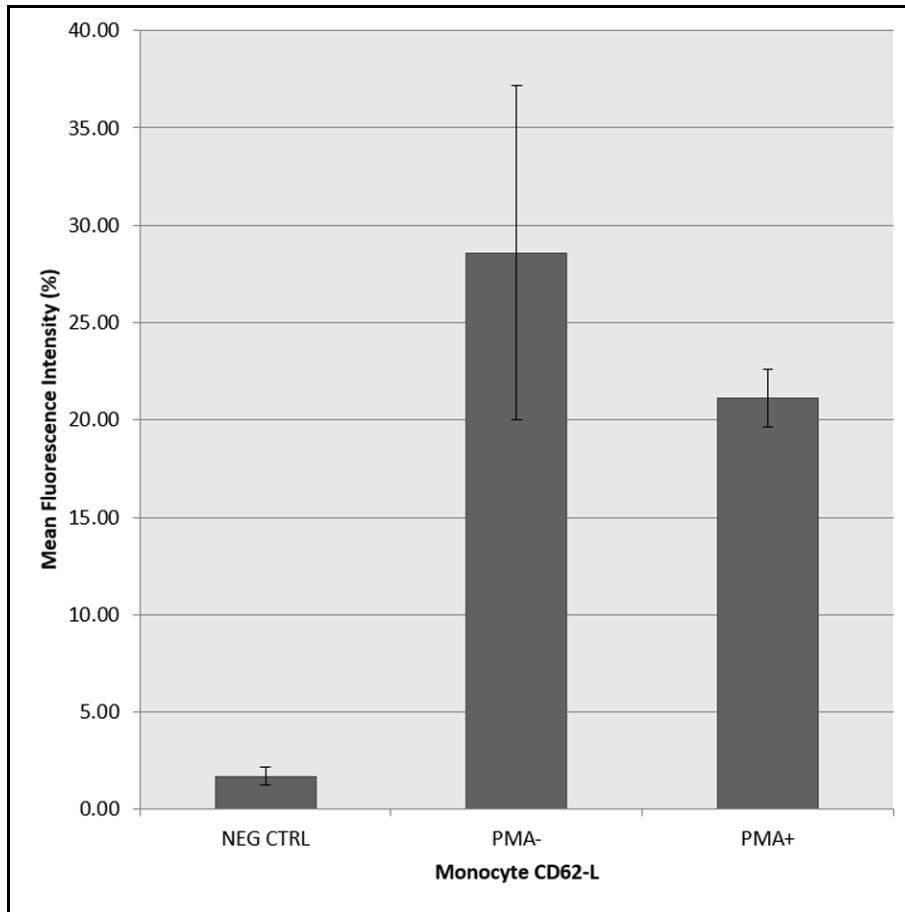
187 **3.4 The Effect of PMA on CD62L Cell Surface Expression of Neutrophils and** 188 **Monocytes**

189 As can be seen from figures 3 and 4, neutrophils and monocytes displayed decreasing
 190 expression of CD62L following treatment with PMA (3.14nM), (un-stimulated neutrophils:
 191 20.86 ± 6.86 ; PMA treated neutrophils: 17.28 ± 8.88 ; un-stimulated monocytes: 28.59 ± 8.57 ;
 192 PMA treated monocytes: 21.12 ± 2.49). **Negative controls of PBS and EDTA were used to**
 193 **show background noise.** Although trends of decreasing neutrophil and monocyte CD62L cell
 194 surface expression was observed following treatment with PMA, these did not show any
 195 statistical differences ($p=0.098$ and $p=0.103$ respectively). Interestingly, monocytes
 196 appeared to express slightly more CD62L on their cell surfaces in comparison to neutrophils,
 197 prior to treatment of cells with PMA. A possible explanation for this could be that monocytes
 198 are morphologically bigger than neutrophils.



199

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

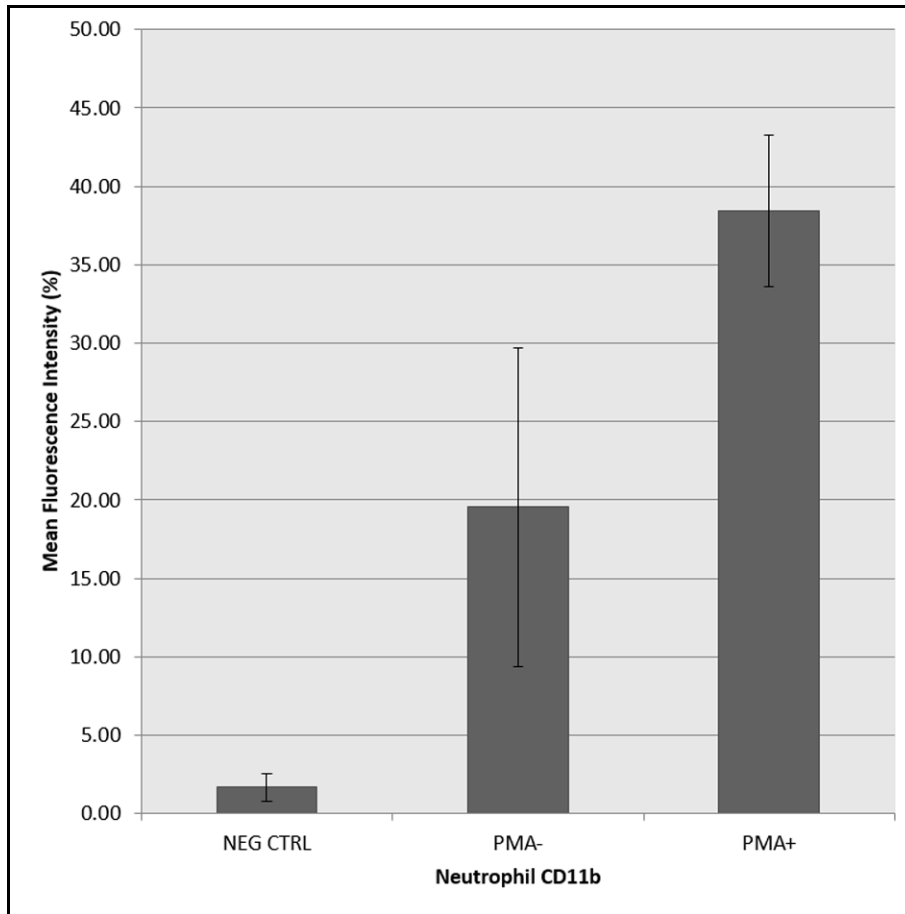


203

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

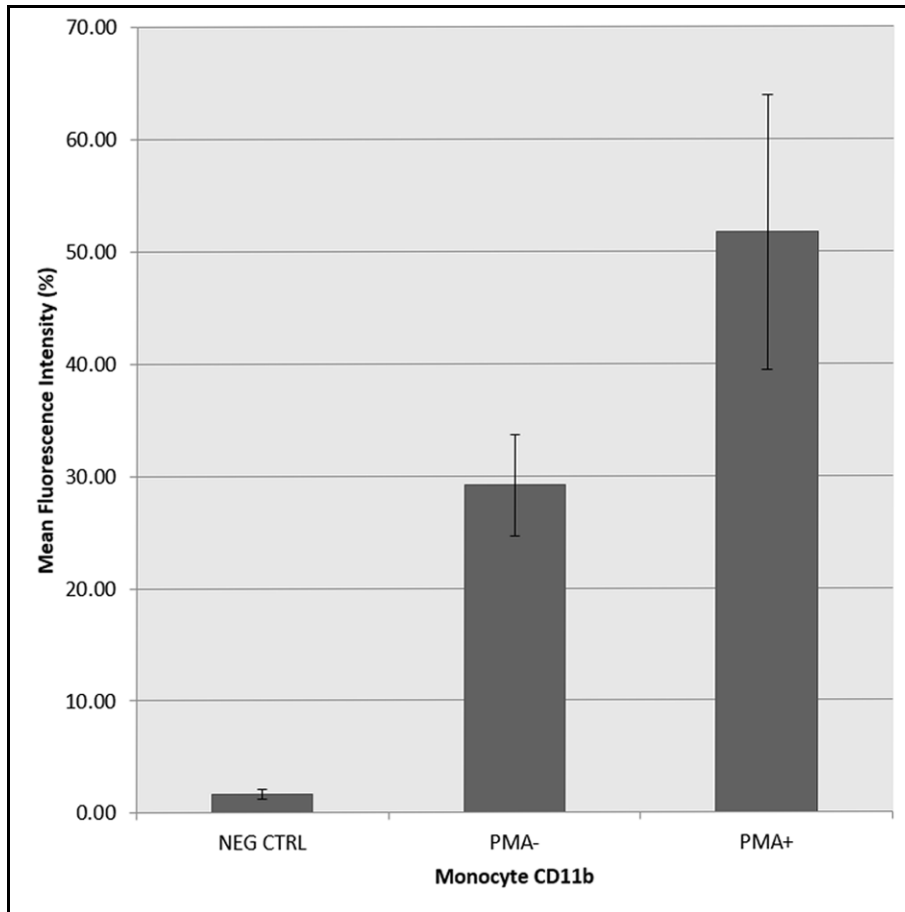
207 **3.5 The Effect of PMA on CD11b Cell Surface Expression of Neutrophils and** 208 **Monocytes**

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



219

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



223

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

227 4. DISCUSSION

228

229 A preliminary part of this research was to verify methods for the isolation of leukocytes, prior
 230 to undertaking further studies. Isolating leukocytes employing a Ficoll-Hypaque density
 231 gradient technique was of importance during these investigations. Optimisation of this
 232 technique allowed for the isolation of concentrated fractionated leukocyte sub-populations of
 233 high purity for use during investigative analysis. Data collected from the isolation of
 234 leukocyte studies demonstrated high percentage yields for both PMN and MN cells. With
 235 regards to PMNs an average yield of 77% (± 3.33) was achieved, with MN cells providing an
 236 average yield of 65% (± 5.01). The efficacy of this leukocyte isolation protocol was also
 237 complemented by the very low erythrocyte contamination, which was found to be 0.04% ($\pm$
 238 0.003) (Figure 1). All blood cell counts performed during this study were analysed using a
 239 highly reliable Coulter[®] MicroDiff¹⁸ analyser. This preliminary study demonstrated the high
 240 degree of reproducibility of this method for the isolation of PMNs and MN cells, prior to
 241 further investigations using gated neutrophil and monocyte cell preparations for flow
 242 cytometric analysis on leukocyte function.

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

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

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

276 Although no power calculations were performed as part of the statistical analysis pertaining
277 to the study, it is acknowledged that a major limiting factor of this study was the relatively
278 small number of subjects recruited. In order to fully appreciate the effects of PMA on the
279 parameters measured, more participants could have been recruited. This in-turn would have
280 been beneficial to some of the statistical trends that were observed, that otherwise may have
281 resulted in significant differences. It is also important to consider that patients which
282 participated in the study were within normal ranges for haematological values and therefore
283 defined as 'healthy'. However, at the time of the investigation it was not possible to compare
284 findings with possible patient implications (e.g. diabetes) that may influence leukocyte
285 activation. Due to time and financial restrictions, it would have also been interesting to
286 undertake similar investigations involving other chemotactic agents such as fMLP, which is a
287 bacterial peptide that interacts with its specific plasma membrane receptor, formyl peptide
288 receptor, and is considered a potent stimulant of phagocytic leukocytes. Other leukocyte
289 parameters such as elastase and the production of reactive oxygen species, such as
290 hydrogen peroxide or superoxide, could have also been considered for the study. Despite
291 these limitations, the work undertaken in the present study provides a sound platform to
292 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

- 427 PMA – Phorbol 12-myristate 13-acetate
428
429 fMLP – N-Formylmethionine-leucyl-phenylalanine
430
431 LPS – lipopolysaccharide
432
433 HEV – high endothelial venules
434
435 ROS – reactive oxygen species
436
437 PBS – phosphate buffered saline
438
439 SD – standard deviation
440
441 MFI – mean fluorescence intensity
442
443 PMN – polymorphonuclear
444
445 MN – mononuclear