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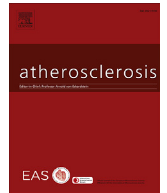

Monocyte inflammatory profile is specific for individuals and associated with altered blood lipid levels

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ABSTRACT

Background and aims: Atherogenesis is dependent upon monocyte influx into the vessel wall. In humans, three monocyte subsets exist, the number and function of which are significantly altered in cardiovascular disease (CVD). Whether such alterations arise in individuals with a perturbed lipid profile remains largely unanswered, but is important to delineate, as adoption of a pro-inflammatory state may promote plaque formation. Here, we compared the inflammatory status of monocyte subsets and determined whether monocyte inflammatory changes are evident in individuals with a perturbed lipid profile.
Methods: Monocyte subset cytokine production, inflammatory and anti-inflammatory marker expression were determined by whole blood flow cytometry and related to participants' lipid levels.
Results: The intermediate and non-classical monocytes were more inflammatory than classicals as seen by their higher cytokine production (TNF-α, IL-1β, IL-6) and M1 marker (CD86) expression, but lower levels of M2 markers (CD93, CD163). More importantly, a considerable variation was seen between participants, with all monocytes of one individual being more inflammatory than those of another. Many inter-individual differences were related to participants' lipid levels. IL-1β production correlated negatively with Apo A1 and HDL-C. CD86 and TLR2 correlated positively with Chol:HDL-C but negatively with HDL-C and Apo A1:Apo B. Interestingly, CD163 expression correlated positively with Chol:HDL-C but negatively with Apo A1:Apo B.
Conclusions: Our data indicates that priming of all monocytes to an inflammatory state occurs in individuals with a perturbed lipid profile, overriding the normal functional distinction attributed to the different monocyte subsets. As such, all monocytes may be important in CVD.
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1. Introduction

Cardiovascular disease (CVD) remains a major problem worldwide [1]. Despite optimal treatment of CVD, there remains a significant residual risk of patients experiencing a clinical event [2]. A major contributor to this residual risk is thought to be inflammation as it is fundamental in plaque development, progression and, ultimately, plaque destabilisation/rupture [3]. Large clinical trials are currently underway to assess the effect of anti-inflammatory agents on CVD event rates [4]. It is recognised, however, that such agents will be associated with their own risks [5], and thus more specific approaches may be required.

A key potential target is the monocyte, as monocyte-derived cells (macrophages) in the plaque are pivotal in both lipid handling and inflammation [6]. In murine models, the continuous influx of monocytes into the plaque is associated with increased plaque size [7] and, accordingly, inhibiting monocyte influx into the plaque reduces its progression [8].

Monocytes are heterogeneous with three different subsets identified in humans, a major subset, the classical: CD14++CD16- (~85%) and two minor subsets, the intermediate: CD14++CD16$^+$ (~5%) and non-classical: CD14+CD16++ (~10%) [9]. Note, this subset division is

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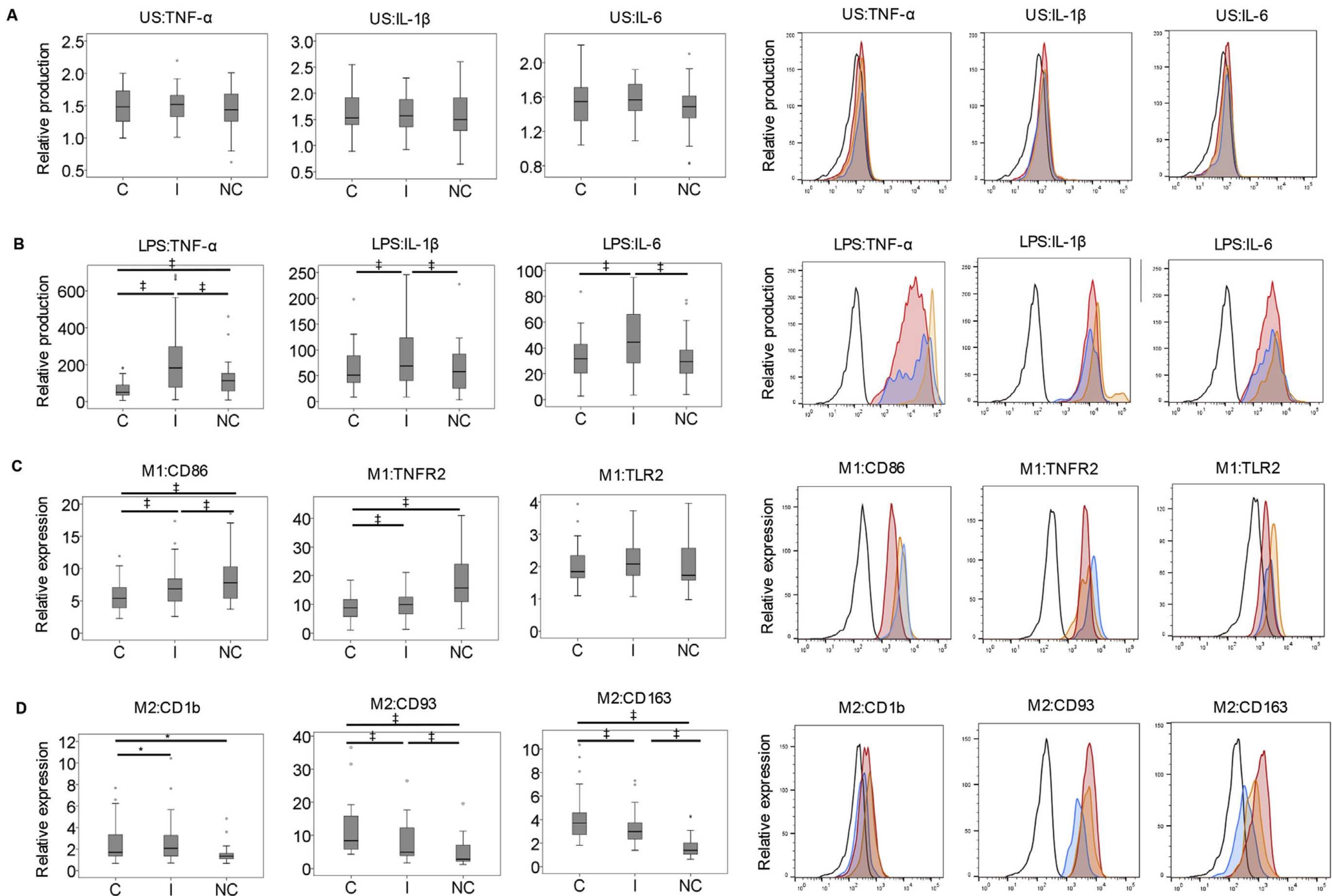


Fig. 1. Comparison of intracellular cytokine production and M1 and M2 marker expression between monocyte subsets.
(A) TNF-α, IL-1β and IL-6 production by unstimulated (US) monocyte subsets. (B) TNF-α, IL-1β and IL-6 production by LPS (1 μg/ml) stimulated monocyte subsets, measured by flow cytometry, n = 30. (C) Expression of M1 markers, CD86, TNFR2 and TLR2 by monocyte subsets, n = 26. (D) Expression of M2 markers, CD1b (n = 23), CD93 and CD163 (n = 26) by monocyte subsets, measured by flow cytometry. C: classical; I: intermediate; and NC: non-classical. Data are presented as box and whisker plots, with outliers denoted by circles and representative histograms. Black lined histograms: isotype control, red histograms: classical monocytes, orange histograms: intermediate monocytes and blue histograms: non-classical monocytes. Statistical calculations of significance were performed using ANOVA followed by the *post hoc* Tukey's test for significant differences between any 2 monocyte subsets: $^{‡}p < 0.01$; $^{*}p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

equivalent to Mon1, Mon2 and Mon3, respectively, as per the recent consensus document [10]. Of these subsets, the proportion of intermediate monocytes is elevated in CVD patients [11,12] and is associated with lipid levels [11,13], plaque morphology [14,15] and occurrence of major clinical events [11,16]. The intermediate subset is, therefore, thought to be a potential treatment target in CVD, akin to other inflammatory conditions [12,16–18]. It is unclear however, whether these associations translate to changes in their function and whether mechanistically, intermediates are the only subset contributing to atherosclerosis.

As the intermediates remain a minor monocyte subset in CVD (<10%), the function of the other subsets, particularly the classical subset given it is the largest proportion, may be important in atherosclerosis progression. Despite speculation in the literature that the intermediate subset is 'pro-atherogenic' [19], in part due to its inflammatory nature [20–22], we found that it was no more inflammatory in CVD patients than in controls, whereas the classical subset was; suggesting that the classical subset also contributes to CVD [23]. With low-density lipoprotein cholesterol (LDL-C) instigating CVD development, here we compared monocyte subset inflammatory profile in a cohort of individuals who were otherwise healthy but differed in their lipid profile. This was done by assessing the expression of inflammatory/pro-atherogenic factors such as cytokine production and expression of CD86, TNFR2 and TLR2 (which are also found on M1 macrophages) [24]. This was counterbalanced by assessing expression of M2 markers, including CD1b, CD93 and CD163 [24]. Results were assessed between subsets, and between participants, as well as for participants relative to their lipid levels.

2. Materials and methods

2.1. Study population

This study was approved by the Western Sydney Local Health District (WSLHD) Human Research Ethics Committee. Informed signed consent was obtained from all participants. We recruited individuals (n = 30) who were in generally good health but differed in lipid levels -with a wide range of lipid levels achieved by including individuals visiting the Westmead Lipid Clinic. Exclusion

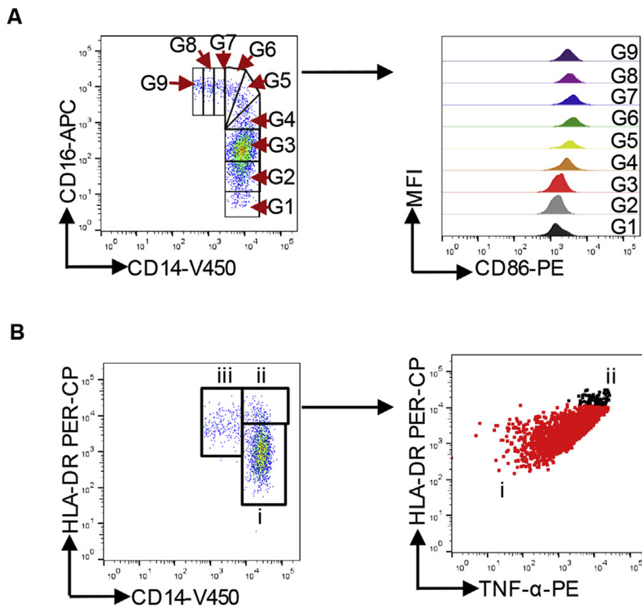


Fig. 2. Differential expression of M1 marker, CD86 on monocyte subsets and comparison of TNF-α production between monocyte subsets.
(A) Division of whole blood monocytes into 9 gates (G1-G9) based on an increasing CD16 expression and a decreasing CD14 expression. The expression of M1 marker, CD86 on whole blood monocyte population (G1-G9). Coloured plots representing G1 to G9 were created using Flow Jo software. (B) Division of whole blood monocytes into 3 gates, i) classical, ii) intermediate and iii) non-classical, based on HLA-DR and CD14 expression. The production of TNF-α upon LPS stimulation (1 μg/ml) is shown on a dot plot. Coloured dots represent i) classical (red) and ii) intermediate (black) monocytes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

criteria included: a documented history of CVD, diagnosed hypertension, diabetes mellitus (Type I or II), a current acute or chronic inflammatory disease (C-reactive protein (CRP) > 5.0 mg/L), being a current smoker, and/or taking lipid-lowering or anti-inflammatory medication.

2.2. Biochemical and lipid measurements

Peripheral blood samples were collected from overnight-fasted participants. Leukocyte counts, cholesterol (Chol), high-density lipoprotein cholesterol (HDL-C), LDL-C, triglyceride, apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B), and CRP were measured using standard laboratory methods at the Institute for Clinical Pathology and Medical Research (ICPMR) at Westmead Hospital.

2.3. Assessment of intracellular cytokine production

The measurement of cytokine production was performed by whole blood flow cytometry on sodium heparinised anti-coagulated blood. Peripheral whole blood aliquots were diluted 1:1 with RPMI 1640 media (Lonza). The cells were stimulated with lipopolysaccharide from *E. coli* at 1 μg/ml (011, Sigma-Aldrich) in the presence of brefeldin-A at 10 μg/ml (B-7651, Sigma-Aldrich) for 4 h at 37°C, 7% CO_2. Unstimulated (brefeldin-A only) wells were used as a control. Subsequently, aliquots (100 μl) were stained with surface monoclonal antibodies anti-CD14-V450 (MΦP9, BD), anti-CD16-APC (3G8, Abcam) and anti-HLA-DR-Per-CP (L243, BioLegend). Cells were lysed with FACS™ lysing solution (BD), centrifuged and incubated with FACS™ Permeabilising solution (BD) for 10 min. After washing, the cells were incubated with intracellular PE antibodies against interleukin (IL)-1β (AS10, BD), IL-6 (AS12, BD), tumour necrosis factor (TNF)-α (6401.1111, BD) or a corresponding isotype control. Samples were resuspended in 1% formaldehyde prior to flow-cytometric analysis.

2.4. Assessment of macrophage polarisation surface markers on monocyte subsets

Surface marker assessment was performed by whole blood flow cytometry on K_2EDTA-anti-coagulated blood. Whole blood aliquots (50 μl) were stained with anti-CD14-V450, anti-CD16-APC and anti-HLA-DR Per-CP to identify monocyte subsets. PE-conjugated antibodies against M1 markers [23–26], CD86 (2331 (FUN-1), BD), TNF receptor (TNFR)-2 (22235, R&D Systems), Toll-like receptor (TLR)-2 (TL2.1, BioLegend), CD319 (162.1, BioLegend) and M2 markers [23–26], CD1b (737249, R&D Systems), CD93 (VIMD2, BioLegend), CD163 (GHI/61, BD) and their corresponding isotype controls were used to determine surface marker expression. The cells were fixed (and red blood cells lysed) by the addition of 250 μl Optilyse C (Beckman coulter).

2.5. Flow cytometry

Flow cytometry was used to detect intracellular cytokine production and monocyte marker expression. For both methods, data was collected on a BD FACS™ Canto II flow cytometer (BD) using FACSdiva software (v6.0, BD). At least 5000 events - based on cells falling in a strong CD14 positive gate on a SSC-A vs. CD14 density plot - were recorded. CompBeads (BD), were used for fluorescence compensation before data analysis which was performed using FlowJo® software (v10.1r5, Tree Star, USA). The gating strategy for defining monocytes for cytokine production and surface marker assessment is presented in Supplemental Fig. 1. The relative level of expression was determined by the ratio of the mean fluorescence intensity (MFI) of the marker of interest over the MFI of the Isotype control as previously reported [23].

2.6. Statistical analysis

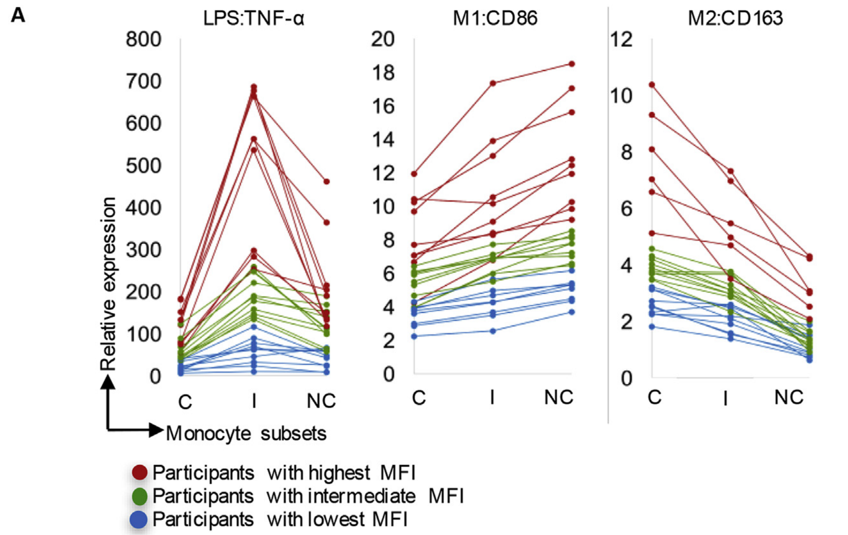
SPSS software (v22, IBM Corporation) was used for statistical analysis and data are shown as mean ± SD unless otherwise stated. Comparisons between monocyte subsets were performed using ANOVA followed by *post-hoc* Tukey's test. Comparisons between genders were conducted using the Student's *t*-test for normally distributed data. Associations between data were assessed using Spearman's rank correlations. All tests were two-tailed.

3. Results

3.1. The inflammatory profile of intermediate and non-classical monocytes is greater than that of classical monocytes

Monocyte cytokine production was assessed by whole-blood intracellular staining. Without stimulation, monocytes (all subsets) produced minimal (and comparable) levels of TNF-α, IL-1β and IL-6 (Fig. 1A). Upon LPS stimulation, the intermediate monocytes produced significantly more TNF-α, IL-1β, and IL-6 than both classical and non-classical subsets (Fig. 1B: $p < 0.01$). For the M1 markers, all subsets expressed CD86, TNFR2 and TLR2 (Fig. 1C). Non-classical monocytes expressed the highest levels of CD86 followed by the intermediate subset (Fig. 1C: both $p < 0.01$). The expression of TNFR2 was highest on non-classical monocytes (Fig. 1C: $p < 0.01$), with a similar expression found between classical and intermediate subsets. All monocyte subsets expressed TLR2 at comparable levels (Fig. 1C). The expression of CD319 was minimal

A



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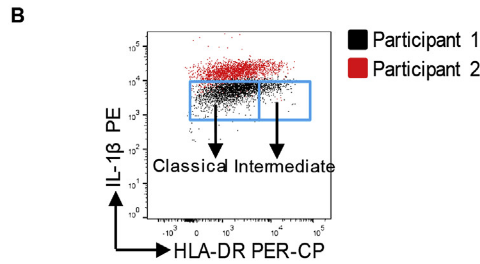

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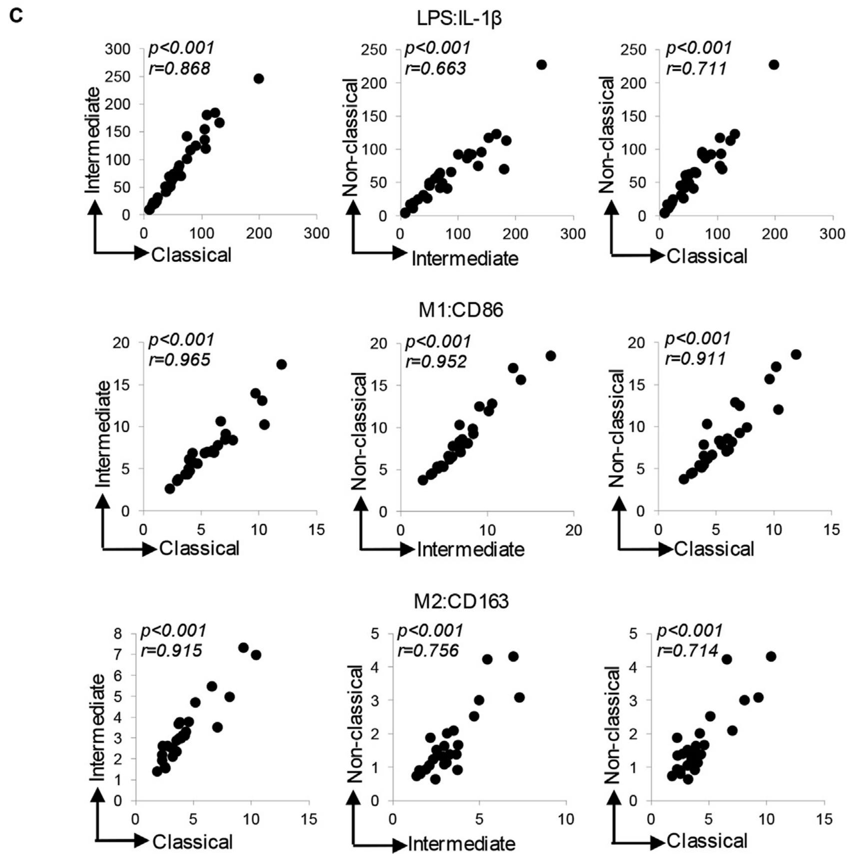

on all subsets (data not shown). With the M2 markers, CD1b was expressed at a similar level by the classical and intermediate subsets but at a lower level on the non-classical monocytes (Fig. 1D: $p < 0.05$). The expression of CD93 and CD163 was higher on the classical subset compared to both the intermediate and non-classical subsets and significantly higher on the intermediate than non-classical subset (Fig. 1D: all $p < 0.01$).

3.2. The functional change is gradual between the monocyte subsets rather than distinct

Given that monocytes differentiate from the classical, through the intermediate to the non-classical subset, we next assessed whether the inflammatory differences seen between the monocyte subsets were distinct or occurred in a gradual manner. We analysed the expression of CD86 by subdividing the monocytes into 9 gates. Following from G1 to G9, a gradual increase in CD86 expression was observed (Fig. 2A). The gradual variation in inflammatory profile through the stages of differentiation was clearly evident on a flow cytometry density plot of TNF-α production, where the level of TNF-α produced by the intermediates was not a distinct jump in expression compared to that of the classicals (Fig. 2B) but rather, an incremental increase.

3.3. Monocyte inflammatory profile varies greatly between participants

Having assessed monocyte subset inflammatory profile for the participants as a whole, we next examined how the monocyte profile differed between individual participants. There were considerable differences between them for all cytokines and markers examined. Importantly, the higher cytokine (TNF-α) production or surface marker expression (CD86, CD163) by one participant compared to another was, in general, evident for all their monocyte subsets (Fig. 3A). This was further evident at the single cell level, as seen on a flow cytometry dot plot where the relative production of IL-1β (by the classical and intermediate monocytes) was elevated in one participant compared with another (Fig. 3B) and, importantly, was as a result of increased expression by all cells. Interestingly, we also observed that the level of inflammation (cytokine production or marker expression) for one subset correlated with that of the next subset (Fig. 3C: all $p < 0.001$).

3.4. Altered lipid levels may be one-factor promoting monocyte subset inflammation

We then assessed whether individuals with a perturbed lipid profile displayed an altered monocyte phenotype and if this explained the variation seen between participants. To ensure that relationships were based on this alone, data for individuals with high fasting glucose (>5.4 mmol/L) or triglycerides (>2.0 mmol/L) were not included in this analysis. There were many correlations between cytokine production or surface marker expression and cholesterol levels (Table 1). Notably, for cytokine production, IL-1β production positively correlated with Chol:HDL-C (all $p < 0.05$) - Table 1 but negatively (in a non-linear relationship) with participants' Apo A1 (Fig. 4A: all $p < 0.001$) and HDL-C levels (Fig. 4A: all $p < 0.01$). For M1 marker expression, CD86 correlated positively with Chol:HDL-C and negatively (in a non-linear relationship) with Apo A1:Apo B (Fig. 4B: all $p < 0.01$). TLR2 expression on all monocyte subsets correlated positively with Chol:HDL-C but negatively with Apo A1:Apo B (Fig. 4C: $p < 0.001$). TNFR2 expression negatively correlated with HDL (classical and intermediate subsets: $p < 0.05$, non-classical: $p < 0.01$) and Apo A1 (classical and non-classical subsets: $p < 0.05$, intermediate: $p = 0.01$) – Table 1. CD319 positively correlated with Chol:HDL (classical and intermediate: $p < 0.01$, non-classical: $p = 0.001$) but negatively correlated with HDL-C (all subsets: $p < 0.05$), ApoA1 (classical: $p < 0.05$ and intermediate: $p < 0.01$) and Apo A1:Apo B (classical and intermediate: $p < 0.01$, non-classical: $p < 0.001$) – Table 1. There were fewer correlations between the M2 markers and lipid levels. CD163 expression on all monocyte subsets, correlated positively with Chol:HDL-C (classical: $p < 0.01$, intermediate: $p < 0.05$ and non-classical: $p = 0.001$) but negatively (in a non-linear relationship) with Apo A1:Apo B (Fig. 4D: classical: $p < 0.01$, intermediate: $p < 0.05$ and non-classical: $p = 0.001$). Note, while there was a spread in participants' ages (Supplemental Table 1), there was no association between age (or gender) and monocyte inflammatory profile (data not shown).

4. Discussion

With an altered lipid profile instigating CVD development, this study was designed to examine the monocyte subset inflammatory profile in individuals with varying lipid profile and assess whether they display an altered monocyte phenotype. While our findings concur that intermediate monocytes are more inflammatory than classical monocytes, they imply that a priming of all monocytes to an inflammatory state is occurring in individuals with a perturbed lipid profile, which overrides the normal functional distinction of the monocyte subsets.

Intermediate monocytes are proposed to be of major clinical importance in CVD, in part because of their inflammatory status [20–22], However, assessment of subset function has primarily been conducted only on small numbers (3–4 participants), and usually with subset isolation, which may have influenced the native state of the cells [22,26,27]. In this study, we assessed monocyte inflammatory status for 30 participants using a whole blood intracellular staining approach [10,20]. That intermediate monocytes produced the highest level of cytokines is consistent with their recognised inflammatory nature [20–22,28]. However, a

Fig. 3. Comparison of monocyte subset inflammatory profile between study participants.
(A) Greater variation in cytokine production and surface marker expression between the participants as well as between the monocyte subsets. Monocyte subset production of TNF-α upon LPS stimulation, CD86 (M1) and CD163 (M2) expression. Each line on the graph is representative of relative cytokine production or surface marker expression (MFI) from one participant. Participants with the highest MFI were ranked based on monocyte subsets expressing the highest TNF-α, CD86 and CD163. Red lines show participants with highest MFI, TNF-α (n = 10), CD86 and CD163 (n = 9), green lines show participants with intermediate MFI, TNF-α (n = 10), CD86 and CD163 (n = 9) and blue lines show participants with lowest MFI, TNF-α (n = 10), CD86 and CD163 (n = 8). C: classical; I: intermediate and NC: non-classical. (B) Differential production of IL-1β upon LPS stimulation on monocyte subsets. The production of LPS-stimulated IL-1β on the classical and intermediate subsets based on HLA-DR expression. The black and red dots indicate the monocytes (classical and intermediate) of participant 1 and 2, respectively. (C) Monocyte subset correlations for cytokine production and surface marker expression. The production of IL-1β upon LPS stimulation; Classical *vs.* Intermediate, Intermediate *vs.* Non-classical and Classical *vs.* Non-classical (all $p < 0.001$; n = 30). (D) Expression of M1 marker, CD86 (all $p < 0.001$; n = 26). (E) Expression of M2 marker, CD163 (all $p < 0.001$; n = 26). Data are presented as scatter plots. The statistical significance of correlation was determined by Spearman's rank correlation. All tests were two tailed. r represents correlation coefficient. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Lipid correlations with monocyte subset cytokine production and surface marker expression.

			Cholesterol (mM)		LDL-C (mM)		HDL-C (mM)		Chol:HDL-C ratio		Apo A1 (g/L)		Apo B (g/L)		Apo A1: Apo B ratio	
			r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Cytokines	C:TNF-α	n = 22	−0.090	0.689	−0.110	0.625	−0.124	0.582	−0.023	0.918	−0.263	0.236	−0.108	0.633	−0.037	0.871
	I:TNF-α		−0.118	00.601	−0.079	0.726	−00.190	0.398	0.026	0.908	−0.284	0.201	−0.056	0.805	−0.078	0.728
	NC:TNF-α		−0.293	00.185	−0.225	0.315	−0.286	0.196	−0.022	0.922	**−0.501***	**0.018**	−0.190	0.396	−0.068	0.763
	C:IL-1β		0.024	0.914	0.216	0.335	**−0.607****	**0.003**	**0.484***	**0.022**	**−0.693****	**0.000**	0.041	0.855	**−0.482***	**0.023**
	I:IL-1β		−0.076	0.736	0.155	0.491	**−0.615****	**0.002**	**0.462***	**0.031**	**−0.686****	**0.000**	0.032	0.887	**−0.441***	**0.040**
	NC:IL-1β		−0.027	0.907	0.169	0.452	**−0.665****	**0.001**	**0.522***	**0.013**	**−0.688****	**0.000**	0.088	0.699	**−0.522***	**0.013**
	C:IL-6		0.052	0.818	−0.006	0.980	−0.005	0.984	−0.016	0.942	−0.199	0.375	0.027	0.907	−0.080	0.725
	I:IL-6		−0.119	0.599	−0.119	0.598	−0.125	0.578	0.013	0.954	−0.260	0.243	−0.008	0.970	−0.075	0.740
	NC:IL-6		−0.043	0.849	−0.098	0.665	−0.042	0.853	−0.026	0.908	−0.250	0.261	−0.008	0.970	−0.084	0.710
M1 markers	C:CD86	n = 20	0.334	0.151	0.433	0.056	**−0.519***	**0.019**	**0.613****	**0.004**	−0.410	0.073	0.325	0.162	**−0.612****	**0.004**
	I:CD86		0.282	0.229	0.396	0.084	**−0.533***	**0.016**	**0.611****	**0.004**	−0.433	0.056	0.322	0.166	**−0.610****	**0.004**
	NC:CD86		0.209	0.376	0.346	0.135	**−0.546***	**0.013**	**0.596****	**0.006**	**−0.457***	**0.043**	0.233	0.323	**−0.571****	**0.008**
	C:TNFR2		0.130	0.586	0.359	0.120	**−0.526***	**0.017**	**0.476***	**0.034**	**−0.485***	**0.030**	0.095	0.691	−0.435	0.055
	I:TNFR2		−0.099	0.677	0.186	0.432	**−0.510***	**0.022**	0.341	0.141	**−0.560***	**0.010**	−0.110	0.645	−0.296	0.206
	NC:TNFR2		0.055	0.818	0.307	0.187	**−0.572****	**0.008**	**0.532***	**0.016**	**−0.478***	**0.033**	0.114	0.631	**−0.454***	**0.044**
	C:CD319		0.163	0.491	0.436	0.055	**−0.520***	**0.019**	**0.639****	**0.002**	**−0.479***	**0.033**	0.189	0.424	**−0.604****	**0.005**
	I:CD319		0.133	0.578	0.424	0.063	**−0.555***	**0.011**	**0.647****	**0.002**	**−0.627****	**0.003**	0.150	0.527	**−0.643****	**0.002**
	NC:CD319		**0.462***	**0.040**	**0.598****	**0.005**	**−0.480***	**0.032**	**0.704****	**0.001**	−0.436	0.054	0.340	0.143	**−0.723****	**0.000**
	C:TLR2		**0.507***	**0.023**	**0.595****	**0.006**	**−0.470***	**0.036**	**0.786****	**0.000**	−0.286	0.222	**0.516***	**0.020**	**−0.758****	**0.000**
	I:TLR2		**0.460***	**0.041**	**0.500***	**0.025**	−0.412	0.071	**0.732****	**0.000**	−0.248	0.293	**0.492***	**0.028**	**−0.718****	**0.000**
	NC:TLR2		**0.481***	**0.032**	**0.540***	**0.014**	**−0.493***	**0.027**	**0.775****	**0.000**	−0.272	0.247	**0.472***	**0.036**	**−0.737****	**0.000**
M2 markers	C:CD1b	n = 13	0.052	0.865	0.251	0.409	−0.110	0.720	0.267	0.378	−0.129	0.674	0.418	0.156	−0.220	0.471
	I:CD1b		−0.047	0.879	0.237	0.436	−0.173	0.571	0.256	0.399	−0.193	0.528	0.379	0.201	−0.203	0.505
	NC:CD1b		0.107	0.727	0.314	0.296	−0.283	0.348	0.338	0.258	−0.327	0.275	0.423	0.150	−0.330	0.271
	C:CD93	n = 20	0.242	0.303	0.249	0.290	−0.326	0.161	0.425	0.062	−0.108	0.652	**0.635****	**0.003**	−0.411	0.072
	I:CD93		0.090	0.705	0.189	0.424	**−0.457***	**0.043**	**0.459***	**0.042**	−0.293	0.210	**0.564****	**0.010**	−0.426	0.061
	NC:CD93		0.261	0.266	0.333	0.151	−0.312	0.180	0.408	0.074	−0.277	0.237	**0.532***	**0.016**	**−0.445***	**0.049**
	C:CD163		**0.618****	**0.004**	**0.737****	**0.000**	−0.262	0.265	**0.611****	**0.004**	−0.141	0.552	**0.510***	**0.022**	**−0.602****	**0.005**
	I:CD163		**0.565****	**0.009**	**0.619****	**0.004**	−0.180	0.448	**0.481***	**0.032**	−0.011	0.962	**0.450***	**0.047**	**−0.465***	**0.039**
	NC:CD163		**0.527***	**0.017**	**0.603****	**0.005**	−0.432	0.057	**0.688****	**0.001**	−0.219	0.354	**0.483***	**0.031**	**−0.666****	**0.001**

C: classical; I: intermediate; NC: non-classical; r: correlation coefficient; *p*: level of significance (2-tailed), $^{**}p \leq 0.01$; $^{*}p \leq 0.05$ (Spearman's rank correlations). Significant values are shown as bold.

drawback was that we were not able to assess anti-inflammatory cytokine production as IL-10 was not detected at the time point used.

Assessment of M1/M2 marker expression on monocytes gave a pro- and anti-inflammatory balance, similar to studies performed in obesity and diabetes [29,30]. The classical subset was seen to be the most anti-inflammatory. Indeed, our findings, from cytokine production and surface marker expression combined, present a picture similar to previous studies [20–22,26,27] which show that CD16 + monocytes (intermediates and non-classicals) are more inflammatory than classical monocytes and furthermore, extend these findings to show that this remains the case even when including individuals with an altered lipid profile. Consistent with the fact that monocytes differentiate from the classical to non-classical subset through the intermediate subset [31], changes in marker (or cytokine) expression occured gradually as the cells transited from one subset to another.

A unique finding of this study was the large variation in the monocyte inflammatory profile between the individuals. Notably, the higher cytokine production (or marker expression) by one individual compared to another was evident for not just one monocyte subset, but for them all. That particular functions may be increased in all monocytes, rather than being the domain of one subset in particular, is a paradigm not yet fully appreciated in the literature - where, though the developmental relationship is appreciated, distinct roles for the subsets are proposed [9,21,26,32].

The difference in the classical monocytes of one individual compared to another not only indicates that monocytes became more inflammatory prior to differentiation into the intermediate subset, but suggests that monocytes in some individuals are likely to be entering the circulation in an inflammatory state. Monocytes are now described (in various models) to undergo functional reprogramming, that is, they are primed (in the circulation) in response to systemic signals to alter their function prior to entry into tissues [33,34]. Our results here raise the question as to whether this may be occurring at the bone marrow level.

A key finding was that participants' cytokine production (or surface marker expression) by one subset correlated with that of the next. This indicates that the inflammatory state acquired throughout differentiation (from classical to non-classical) is dictated by the inflammatory profile of the emerging classical subset. Thus, though the intermediate subset is considered inflammatory, the degree to which it becomes inflammatory is not a factor particular to the subset itself.

Interestingly, the inflammatory state of the monocyte subsets was related to the participants' lipid levels. Several associations were found for IL-1β. The association with Chol:HDL-C indicates that monocytes display an increased inflammatory state with increased cardiovascular risk. However, the inverse correlations of IL-1β with HDL-C and Apo A1, but not with cholesterol itself, or with Apo B, suggests that it is the level of HDL-C that impacts IL-1β production. Indeed, HDL-C and Apo A1 have been shown to inhibit monocyte cytokine production *in vitro* [35–38]. Interestingly, the inverse relationships were non-linear, suggesting that there is a point at which increased HDL-C (and Apo A1) affords no additional anti-inflammatory protection. This is similar to clinical findings where there is likewise a point at which, with increased HDL-C, a further reduction in cardiovascular risk is not observed [39–41]. As

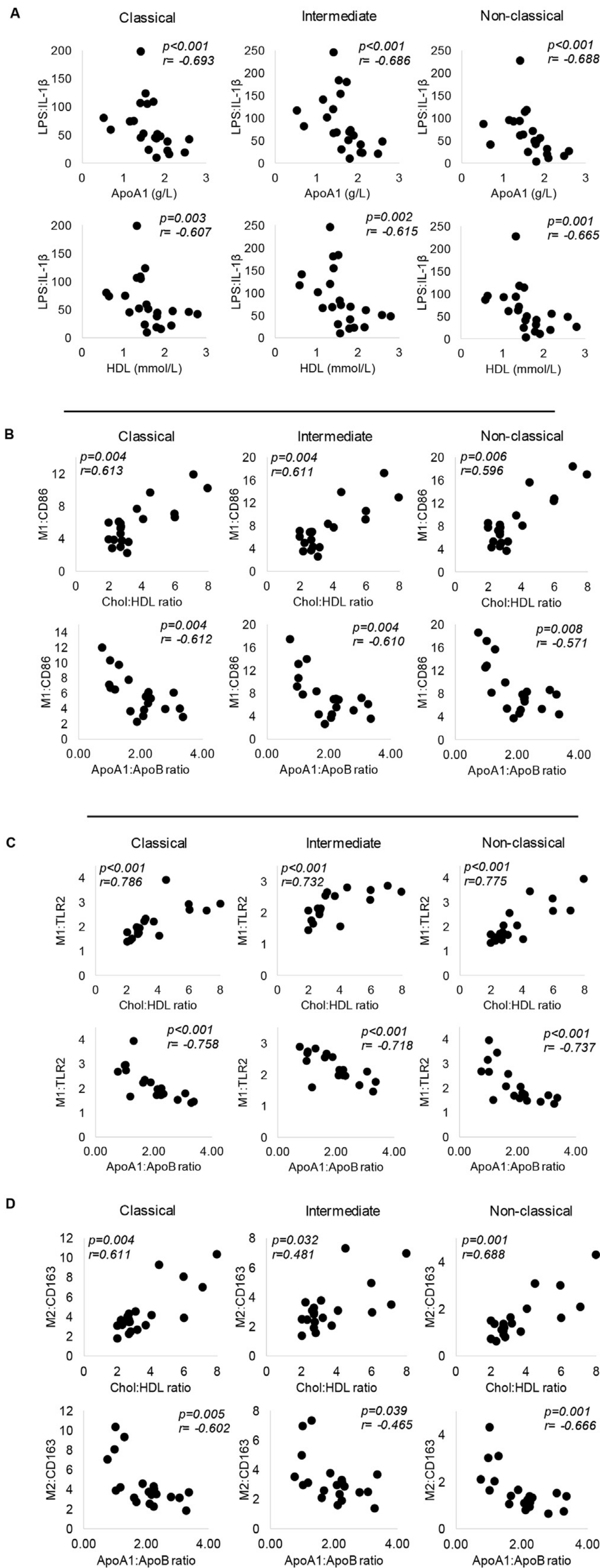

Fig. 4. Lipid correlations for cytokine production and surface marker expression on all monocyte subsets.
(A) Classical, Intermediate and Non-classical correlations of IL-1β (upon LPS stimulation) with Apo A1 and HDL-C, n = 22. (B and C) Classical, Intermediate and Non-classical correlations of M1 markers (CD86 and TLR2) with Chol:HDL-C ratio and Apo A1:Apo B, n = 20. (D) Classical, Intermediate and Non-classical correlations of M2 marker (CD163) with Chol:HDL-C ratio and Apo A1:Apo B, n = 20. Data are presented as scatter plots. The statistical significance of correlation was determined by Spearman's rank correlation. All tests were two-tailed. r represents correlation coefficient.

such, elevating HDL-C may not dampen IL-1β production. The lack of association between lipids and TNF-α or IL-6 may be because their production is regulated by different pathways [5].

Similarly, the inflammatory markers (M1: CD86, TNFR2, TLR2 and CD319) displayed positive correlations with Chol:HDL-C, with TNFR2 and CD319 also displaying inverse relationships with HDL-C, Apo A1 and Apo A1:Apo B. This further demonstrates that increased cardiovascular risk is associated with increased monocyte inflammation, the degree of which is likely regulated by Apo A1 and HDL-C.

Interestingly, the associations with TLR2 stood out as unique as its expression also correlated with total cholesterol, LDL-C and Apo B levels, but not Apo A1. Indeed, previous studies have shown that minimally modified LDL-C can activate CD14, TLR4 and TLR2 inducing inflammatory cytokine production [42]. The increased TLR2 (on all monocyte subsets) in individuals with high cholesterol may promote plaque development as TLR activation in mice has been found to promote monocyte recruitment [43], the formation of M1 macrophages [44–47], and foam cell formation [44,48]. The lack of association with Apo A1 suggests that the anti-inflammatory properties of Apo A1 do not extend to TLR2. Compared with the M1 markers, relatively few correlations were evident with the M2 markers, implying that a more inflammatory monocyte profile in the circulation would primarily be due to increased inflammation, rather than suppression of an anti-inflammatory response. However, surprisingly, positive correlations were evident between CD163 and cholesterol, LDL-C and Apo B levels. Given that macrophages express higher levels of CD163 than monocytes [49], then the observed increase in expression may be a factor of an increased maturation of the monocyte, rather than a reflection of an M2 function, particularly as minimal association was seen with the other M2 markers. The lack of association with HDL-C or Apo A1 suggests that increased cholesterol may be driving monocyte maturation.

A key finding is that the correlations between lipids and monocyte inflammation were mostly evident for all three of the monocytes subsets indicating that, all monocytes, not just the minor intermediate subset, were circulating inflammatory in individuals with perturbed lipid levels. The impact of this on plaque development is yet unclear. However, monocyte adoption of an inflammatory phenotype would be expected to promote plaque development, as they would enter the vessel wall primed to become an inflammatory macrophage.

In summary, our results are consistent with the understanding that intermediate and non-classical monocytes are more inflammatory than classical monocytes. However, these inter-subset differences were overridden by inter-participant differences with all monocytes in one individual being more inflammatory than those of another. The acquired heightened inflammatory state is likely occurring at the bone marrow level and recapitulated across the monocyte subsets. Perturbed lipid levels may be a key factor promoting increased monocyte inflammation. This highlights a need to intervene early, as such changes (increased monocyte inflammation, not just lipids) in these individuals could be promoting plaque development.

While it has been proposed that the minor intermediate subset is a potential treatment target for CVD [11,16], we propose that all monocytes are likely to be contributing to the disease. Given that

most monocyte inflammatory associations were found with HDL-C and Apo A1 rather than LDL-C, lowering LDL-C alone without improving functional HDL-C (and Apo A1 levels) is not likely to fully reverse monocyte inflammation and thus pro-atherogenic functions will persist. Indeed, this may be one contributing factor to the residual CVD risk that remains after optimal treatment.

Conflict of interest

The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

V.P. designed and performed the experiments, analysed the results, performed statistical analysis, made the figures and wrote the manuscript. H.M. planned the project, and with H.W. supervised the work and contributed to data interpretation and manuscript writing. S.L. identified suitable patients and with V.P. collected patient information. The project was conducted under J.F. All authors critically reviewed drafts and approved manuscript submission.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2017.05.026.

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