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NATIVE LDL PROMOTES DIFFERENTIATION OF HUMAN MONOCYTES TO MACROPHAGES WITH AN INFLAMMATORY PHENOTYPE

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Effect of native LDL on macrophage polarisation

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Summary

Recruitment of monocytes in atherosclerosis is dependent upon increased levels of plasma lipoproteins which accumulate in the blood vessel wall. The extracellular milieu can influence the phenotype of monocyte subsets (classical: CD14⁺⁺CD16⁻, intermediate: CD14⁺CD16⁺ and non-classical: CD14^{dim}CD16⁺⁺) and macrophages (M1 or M2) and consequently the initiation, progression and/or regression of atherosclerosis. However, it is not known what effect lipoproteins, in particular native low-density lipoproteins (nLDL), have on the polarisation of monocyte-derived macrophages. Monocytes were differentiated into macrophages in the presence of nLDL. nLDL increased gene expression of the inflammatory cytokines *TNF α* and IL-6 in macrophages polarised towards the M1 phenotype and decreased the M2 phenotype surface markers, CD206 and CD200R and the anti-inflammatory cytokines TGF β and IL-10. Compared to the classical and intermediate subsets, the non-classical subset-derived macrophages had a reduced ability to respond to M1 stimuli (LPS and IFN γ). nLDL enhanced the *TNF α* and IL-6 gene expression in macrophages from all monocyte subsets, indicating an inflammatory effect of nLDL. Further, the classical and intermediate subsets both responded to M2 stimuli (IL-4) with upregulation of TGF β and SR-B1 mRNA; an effect, which was reduced by nLDL. In contrast, the non-classical subset failed to respond to IL-4 or nLDL, suggesting it may be unable to polarise into M2 macrophages. Our data suggests that monocyte interaction with nLDL significantly affects macrophage polarisation and phenotype and that this interaction appears to be subset dependent.

Keywords

Monocyte subsets, macrophages, native LDL, polarisation

Abbreviations

CVD	Cardiovascular disease
LDL	Low Density lipoprotein
nLDL	Native Low Density lipoprotein
ROS	Reactive oxygen species
M-CSF	Macrophage – Colony stimulating factor
IL -	Interleukin -
TNF α	Tumor Necrosis Factor alpha
oxLDL	Oxidised low density lipoprotein
IFN γ	Interferon gamma
TH1	T-1 helper cells
LPS	Lipopolysaccharide
TGF- β	Transforming Growth Factor beta
ABCA-1	Adenosine-triphosphate-binding cassette protein 1
SR	Scavenger receptor
TLR	Toll-like receptor
SRB1	Scavenger receptor B1
PBMCs	Peripheral blood mono-nuclear cells
PBS	Phosphate buffered solution
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
RBC	Red blood cells
PBS	Phosphate buffered solution

Introduction

A hallmark of atherosclerosis is increased levels of cholesterol-rich plasma lipoproteins, which become deposited in the artery wall, leading to monocyte-macrophage recruitment. Macrophage cholesterol homeostasis, involving the balance of cholesterol uptake and efflux, is critical in the progression and/or regression of atherosclerosis. Upon differentiation of monocytes to macrophages, the affinity of modified low density lipoprotein (LDL) particles such as oxidized (ox)LDL for the LDL-receptor is lost as these modified particles are recognized by toll like receptor (TLR) - 4 and scavenger receptors (SRs), including CD36 and SR-A (1). This is a well-studied pathway which results in the internalization of modified LDL particles, creating foam cells and ultimately driving the progression of atherosclerotic plaques. However, the influence of native (n)LDL on monocytes as they mature into macrophages is unknown.

Monocytes make up about 10% of leukocytes in human blood and depending on their subset type, either circulate freely or patrol blood vessels for several days. Some then differentiate to either macrophages or dendritic cells upon tissue infiltration (2). Although human monocytes were once thought to be homogenous, the heterogeneity of monocytes, based on the expression of the cell surface markers CD14 (co-LPS receptor) and CD16 (FcγIII receptor) is now commonly used to characterise three bonafide monocyte subsets: classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺CD16⁺) and non-classical (CD14^{dim}CD16⁺⁺) (3). These subsets differ in many respects, including in their expression of adhesion molecules, chemokine receptors and functionality (4). The ability of monocytes to differentiate to various cell types could reflect their 'plasticity' in response to the extracellular milieu, but may also relate to the intrinsic properties of each subset. The role of each subset in human disease is not well characterized beyond associations made between some subsets counts and disease. For example, patients with coronary artery disease (5-7) or vulnerable plaque (8) have higher numbers of CD16⁺ monocytes. On the other hand, a decrease in these monocytes is associated with plaque stabilization (9). Furthermore, high cell counts of CD16⁺ monocytes appear to predict cardiovascular events (3) and the monocyte subsets counts after stroke (10) and myocardial infarction (11) positively predict clinical course and prognosis.

There is some evidence to suggest that each monocyte subset interacts differently with lipids. Differences in the expression of SRs and the subsequent uptake of native and oxLDL cholesterol in different monocyte subsets have been reported (12-14). Small epidemiological studies show a positive correlation between LDL cholesterol and CD16⁺ monocyte counts (3, 15-17). There are also reports on the effect of statins on monocyte subsets but these have been ambiguous, where either no change or an increase in CD16⁺ monocytes have been reported (9, 17, 18). However, little is known neither about how plasma nLDL particles affect monocyte subsets counts nor on the intrinsic ability of each subset to differentiate and polarise to specific macrophage subsets. While there is intense study on macrophage inflammatory processes in their post-polarised state involving TLR4 (19), little has been done in their pre-polarised state and the predetermined capacity of each monocyte subset to differentiate and polarise into macrophages, nor on how extracellular lipoproteins affect this capacity.

In respects to how macrophages function once they are formed, the current simplified classification of their phenotypes, mirroring the Th1/Th2 dichotomy, is the discrimination between M1 (classically activated) and M2 (alternatively activated) macrophages. M1 macrophages can be induced by Interferon - γ (IFNγ) alone, or with microbial stimuli such as Lipopolysaccharide (LPS), and have the capacity to increase and sustain the ongoing inflammatory response by producing mediators such as Tumor Necrosis Factor - α (TNFα), Interleukin (IL)-6, IL-1β and IL-12. They remove bacteria, protozoa and viruses through the production of cytotoxins (e.g. reactive oxygen species; ROS). However, continuous

induction of M1 can cause tissue damage and impair wound healing. Alternatively, M2 macrophages, originally discovered as a response to IL4 (20), are composed of expanding subtypes: M2a polarised by IL-4 or IL-13, M2b by some immune complexes and M2c by IL-10 or glucocorticoids (21). Despite these subtype differences, M2 macrophages in general promote tissue repair and healing, are prominent producers of anti-inflammatory cytokines (e.g. IL-10 and Transforming Growth Factor – β (TGF β)) and are critical for suppressing the immune response. This study sought to determine if exposure of human monocytes to nLDL, as seen in the circulation of people at risk of cardiovascular disease (CVD), influences the ability of monocytes to differentiate into macrophages and also, how these macrophages polarize when incubated with the M1 or M2 stimuli mentioned above. Further, we examined if different monocyte subsets behave differentially in response to nLDL and their maturation to macrophage subsets, which adds to our knowledge on the relationship between monocyte subsets and CVD.

Methods

Isolation of LDL

Plasma from healthy donors was obtained from the Australian Red Cross Blood Service. Volume of plasma was 240 mL extracted from an individual blood donation. Plasma density was adjusted to 1.109g/L by the addition of 0.018g KBr/mL and spun for 18 hrs at 65,000rpm at 4°C. The upper fraction containing VLDL was removed and the density of the lower fraction containing LDL and HDL was adjusted to 1.063g/L by the addition of 0.064 g/mL KBr and spun for 18 hrs at 65,000 rpm at 4°C. The upper fraction containing LDL was removed and dialysed against PBS + 0.001% ethylenediaminetetraacetic acid (EDTA) for 3 days in a cold room in dark conditions with the buffer replaced daily. Isolated LDL was stored at -80°C until required.

Isolation of human monocytes

Human monocytes were obtained from buffy coats (60 mL from an individual donor containing white blood cells, red blood cells, platelets and plasma) from healthy volunteers provided by the Australian Red Cross Blood Service (Ethics #14-11vic-08). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density gradient centrifugation. Monocytes were isolated from PBMCs using a human pan monocyte negative selection kit (Miltenyi Biotec, Germany) as per the manufacturer's directions. Flow cytometry using CD14⁺/CD16⁺ expression was used to assess purity which was greater than 90%.

Monocyte subset isolation

PBMCs were resuspended in sorting buffer (0.01% EDTA and 0.2% FCS in PBS; 10 μ L per 10⁶ cells) and stained with anti-human antibodies (BD Pharmingen, CA, USA) specific for anti-HLA-DR (TU36, FITC), anti-CD14 (M5E2, PacB), anti-CD16 (3G8, PE CY7), anti-CD2 (RPA-2.10, PE), anti-CD19 (HIB19, PE), anti-CD15 (VIMC6, PE), anti-CD56 (MY31, PE) and anti-NKp46 (BAB281, PE) for 25 mins in darkness at 4°C. Cells were sorted using the FACS Aria I (BD Biosciences, San Jose, CA) and the cell solution was gated by size and granularity (FSC and SSC) for the selection of the desired cell populations (Supp Figure 1). Gating against cell doublets was achieved through FSC-Area/Height. Isolated monocyte populations were >98% pure.

Monocyte to macrophage differentiation and polarisation

Monocytes (1x10⁶/mL) were re-suspended in culture medium (RPMI 1640; supplemented with penicillin 100IU/mL, streptomycin 100g/mL, 1% MEM NEAA and 10% FCS) in the presence and absence of nLDL (200 μ g/mL) for 7 days. The differentiation into macrophages was promoted by the addition of M-CSF; 100ng/ μ L. After 7 days, cells were either untreated (M0) or treated with LPS (100ng/mL) plus IFN γ (20ng/mL) or IL-4 (20ng/mL) to induce polarisation into the M1 and M2 phenotypes, respectively. Cells

were polarised for either 6 hrs for real time-PCR or 24 hrs for flow cytometry (surface marker expression) analysis.

Quantitative Real-Time PCR

Total RNA was extracted from macrophages using TRIzol (Life Technologies, NY, USA) and treated with RNase-free DNase (Promega, Madison, WI). Total RNA (2µg) was transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI). cDNA (100ng) was used to determine target gene mRNA levels using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using fast Syber Green chemistry (Roche Diagnostics, IN, USA), according to manufacturer's specifications. Gene expression for each gene of interest relative to 18S in each sample was determined using the comparative ($\Delta\Delta CT$) method. Primers were ordered from GeneWorks (Thebarton, SA, Australia) and sequences are available upon request.

Surface marker expression

Protein expression of CD64, CD200R and CD206 was determined in macrophages using flow cytometry with the FACS Calibur using Cell Quest Pro software (BD Biosciences, CA, USA). Harvested cells were stained with anti-CD64 (10.1; FITC, R&D systems, Australia) to detect M1 macrophages or 5ng/µl of anti-CD206 (19.2; FITC, BD Biosciences, Australia) or anti-CD200R (380529; APC, R&D systems, Australia) to detect M2 macrophages. Corresponding isotype controls were used; IgG1 control (11711; FITC, R&D systems, Australia) for anti-CD64, IgG1κ control (MOPC-21; FITC, BD Biosciences, Australia) for anti-CD206 and IgG control (133303; APC, R&D systems, Australia) for CD200R. 10,000 cells were counted per sample and the read-out was analysed using CellQuest Pro software (BD Biosciences, San Diego, CA).

Statistics

All values are expressed as mean $\pm$ SEM with the number of monocyte isolations from each individual blood donor denoted by n, each performed in triplicates, $P < 0.05$ was considered statistically significant. All statistical calculations were performed using GraphPad Prism Version 6.0. Student's unpaired t-test, 1-way or 2-way ANOVA was used to compare mean pEC_{50} values between two or between three or more experimental groups, respectively. A Tukey post hoc analysis was performed when the ANOVA indicated statistical differences. Please note that M0 macrophages were used as a reference only to show that macrophage polarization was achieved using the appropriate stimuli. As M0 and M1 or M2 are different cells, we only compared the LDL treatment in the respective cell type.

Results

Native LDL increases *TNF α* and *IL-6* gene expression in M1 macrophages

In the setting of CVD, monocytes are exposed to high levels of nLDL in circulation before they enter the atherosclerotic lesion. Thus we aimed to determine if incubating monocytes in nLDL affects the ability of monocytes to differentiate into macrophages and the ability of these cells to become M1 macrophages. Monocytes incubated with or without nLDL during the differentiation period and then polarised to an M1 phenotype using LPS (100ng/mL) plus IFN γ (20ng/mL) or cultured without any further stimuli to reflect a resting macrophage (M0). Using M1 cell surface marker CD64, we found that while LPS and IFN γ upregulated CD64 expression as expected in reference to M0. Exposure of the monocytes to nLDL had no effect on CD64 expression when macrophages were polarized to an M1 phenotype (Figure 2A).

Since cell surface markers only provide limited information on the overall phenotype we next examined the expression of the inflammatory genes *TNF α* and *IL-6* (22, 23), associated with M1 macrophages. Consistent with the enhanced cell surface expression of M1 markers, LPS + IFN γ incubation significantly

induced the expression of *TNF α* and *IL-6* compared to untreated macrophages (M0) (Figure 2B, C). Interestingly, incubation with nLDL, while having no additive effect on abundance of cell surface M1 markers, significantly enhanced the expression of *TNF α* and *IL-6* when the macrophages were challenged with LPS + IFN γ . Furthermore, nLDL appeared to be having a stimulator function in its own right, as compared to control (M0), as there was a significant induction of *IL-6* gene expression and a trend to increase *TNF α* gene expression (Figure 2B, C). Similar results were seen in respects to enhancing M1 polarization when macrophages were exposed to oxLDL (Figure 2D, E).

Native LDL decreases M2 macrophage expression and *TGF β* and *IL-10* gene expression

Since incubation with nLDL increased the inflammatory profile of M1 macrophages (Figure 2B, C), we decided to examine if nLDL could modulate M2 macrophages. Incubation of monocytes with nLDL had no effect on the expression of M2 cell surface markers CD200R and CD206 on M0 macrophages (Figure 3A, B). As expected, when macrophages were incubated with IL-4 there was a marked upregulation of CD200R and CD206, suggesting polarisation to the M2 phenotype. Interestingly, incubation of monocytes with nLDL significantly inhibited the expression of CD200R and CD206 in response to IL-4 (Figure 3A, B). This suggests that nLDL prevented complete polarisation to the M2 phenotype.

To confirm if these macrophages were in some way functionally impaired and unable to produce anti-inflammatory cytokines, we examined *TGF β* and *IL-10* gene expression in response to IL-4 (22, 23). Consistent with the M2 phenotype, IL-4 significantly induced the expression of *TGF β* and *IL-10* (Figure 3C, D). However, similar to what was observed with the cell surface markers, incubation of the monocytes with nLDL completely prevented the induction of *TGF β* and *IL-10* (Figure 3C, D). Therefore our data suggests that exposure of monocytes to nLDL can significantly impair their ability to respond to Th2 cytokines such as IL-4 to mount a resolving or anti-inflammatory response. Conversely, treatment of macrophages with oxLDL increased the surface expression of CD200R, but similar to nLDL suppressed *TGF β* gene expression (Figure 3E, F).

Native LDL increases *TNF α* and *IL-6* gene expression in M1 macrophages regardless of monocyte origin

Monocytes are a heterogeneous population of cells and it is possible that particular subsets could respond differentially in respect to either the stimuli used to polarise (i.e. LPS + IFN γ) or to nLDL. To address this we first isolated human monocyte subsets (i.e. classical CD14⁺⁺/CD16⁻, intermediate CD14⁺/CD16⁺ and non-classical CD14^{dim}/16⁺⁺) by FACS (Supp Figure 1). Using the same differentiation process as with the total population of monocytes (Figure 2) we first showed that all monocyte subset derived M0 macrophages were able to take up nLDL using BODIPY-LDL (Supp Figure 2). We then examined the ability of each subset to polarise into the M1 phenotype as assessed by the expression of *TNF α* and *IL-6*. While each monocyte subset did respond to LPS + IFN γ by upregulating *TNF α* and *IL-6* gene expression, this was more pronounced in the classical and intermediate subsets, likely reflecting their expression of the LPS co-receptor CD14 (Figure 4). However, it was abundantly clear that incubation of each subset with nLDL, significantly enhanced their response to LPS + IFN γ , evidenced by an induction of *TNF α* and *IL-6* gene expression compared to LPS + IFN γ alone (Figure 5A-F). Thus, while there appears to be a difference in the ability on the non-classical subset to respond to LPS + IFN γ compare to the other two CD14⁺ subsets, nLDL does augment the response to these stimuli in all monocyte subsets, suggesting an inflammatory/priming role of circulating nLDL.

Classical and intermediate but not non-classical monocytes polarise into M2 macrophages

Next we examined the ability of each monocyte subset to mature into M2 macrophages in response to IL-4. We made the interesting observation that the classical and intermediate subsets both responded to

IL-4 and upregulated the M2 genes *TGF β* and *SRB1*, while the non-classical subset failed to respond and appeared to be unable to polarise into an M2 macrophage (Figure 6). The suppressive nature of nLDL on M2 polarisation that we observed in the total monocyte pool (Figure 2) was also observed in the classical and intermediate subsets in respect to *TGF β* and *SRB1* gene expression (Figure 7A, B, E, F). Consistent with the data in Figure 5, the non-classical subset was completely unresponsive to IL-4 treatment and did not upregulate *TGF β* or *SRB1* and nLDL did not affect expression of either gene as expected (Figure 7C, F).

Discussion

We have identified a role for nLDL in modifying the ability of monocyte derived macrophages to respond to stimuli that shapes their polarisation into inflammatory or resolving subsets (i.e M1 and M2). In general, we discovered that nLDL augmented the M1 response and removed the ability to polarise into M2 macrophages. Further, we isolated the three specific monocyte subsets to determine their intrinsic ability to polarise into the macrophage subsets. Importantly, our findings indicate that while each subset could polarise to an M1 phenotype, this was much more prominent in the cells which expressed CD14. Somewhat surprisingly only the classical and intermediate monocytes could mature into M2 macrophages, with a complete lack of response seen in the non-classical monocytes. We also found that incubation with nLDL enhanced the inflammatory M1 response in each subset, albeit to varying degrees and prevented polarisation to the M2 phenotype in all cells. Our findings raise novel insights as to how monocytes as a whole and more importantly their particular subsets mature into macrophages and how they respond to extracellular stimuli in the context of Th1 and Th2 responses. We also provide a potential mechanism as to how high levels of circulating nLDL might influence monocyte to macrophage differentiation and behavior in the setting of hypercholesterolemia which is a major risk-factor of CVD.

The effect of LDL particles on macrophages within the atherosclerotic lesion have garnered the lions share of attention in the setting of cardiovascular disease. However, this is mainly in relation to modified LDL particles such as minimally modified LDL or oxLDL that stimulate inflammatory responses through interactions with macrophage receptors including TLR4 and scavenger receptors (22). While this is an important process of the maturing atherosclerotic lesion and the formation of cholesterol rich macrophage foam cells, our study sought to understand what occurs upstream when monocytes begin to differentiate into macrophages. Initially we decided to examine how exposure of a heterogeneous pool of human monocytes would respond when incubated with nLDL. This in some respects mimics what is seen in people with elevated plasma LDL cholesterol, where monocytes are constantly exposed in the circulation before they enter into a tissue to differentiate into a specific macrophage subset based on the extracellular milieu. Monocytes in circulation likely take up nLDL through non-traditional mechanisms such as macropinocytosis and micropinocytosis (24). Therefore it is conceivable that monocytes, before they become macrophages already have a load of cholesterol. Certainly there is significant evidence for this occurring in experimental models of atherosclerosis, where circulating monocytes in high fat fed zebra fish carry a lipid load (25-27). We found that exposure of monocytes to nLDL during the differentiation process resulted in an enhanced response to the M1 promoting stimuli LPS and IFN γ , while abolishing the ability of these cells to respond to the M2 stimuli IL-4. This suggests that plasma lipoproteins can influence the circulating monocyte and tip the balance towards an inflammatory macrophages rather than resolving macrophages.

The concept that atherosclerosis is a form of non-resolving inflammation has been recently discussed(28, 29). Unlike acute inflammatory responses, the chronic response in atherosclerosis appears to lack the necessary switch from inflammatory macrophages to anti-inflammatory cells. It has been

previously shown that nLDL isolated from hypercholesterolemic patients with coronary artery disease promotes monocyte adhesion(30). Together with our findings it suggests that recruitment of these LDL exposed/activated monocytes into the lesion results in an accumulation of macrophages that are incapable of switching into the M2 phenotype. Thus there is now a growing body of evidence to suggest that there is a major failure in the inflammatory response that is evident at the level of plaque macrophages(28, 29), but is likely initiated in precursor cells from stem cells in the bone marrow to circulating monocytes(31).

We acknowledge that our experiments only examined a role for nLDL and not a mixture of the complete lipoproteins in the plasma. However, we have previously shown that HDL has anti-inflammatory functions of circulating monocytes (32) and neutrophils (33), while others have described an M2 promoting role of HDL in murine macrophages (34). It should also be noted that lipoproteins can influence the production of monocytes, where LDL promotes (35) and HDL inhibits (36, 37), thus the influence of lipoproteins on monocyte fate could begin as early as the hematopoietic progenitors (31).

It is well established that monocytes are a heterogeneous population of cells, where the major subset is the CD14⁺ classical monocytes. While each monocyte subset is thought to have distinct properties, there is no clear consensus of their inflammatory and/or resolving profiles (2, 38). Given that the classical monocytes make up approximately 80% of the circulating monocytes and in our studies changes in the intermediate and non-classical subsets could have been masked. Therefore, we overcame this concern by isolating the monocyte subsets and assessing their ability to mature into the specific macrophage phenotypes. Interestingly all the monocyte subsets were able to polarise into M1 macrophages, albeit to varying degrees. The CD14⁺ monocyte-derived macrophages displayed a robust upregulation of TNF α and IL-6, whereas the CD14^{dim}CD16⁺⁺ non-classical-derived macrophages only displayed a modest increase in the expression of these inflammatory genes in response to the M1 stimuli. While our studies were unable to unravel the mechanism explaining this lack of induction of inflammatory gene expression in the non-classical monocyte-derived macrophages, it is possible that this is due to the lack of CD14 on these cells, which is the co-receptor for TLR4 in sensing LPS. These findings are comparable to the genetic read outs in human monocyte subsets stimulated with LPS, where the two CD14⁺ subsets respond to LPS to differing degrees, whereas the CD14^{dim}CD16⁺⁺ cells weakly respond to LPS (39). Further, since there is a lack of CD14 on the non-classical cells, this could impair an important autocrine loop, where after the engagement of CD14/TLR4 with LPS and the internalization of this complex, which is CD14 dependent (40, 41), IRF3 is phosphorylated to promote more IFN α / β to stimulate inflammatory gene expression (42). Importantly, when each of these monocyte subsets were bathed in nLDL to mimic what is seen with circulating monocyte subsets *in vivo*, there was a significant upregulation of the inflammatory gene expression. Consistent with this finding, an earlier study also identified an increase in TNF α expression in PBMCs following incubation with nLDL (43), implying that prior to differentiating into macrophages LDL promoted an inflammatory phenotype. Together these findings suggest that nLDL can indeed promote inflammation in monocyte/macrophages to contribute to atherogenesis.

Along with the apparent defects in M1 polarisation, the CD14^{dim}CD16⁺⁺ monocyte-derived macrophages were also unable to polarise into M2 macrophages in respects to the induction of TGF β and SR-B1. The mechanism behind this is unknown and may arise when these monocytes differentiate into macrophages. An earlier study exploring cytokine expression identified that the CD16⁺ monocytes (albeit a mixed population of intermediate and non-classical subset) express higher levels of inflammatory cytokines (44, 45), and thus may already be pre-programmed. Perhaps another explanation is that these CD14^{dim} non-classical monocytes have a patrolling function *in vivo* meaning they may never exit the vessels and become macrophages (39). Overall it suggests that the non-classical monocytes are inert in regards to their ability to mature into specific macrophages. Incubation of the CD14 expressing classical

and intermediate monocyte subsets with nLDL resulted in suppression of the M2 phenotype, particularly in respects to *TGFβ* expression.

The majority of studies looking at the correlation of monocyte subsets to CVD have found that CD14⁺CD16⁺ intermediate monocytes are expanded (46, 47). However, the intermediate monocytes did not respond differently to the classical cells in our experiments in respects to polarisation to each macrophage phenotype or to LDL incubation. Thus, more work is required to understand the relationship between the intermediate subset and disease progression and severity. However, our findings do provide an insight as to why there is a failure of atherosclerotic lesion regression unless there is sufficient control of plasma LDL levels in chronic hypercholesterolemia such as that often observed in CVD. In general it is thought that LDL requires modification to assert atherogenic effects, but this is generally studied at the macrophage level where scavenger receptors play a dominant role. However, monocytes in circulation are exposed to nLDL which they can take up through the LDL receptor or via macropinocytosis and micropinocytosis (24). Our data reveals that monocyte interaction with nLDL has a significant effect on the ability of the resulting macrophage to respond to specific stimuli and mature into a specific phenotype. This suggests that the atherogenic fate of the macrophage precedes this time in the myeloid lineage. Further, it is likely that changes in lipoprotein levels and ratios alters the fate of these cells (i.e. the myeloid lineage) at the beginning at the level of the haematopoietic stem cell (31, 35, 36). Our findings provide another dimension to our understanding of why patients with elevated levels of nLDL are at a higher risk of developing atherosclerotic cardiovascular disease.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Figure 1: Experimental overview. Isolated human monocytes (total or subsets) were differentiated into macrophages with M-CSF in the presence or absence of nLDL. After 7 days macrophages were stimulated with M1 polarizing cytokines (LPS and IFN γ) or the M2 promoting cytokine (IL-4) for the specified amount of time for the experiment.

Figure 2: Native LDL promotes inflammatory gene expression in M1 macrophages. Macrophages were either untreated (M0) or polarised into the M1 phenotype with LPS (100ng/mL) and IFN γ (20ng/mL). Flow cytometry analysis or real-time PCR analysis was performed in the presence and absence of: nLDL (200 μ g/mL) to determine the expression of **A** CD64, **B** *TNF α* and **C** *IL-6* or oxLDL (50 μ g/mL) to determine **D** CD64 or **E** *IL-6*. Values expressed as **A** and **D** % control (M0) or **B**, **C** and **E** as fold change from M0 and presented as mean $\pm$ SEM, Student's t-test was used to compare LPS+IFN γ with LPS+IFN γ + LDL only, $^*P<0.05$ and, $n=6-7$.

Figure 3: Native LDL suppresses polarisation of M2 macrophages. Monocyte-derived Macrophages were either unstimulated (M0) or stimulated with IL-4 (20ng/mL)(M2). Flow cytometry analysis or real-time PCR was performed in the presence and absence of: nLDL (200 μ g/mL) to determine the expression of **A** CD200R, **B** CD206, **C** *TGF β* and **D** *IL-10* or oxLDL (50 μ g/mL) to determine the expression of **E** CD200R and **F** *TGF β* . Values expressed as **A**, **B** and **E** % control (M0) or **C**, **D** and **F** as fold change from M0 and presented as mean $\pm$ SEM where $^*P<0.05$ and $^{**}P<0.01$ determined via a one way ANOVA with Tukey post hoc analysis, $n=6-8$ for flow cytometry and $n=3-5$ for PCR.

Figure 4: Intrinsic M1 potential of monocyte subset derived macrophages. Monocyte subsets (CD14⁺⁺/16⁻, CD14⁺/16⁺, CD14^{dim}/16⁺⁺) were differentiated into macrophages for 7 days in the presence of M-CSF (100ng/mL) and were either untreated (M0) or polarised into the M1 phenotype with LPS (100ng/mL) and IFN γ (20ng/mL). Real-time PCR was performed to determine the relative gene expression of *TNF α* and *IL-6* between macrophages from the three different subsets. Values expressed as fold change from M0 and presented as mean $\pm$ SEM where $^*P<0.05$ and $^{***}P<0.001$ as determined using a two-way ANOVA with Tukeys post hoc test, $n=6-8$.

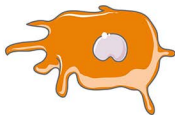
Figure 5: Native LDL propagates inflammatory profile. Monocyte subsets (CD14⁺⁺/16⁻, CD14⁺/16⁺, CD14^{dim}/16⁺⁺) were differentiated into macrophages for 7 days in the presence of M-CSF (100ng/mL) and were either untreated (M0) or polarised into the M1 phenotype with LPS (100ng/mL) and IFN γ (20ng/mL). Real-time PCR was performed to determine the gene expression of **A-C** *TNF α* and **D-F** *IL-6* in macrophages derived from **A&D** classical monocytes (CD14⁺⁺/16⁻), **B&E** intermediate monocytes (CD14⁺/16⁺) and **C&F** non-classical monocytes (CD14^{dim}/16⁺⁺). Values expressed as fold change from M0 and presented as mean $\pm$ SEM, Student's t-test was used to compare LPS+IFN γ with LPS+IFN γ + LDL only, $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, $n=5-7$.

Figure 6: Intrinsic M2 potential of monocyte subset derived macrophages. Monocyte subsets (CD14⁺⁺/16⁻, CD14⁺/16⁺, CD14^{dim}/16⁺⁺) were differentiated into macrophages for 7 days in the presence of M-CSF (100ng/mL) and were either untreated (M0) or polarised into the M2 phenotype with IL-4 (20ng/mL). Real-time PCR was performed to determine the relative gene expression of *TGF β* and *SRB1* between macrophages from the three different subsets. Values expressed as fold change from M0 and

presented as mean $\pm$ SEM where $**=P<0.01$ as determined using a two-way ANOVA with Tukeys post hoc test, n=5-9.

Figure 7: Native LDL inhibits monocyte subset derived M2 macrophage gene expression. Monocyte subsets (CD14⁺⁺/16⁻, CD14⁺/16⁺, CD14^{dim}/16⁺⁺) were differentiated into macrophages for 7 days in the presence of M-CSF (100ng/mL) and were either untreated (M0) or polarised into the M2 phenotype with IL-4 (20ng/mL). Real-time PCR was performed to determine the gene expression of **A-C** *TGF β* and **D-F** *SRB1* in macrophages derived from **A&D** classical monocytes (CD14⁺⁺/16⁻), **B&E** intermediate monocytes (CD14⁺/16⁺) and C&F non-classical monocytes (CD14^{dim}/16⁺⁺). Values expressed as fold change from M0 and presented as mean $\pm$ SEM, a Student's t-test was used to compare IL-4 with IL-4 + LDL only, $*=P<0.05$, $**=P<0.01$ and $***=P<0.001$, n=5-10.

M1 "inflammatory"



M2 "anti-inflammatory"



Macrophage (M0)



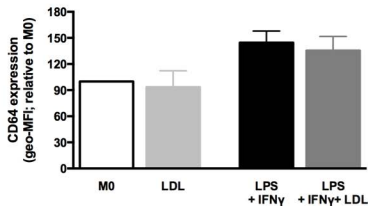
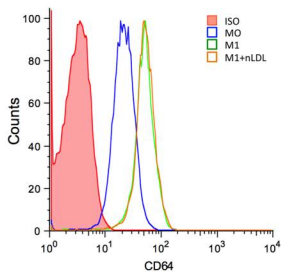
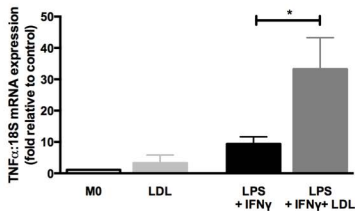
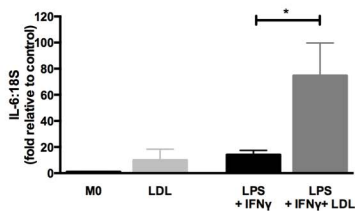
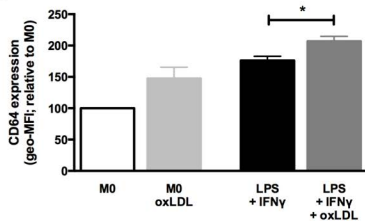
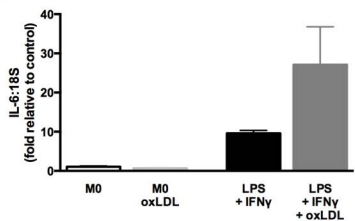
Monocytes

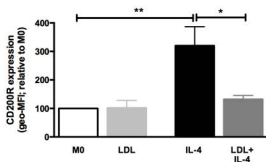
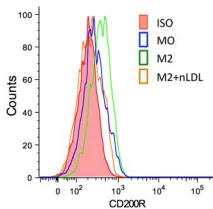
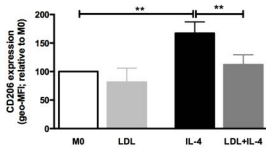
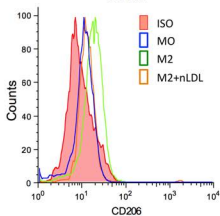
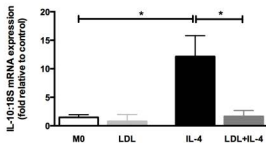
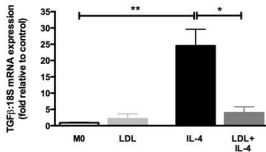
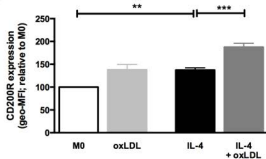
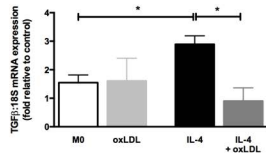
M-CSF +/- nLDL

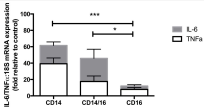
7 days

LPS/IFN γ (M1) or IL-4 (M2)

6-24 hours

A**B****C****D****E**

A**B****C****D****E****F**



Classical



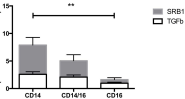
Intermediate



Non-Classical



SRB1/TGF β -18S mRNA expression
(fold relative to control)



Classical



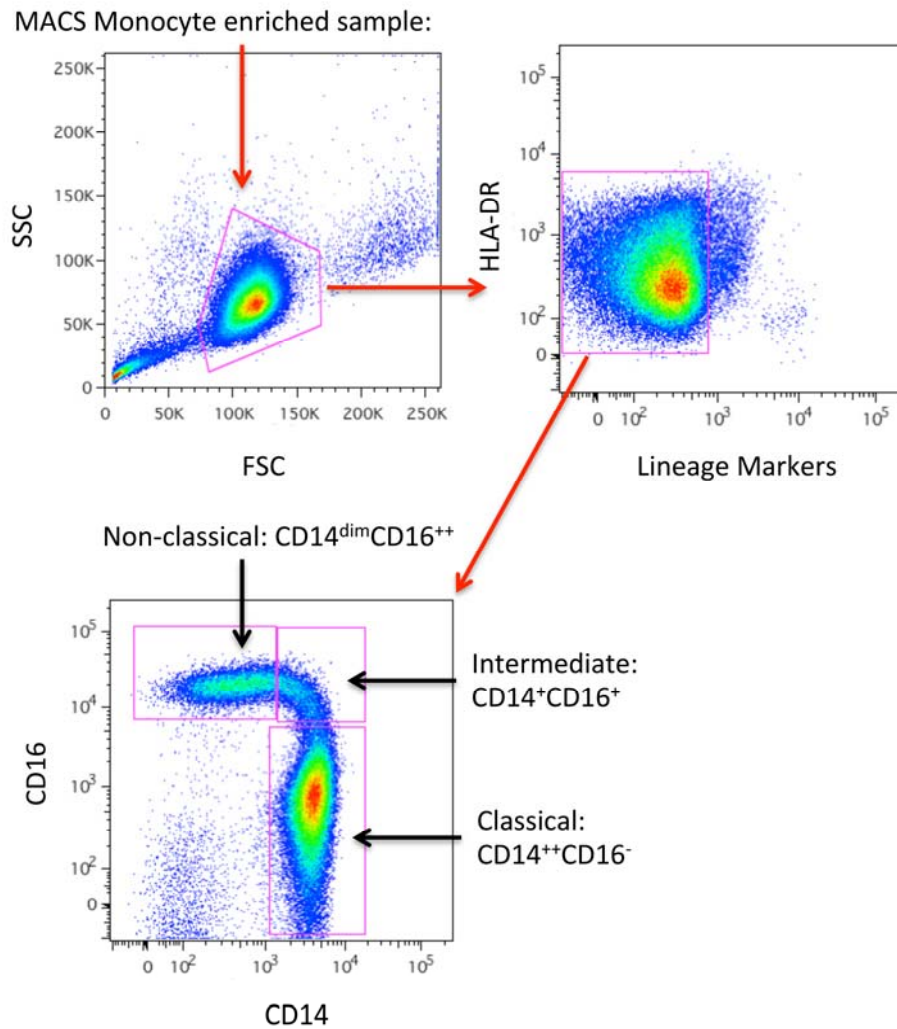
Intermediate



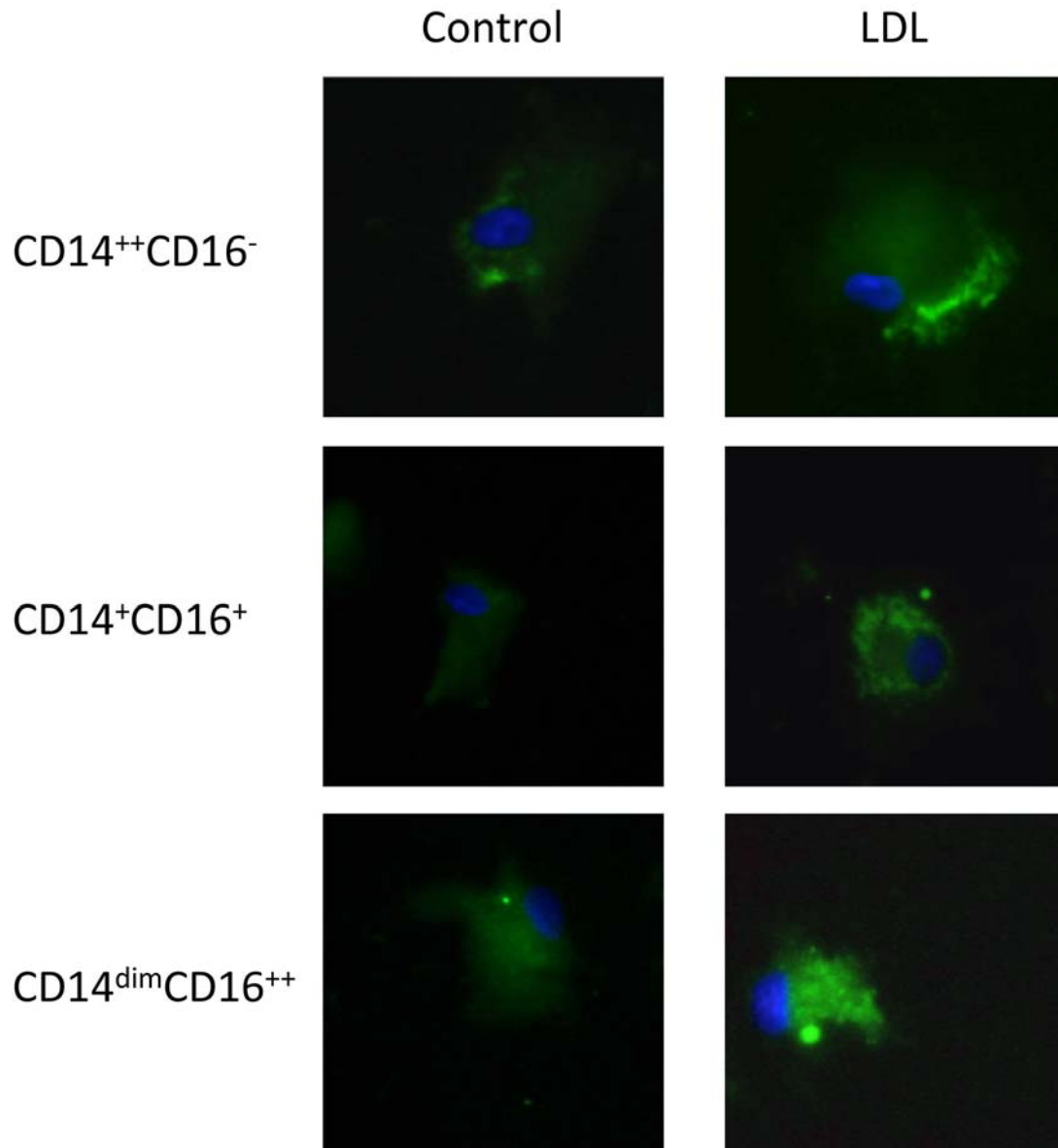
Non-Classical



Supplemental Figures



Supplemental Figure 1: *Experimental overview of monocyte subset isolation via FACS.*



Supplemental Figure 2: Monocyte subset derived M0 macrophages. Human monocyte subsets were purified by FACS and cultured in M-CSF +/- LDL containing media for 7 days to promote macrophage differentiation. The mice that received LDL were then incubated overnight with BODIPY-LDL. The macrophages were then fixed and counterstained with DAPI. Representative images show LDL uptake in all monocyte subset derived macrophages.