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Abstract: Background & Aims: Lipid-rich foam cell macrophages drive atherosclerosis via several mechanisms, including inflammation, lipid uptake, lipid deposition and plaque vulnerability. The atheroma environment shapes macrophage function and phenotype; anti-inflammatory macrophages improve plaque stability while pro-inflammatory macrophages promote rupture. Current evidence suggests a variety of macrophage phenotypes occur in atherosclerotic plaques with local lipids, cytokines, oxidised phospholipids and pathogenic stimuli altering their phenotype. In this study, we addressed differential functioning of macrophage phenotypes via a systematic analysis of in vitro polarised, human monocyte-derived macrophage phenotypes, focussing on molecular events that regulate foam-cell formation.
Methods: We examined transcriptomes, protein levels and functionally determined lipid handling and foam cell formation capacity in macrophages polarised with IFNγ+LPS, IL-4, IL-10, oxPAPC and CXCL4.
Results: RNA sequencing of differentially polarised macrophages revealed distinct gene expression changes, with enrichment in atherosclerosis and lipid-associated pathways. Analysis of lipid processing activity showed IL-4 and IL-10 macrophages have higher lipid uptake and foam cell formation activities, while inflammatory and oxPAPC macrophages displayed lower foam cell formation. Inflammatory macrophages showed low lipid uptake, while higher lipid uptake in oxPAPC macrophages was matched by increased lipid efflux capacity.
Conclusions: Atherosclerosis-associated macrophage polarisation dramatically affects lipid handling capacity underpinned by major transcriptomic changes and altered protein levels in lipid-handling gene expression. This leads to phenotype-specific differences in LDL uptake, cellular cholesterol levels and cholesterol efflux, informing how the plaque environment influences atherosclerosis progression by influencing macrophage phenotypes.

Research Data Related to this Submission

Title: Data for: Macrophage polarisation associated with atherosclerosis differentially affects their capacity to handle lipid

Repository: Mendeley Data

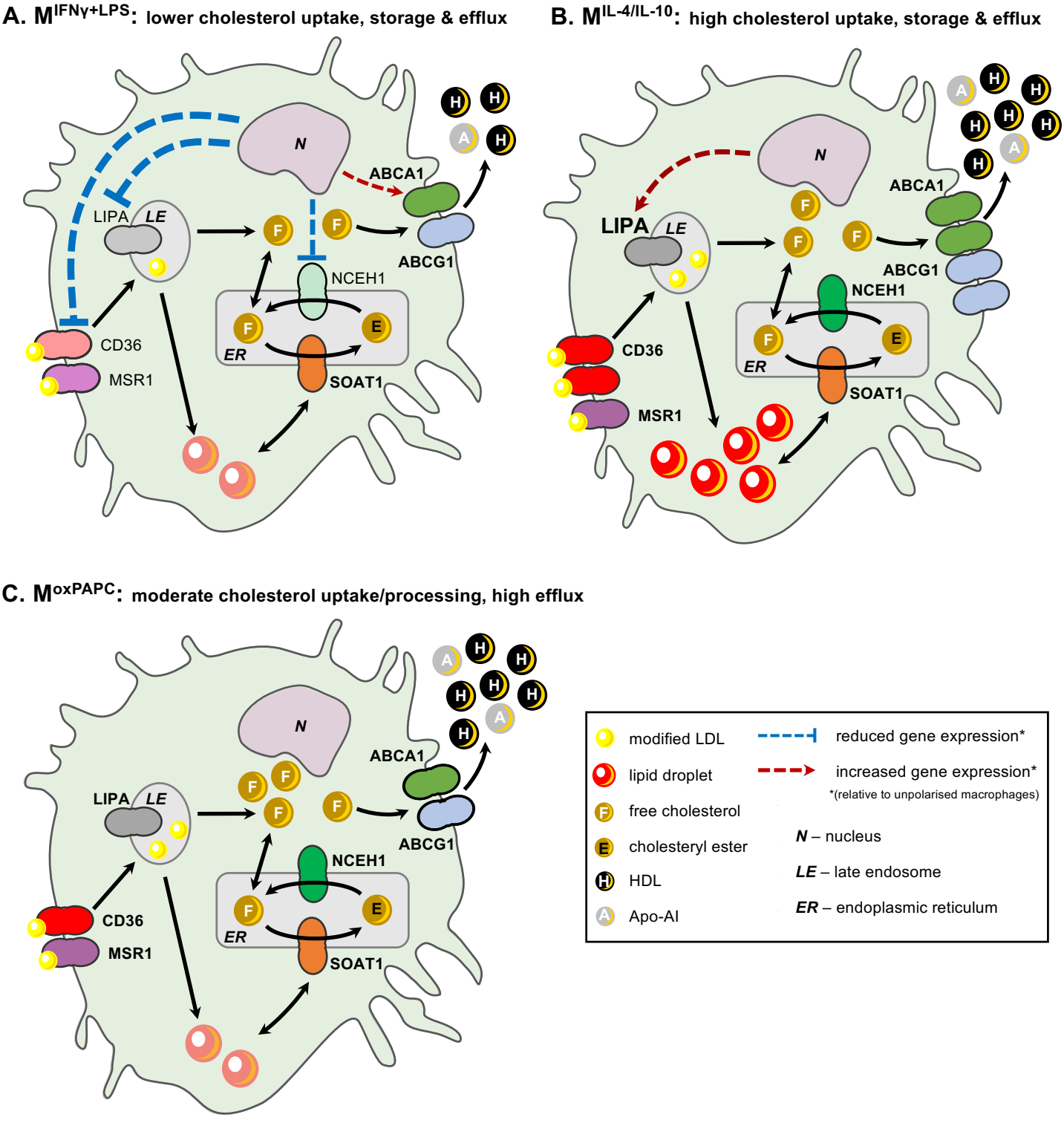
https://data.mendeley.com/datasets/snwtjhx7h3/draft?a=f9f585e0-f497-4c9f-87aa-cb40823f91bc

Highlights:

- Total RNA sequencing of differentially polarised macrophages showed enrichment in genes associated with atherosclerosis and lipid pathways
- Anti-inflammatory macrophages had higher lipid uptake and foam cell formation activities
- Inflammatory and oxPAPC macrophages displayed lower foam cell formation
- Inflammatory macrophages showed low lipid uptake, while higher lipid uptake in oxPAPC macrophages was matched by increased lipid efflux capacity

*Graphical Abstract

Graphical abstract



Macrophage polarisation associated with atherosclerosis differentially affects their capacity to handle lipid

Short Title: Macrophage polarisation & lipid handling capacity

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(ii) **Abstract**

Background & Aims: Lipid-rich foam cell macrophages drive atherosclerosis via several mechanisms, including inflammation, lipid uptake, lipid deposition and plaque vulnerability. The atheroma environment shapes macrophage function and phenotype; anti-inflammatory macrophages improve plaque stability while pro-inflammatory macrophages promote rupture. Current evidence suggests a variety of macrophage phenotypes occur in atherosclerotic plaques with local lipids, cytokines, oxidised phospholipids and pathogenic stimuli altering their phenotype. In this study, we addressed differential functioning of macrophage phenotypes via a systematic analysis of *in vitro* polarised, human monocyte-derived macrophage phenotypes, focussing on molecular events that regulate foam-cell formation.

Methods: We examined transcriptomes, protein levels and functionally determined lipid handling and foam cell formation capacity in macrophages polarised with IFNγ+LPS, IL–4, IL–10, oxPAPC and CXCL4.

Results: RNA sequencing of differentially polarised macrophages revealed distinct gene expression changes, with enrichment in atherosclerosis and lipid-associated pathways. Analysis of lipid processing activity showed IL–4 and IL–10 macrophages have higher lipid uptake and foam cell formation activities, while inflammatory and oxPAPC macrophages displayed lower foam cell formation. Inflammatory macrophages showed low lipid uptake, while higher lipid uptake in oxPAPC macrophages was matched by increased lipid efflux capacity.

Conclusions: Atherosclerosis-associated macrophage polarisation dramatically affects lipid handling capacity underpinned by major transcriptomic changes and altered protein levels in lipid-handling gene expression. This leads to phenotype-specific differences in LDL uptake, cellular cholesterol levels and cholesterol efflux, informing how the plaque environment influences atherosclerosis progression by influencing macrophage phenotypes.

1. Introduction

Arterial macrophages form disease-associated lipid-rich foam cells in atherosclerotic plaques [1]. Progressive atherosclerosis results in cardiovascular diseases, which are a leading cause of death, worldwide [2]. Plaque formation arises from the accumulation of lipid in the artery wall over decades; acute myocardial infarction or stroke can occur following a loss of plaque stability due to cellular changes in the artery wall [3]. Macrophages are a predominant cell type within the artery wall, whose major functions in the plaque are to regulate inflammation, clear apoptotic cells via efferocytosis, regulate plaque stability by secreting matrix metalloproteases (MMPs) or protease inhibitors and to uptake, process, accumulate and efflux lipid [4]. The capacity of macrophages to accumulate or process lipid and to regulate proteases and cytokines in their local environment is a critical determinant of plaque development, lipid core formation and plaque stability [5,6].

Macrophages regulate lipoproteins within their local environment [7]. Uptake of lipoproteins occurs via receptor-mediated endocytosis, such as scavenger receptor, pinocytosis and phagocytosis [4]. Macrophages express scavenger receptors including SR-A1, CD36 and LOX-1 for lipoprotein uptake [8], while phagocytosis mediated internalisation delivers lipids to late endolysosomes where lysosomal acid lipase (LAL) digests cholesteryl esters to produce free cholesterol [9]. In the case of lipoprotein, cellular lipases such as LAL break down the lipoprotein particles that have been taken up, to cholesterol, triglycerides and fatty acids [10–12]. Cholesteryl esters and acylglycerides can be stored in lipid droplets, resulting in foam cell formation. Alternatively, Neutral cholesterol ester hydrolase (NCEH1) can metabolise these molecules into free cholesterol [13–15]; this can be removed from the cell by efflux or converted to fatty acids (oxidised to produce ATP) and glycerol [14,15]. Sterol O-acyltransferase 1 (SOAT1) in the endoplasmic reticulum can re-esterify free cholesterol into cholesterol ester or fatty acids into acylglycerides, for subsequent storage in lipid droplets [4,16–18]. Cholesterol, produced by NCEH1, is then effluxed from the cell by transporters ABCA1, ABCG1 and SR-BI [19] or re-esterified and stored as lipid droplets [19]. The build-up of cellular cholesterol activates transcription factors RXR, LXRα, LXRβ, PPARα and PPARγ [20]. Heterodimerisation of LXR and RXR increases protein levels of ABCA1 and ABCG1, which actively transport HDL loaded-cholesterol out of the cell [19]. The cytosolic accumulation of esterified cholesterol and acylglyceride droplets in macrophages results in the generation of foam cells, driving atherosclerotic plaque formation [9]. In addition to lipid uptake and intracellular processing, lipid and cholesterol content are influenced by the cellular capacity for cholesterol efflux. These efflux pathways in macrophages function to remove excess lipid to prevent its accumulation, which can be cytotoxic [21–23].

The local cellular environment determines macrophage function and phenotype [24]. Traditionally, macrophages have been described as classically activated M1 and alternatively activated M2 macrophages [24]. Pro-inflammatory M1 macrophages arise from exposure to bacterial LPS and interferon-gamma [25]. Cytokines such as IL-4 and IL-13 drive polarisation to M2 macrophages [26]. In arterial atherosclerotic plaques, pro-inflammatory macrophages localise to areas of increased vulnerability, such as the shoulder regions[27]. In these vulnerable areas, they promote rupture by secreting MMPs and inflammatory cytokines thus weakening the cap structure [27]. In contrast, anti-inflammatory macrophages are associated with increased plaque stability, at times with plaque regression and less severe disease [1]. The traditional classification of M1 and M2 macrophages is now widely understood to be simplistic where other "intermediate" or differently polarised phenotypes exist according to the local environment [25]. Macrophages expressing markers for both M1 and M2 phenotypes have been labelled within atherosclerotic plaques [25]. The plaque environment contains

oxidised phospholipid [28], platelet-derived CXCL4 [29] and IL-10 [26] as well as haemoglobin [30], which can each influence macrophages to polarise to different functional states.

The accumulation of lipid within the macrophage, and propensity to form foam cells, is altered according to macrophage phenotype [31]. Pro-inflammatory stimuli have been reported to alter expression of some scavenger receptors [32] [33] and to reduce cholesterol efflux transporter expression [34], reducing their capacity to clear lipid from the plaque. In contrast, IL-4 macrophages are considered to have high lipid handling and foam cell formation capacities [35]. The effect of IL-10, CXCL4 and oxidised phospholipid on human macrophage lipid handling is less well understood.

Compelling evidence shows that a variety of macrophage phenotypes occur in atherosclerotic plaques dependent on their environment. However, it is noteworthy that the majority of our current understanding of macrophage polarisation and its impact on atherosclerosis derives from murine models of atherosclerosis and analysis of mouse bone marrow derived macrophages [36,37]. The relevance to some of these findings to atherogenesis in humans is less clear. For example, whilst *Nos2* and *Arg1* expression are robust markers of proinflammatory vs. alternatively activated macrophages, expression of these genes does not correlate with such macrophage subsets in humans [38–40]. In order to address how the variety of plaque macrophage phenotypes function differentially, we undertook a systematic study to model human macrophages polarised towards factors relevant to the atherosclerotic plaque environment. Using human samples, we sequenced and analysed whole transcriptomes, protein levels and functionally determined lipid handling and foam cell formation capacity in human macrophages polarised with IFNγ+LPS, IL–4, IL–10, oxPAPC (oxidised phospholipid 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine macrophages) and CXCL4. Our data revealed transcriptionally distinct phenotypes, differential lipid uptake, processing and efflux capacities and functional differences to account for low foam cell formation in differentially polarised inflammatory macrophages.

2. Materials and methods

Detailed methods are described in supplementary information. In brief, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood donated by healthy adult donors and monocytes purified by CD14 positive magnetic selection. Monocytes were differentiated into monocyte–derived macrophages (MDMs) over 7 days in M–CSF, resulting in CD68+ macrophages expressing receptors for polarising agents (Supplementary methods; Supplementary Table 1). On day 7, the media was replaced for 24 h with fresh complete media containing the following polarising agents: IFNγ and LPS (R515); IL–4; IL–10; oxPAPC; CXCL4; or unpolarised as controls in each experiment (Supp.Fig.1). RNA was extracted from unpolarised and polarised MDM from 8 separate donors for RNA-seq analysis. RNA-seq data is deposited at DOI: 10.17632/j2hmt7k9fh.1. Differential gene expression was validated by qPCR, and protein by immunofluorescence and flow cytometry. Functional changes in foam cell formation and lipid handling were assessed by oil-red-o lipid staining, acLDL uptake, cholesterol content and efflux determination. Replicate values (n) represent separate human donors.

3. Results

Polarisation causes atherosclerosis–related changes in MDM transcriptomes

We set out to systematically extend the current knowledge of transcriptional changes associated with macrophage polarisation by assessing gene expression changes specifically in *human* macrophage

phenotypes arising from the same donors, treated under a variety of conditions reflecting the arterial plaque environment. Monocyte-derived macrophages from 8 donors were polarised with IFNγ+LPS, IL–4, IL–10, oxPAPC or CXCL4 and examined by RNA–seq for global and specific pathway changes in their transcriptomes that may influence atherosclerosis progression.

Principal component analysis (PCA) clearly showed $M^{IFN\gamma+LPS}$ as the most transcriptionally distinct phenotype with > 5,500 differentially expressed genes (DEGs) compared to unpolarised macrophages and to other phenotypes (Figure 1A). $M^{IFN\gamma+LPS}$ displayed unique and high upregulation of common pro-inflammatory markers (Supplementary Tables 2 and 3). M^{IL-4} was the next most transcriptionally distinct phenotype as evidenced by the PCA, and characterised by *IRF4* (interferon regulatory factor 4) and *MRC1* (mannose receptor C–type 1) upregulation. The PCA shows that $M^{IFN\gamma+LPS}$ and M^{IL-4} represent the extreme ends of the *in vitro* MDM polarisation spectrum, since they exhibited the highest number of differentially expressed genes amongst the polarisation conditions applied. Interestingly, in the PCA M^{IL-10}, M^{oxPAPC} and M^{CXCL4} clustered more closely to unpolarised macrophages. Of note, M^{IL-10}, M^{oxPAPC} and M^{CXCL4} also showed a cytokine/chemokine (and their regulators) expression profile, similar to M^{IL-4}. These included a notably lower upregulation of *CXCL8* (IL–8), *CXCL9*, *CXCL11, IL1B, IL2RA* and *SOCS3* compared to $M^{IFN\gamma+LPS}$, but no changes in *IRF1, NFKB1, NFKB2, RELA* and *RELB* expression (Supplementary Table 4). M^{IL-10} showed upregulation of *CD163* RNA, consistent with recent findings [41], while M^{oxPAPC} was clearly distinguished by increased RNA for *HMOX1* (heme oxygenase 1) and *TXNRD1* (thioredoxin reductase 1).

CXCL4 macrophages showed a remarkably similar transcriptome profile to unpolarised macrophages as indicated by their close clustering in PCA and only 207 DEGs (1.53% of transcripts detected from a total of 13,531 genes) between the two conditions (Figure 1B). These results were largely in agreement with transcriptomic data on human monocyte-derived macrophages with and without CXCL4 polarisation from another study[42], where monocyte differentiation into unpolarised macrophages and M^{CXCL4} with M–CSF or CXCL4 respectively over 6 days resulted in only 460 differentially expressed probes or 375 DEGs (1.77% out of 26,051 probes with signals above the detection limit [42]). S100A8 (S100 calcium binding protein A8) and MMP7 (matrix metallopeptidase 7) have previously been reported as M^{CXCL4} markers [43]; our RNA-seq data showed their expression in M^{CXCL4} was highly variable among donors and was up–regulated in most other phenotypes, especially $M^{IFN\gamma+LPS}$ (Supplementary Tables 2 and 3). Given this low difference between M^{CXCL4} and unpolarised macrophage transcriptomes, we omitted M^{CXCL4} from further analysis (Figure 1C, 1D).

Each MDM phenotype displayed significant enrichment of DEGs in atherosclerosis–related pathways (Supplementary Table 5). In most phenotypes, these pathways included genes clearly involved in macrophage lipid handling, such as *ABCA1* (ATP binding cassette subfamily A member 1), *ABCG1* (ATP binding cassette subfamily G member 1), *LPL* (lipoprotein lipase) and *MSR1* (macrophage scavenger receptor 1) prompting further investigation into the lipid handling capacity of these phenotypes. The RNA-sequencing results were validated by qRT–PCR on parallel samples (Supplementary Figure 3), confirming differences in selected atherosclerosis-related genes.

Reduced foam cell formation capacity in $M^{IFN\gamma+LPS}$ and M^{oxPAPC}

Our transcriptome analyses of macrophage phenotypes revealed differences in lipid handling and other associated pathway enrichment for differentially expressed genes (Supplementary Table 5). We therefore sought to assess foam cell formation capacity of the various polarised macrophages since this underlies the pathophysiology of atherosclerosis [44]. After loading with acLDL, we measured foam cell formation by oil-red-O staining (measured as % control – % acLDL–loaded Area ORO >

Area haematoxylin cells) in $M^{IFN\gamma+LPS}$ and M^{oxPAPC}, while M^{IL-4} and M^{IL-10} showed an increase in foam cell formation, similar to that of unpolarised macrophages (Figures 2A & 2B).

Reduced LDL uptake in $M^{IFN\gamma+LPS}$

Since $M^{IFN\gamma+LPS}$ and M^{oxPAPC} showed reduced foam cell formation compared to other phenotypes, we assessed lipoprotein uptake, which impacts macrophage lipid handling and resultant foam cell formation [45]. We therefore measured fluorescently-labelled acLDL uptake in the different MDM phenotypes by flow cytometry. Analysis of MDMs exposed to AlexaFluor–488-conjugated acLDL showed significantly lower uptake in $M^{IFN\gamma+LPS}$ and non-significant reduction in M^{oxPAPC} ($p = 0.10$). In contrast, M^{IL-4} and M^{IL-10} showed intracellular acLDL fluorescence levels similar to unpolarised macrophages (Figure 3A). Using reactome.org analysis, in $M^{IFN\gamma+LPS}$ we detected a global down–regulation of 'Ligand binding and uptake by scavenger receptors' pathways (Figure 3B) and in key lipid uptake genes (Figure 3C), such as modified LDL receptors *CD36* (cluster of differentiation 36) and *MSR1* (macrophage scavenger receptor 1) [46,47]. This suggests that differential polarisation influences a transcriptional programme that affects downstream pathways regulating LDL uptake. In agreement with this, a reduction in both CD36 and MSR1 scavenger receptors was detected at the cell surface protein level in $M^{IFN\gamma+LPS}$ (Figure 3D).

Cellular free cholesterol content in polarised macrophages after LDL loading

In addition to lipid uptake, macrophage foam cell formation capacity is influenced by the ability of the cell to regulate its internal lipid content. Altering the balance of internal lipid processing and storage may result in changes in total, free and esterified cholesterol content in macrophage phenotypes. While IFNγ-treated macrophages have been shown to contain different levels of free cholesterol and its ester [48], the other phenotypes described in this study have not been assessed in this regard. We therefore measured the abundance of total, free and esterified cholesterol in control and acLDL–loaded cells to determine if intracellular cholesterol processing capacity affected differences in foam cell formation.

Unpolarised macrophages showed a larger change in total cholesterol (73.5 ± 19.7 ng cholesterol / ng protein), higher than for all polarisation-treated macrophage phenotypes (Figure 4A). $M^{IFN\gamma+LPS}$ exhibited no significant change in total cholesterol following loading ($p = 0.10$ for the difference between this condition and unpolarised cells, Figure 4A). We also calculated the cholesterol ester content, but did not observe statistically significant differences in cholesterol ester by phenotype, when compared to unpolarised macrophages (Figure 4B). Similar to the total cholesterol changes, $M^{IFN\gamma+LPS}$ showed no significant differences ($p = 0.11$) in the change of free cholesterol content (Figure 4C).

We examined the 'LDL clearance' pathway for DEGs according to MDM polarisation, to identify transcriptional differences in the cholesterol clearance pathways. In agreement with the cholesterol content determination, $M^{IFN\gamma+LPS}$ showed largely down–regulated expression of the entire clearance pathway (Figure 4D). Among key genes, *SOAT1* was not differentially expressed in any phenotype, whereas, expression of *NCEH1* was significantly reduced in $M^{IFN\gamma+LPS}$ (Figure 4E), in keeping with the cholesterol content assessment. In addition, *LIPA* (lipase A, lysosomal acid type, involved in lipoprotein particle breakdown) showed reduced expression in $M^{IFN\gamma+LPS}$ and increased in M^{IL-4}, likely contributing to the differences in intracellular lipid handling between these phenotypes (Figure 4E).

Reduced cholesterol efflux capacity in $M^{IFN\gamma+LPS}$

Lipid and cholesterol content are influenced by the cellular capacity for cholesterol efflux. Macrophages mainly unload intracellular lipid to apoliprotein AI (Apo–AI) via ABCA1 transporter while high–density lipoprotein (HDL) is capable of accepting lipid from ABCG1, SCARB1 (scavenger receptor class B member 1) [49] and other pathways. Previous reports indicated that pro-inflammatory macrophages had lower cholesterol efflux capacity [34] than anti-inflammatory macrophages [50]. We tested cholesterol efflux via both acceptors, 24 h after macrophage polarisation, to determine the reverse cholesterol transport capacity of each phenotype.

Total TopFluor cholesterol efflux, involving both Apo–AI and HDL, was significantly reduced in $M^{IFN\gamma+LPS}$ while M^{IL-4} did not show a significantly altered efflux compared to unpolarised macrophages (Figure 5A). $M^{IFN\gamma+LPS}$ also showed a significantly lower capacity to efflux cholesterol regardless of whether HDL or Apo–AI was used as cholesterol acceptor (Figure 5B and C). M^{IL-4} showed no significant difference in cholesterol efflux capacity in the presence of Apo–AI compared to unpolarised macrophages (Figure 5C). Following polarisation, expression of key active cholesterol efflux receptors ABCA1 and ABCG1 in 'HDL assembly' and 'HDL remodelling' pathways (Figure 5D) showed an opposite pattern (Figure 6E) from the downstream functional data (Figure 5A–C). Expression of the major passive cholesterol efflux receptor SCARB1 (functions via HDL) was significantly lower in $M^{IFN\gamma+LPS}$, but also uniquely increased in M^{IL-4} (Figure 5E), suggesting that expression of SCARB1 and the aforementioned ABC transporters are under different transcriptional regulation for lipid handling, post-polarisation. Protein levels of ABCA1 and ABCG1 upon MDM polarisation (Figure 5F) were similar to the cholesterol efflux capacities of each of the macrophage phenotypes (Figure 5A–C). SCARB1 cell–surface protein was uniquely up–regulated in M^{IL-4}, matching our observations at the transcript level and further suggesting differences between regulation of ABCA1/ABCG1 and SCARB1 expression.

4. Discussion

Macrophages polarise to a spectrum of phenotypes in response to their environment [24]. This systematic study compared human macrophage lipid handling capacities at the transcriptome, protein and functional levels for cells polarised towards factors relevant to the atherosclerotic plaque environment. Polarisation with IFNγ+LPS, IL–4, IL–10, oxPAPC and CXCL4 identified distinct transcriptional changes, $M^{IFN\gamma+LPS}$ being the most transcriptionally distinct phenotype in agreement with other related studies [51,52]. Transcriptional changes showed enrichment in atherosclerosis and lipid-associated pathways. Analysis of lipid processing activity showed M^{IL-4} and M^{IL-10} to have higher lipid uptake and foam cell formation activities, while inflammatory $M^{IFN\gamma+LPS}$ and M^{oxPAPC} have lower foam cell activity. Functionally, there are key differences between the $M^{IFN\gamma+LPS}$ and M^{oxPAPC} inflammatory macrophage phenotypes that account for their low foam cell formation: $M^{IFN\gamma+LPS}$ exhibit low lipid uptake while a higher lipid uptake in M^{oxPAPC} is compensated by increased lipid efflux activity.

Lipid handling by macrophages is clearly critical in atherogenesis. Thus, we also considered using lipids and lipoprotein particles as stimulating agents alone or in combination with the polarising factors we used to treat MDMs. However, our own pilot data (unpublished) and previous work has shown that lipid exposure of MDMs does not lead to notable transcriptomic changes or modify the impact of the polarising stimuli used in this study [52]. We therefore considered this as unlikely to provide additional insight beyond the current analysis without complicating data interpretation.

While previous studies, and more frequently in murine models, have compared macrophage polarisation in response to specific stimuli, we undertook a systematic approach to directly compare the impact of IFNγ+LPS, IL–4, IL–10, oxPAPC and CXCL4 in human macrophages at the level of transcriptomes, protein through to functional lipid processing analysis. The analysis using 8 individual donors showed a clear distinction in transcriptional programming due to polarisation. All phenotypes showed a unique expression profile compared to unpolarised controls with exception of CXCL4 polarisation, similar to work by Gleissner *et al* [42]. We noted that only a subset of the differentiated macrophages expressed CXCR3, the receptor for CXCL4, in all donors (46.3 ± 16.3 %). This may well have contributed to the minimal transcriptomic difference in this phenotype compared to unpolarised macrophages. Consistent with this, *MMP7* and *S100A8*, which are considered markers for the CXCL4 phenotype [43], were not upregulated consistently in all donors, again suggesting incomplete polarisation.

For the IFNγ+LPS, IL–4, IL–10 and oxPAPC polarisation conditions, the largest divergence in DEGs was between $M^{IFN\gamma+LPS}$ and M^{IL-4} polarisation, in keeping with other findings [51]. DEG enrichment in atherosclerosis and lipid associated IPA canonical pathways suggests a logical rationale for studying differences in lipid handling between macrophage phenotypes.

The lipid handling capacities were similar for M^{IL-4}, M^{IL-10} and unpolarised macrophages where each displayed efficient foam cell formation and acLDL uptake abilities alongside increased expression of *MSR1, CD36, SOAT1, NCEH1, LIPA* and other key genes in these scavenger receptor and LDL clearance pathways. At earlier stages of atherogenesis, M^{IL-4} and M^{IL-10} may prevent atherosclerosis progression by clearing lipid from within the artery wall due to their effective lipid processing capacity alongside effective efflux [4]. Our oil-red-O measurements showed high foam cell formation in these macrophages which may reflect a role in promoting atherosclerosis at later stages of disease where high levels of LDL and other lipids may overwhelm their capacity to process extracellular lipid[20,53]. The cholesterol transporter SCARB1 plays a role in both cholesterol efflux and influx[54]. At the protein level, SCARB1 was most highly expressed in M^{IL-4} compared to other polarisation states while ABCA1 and ABCG1 were significantly increased in both M^{IL-4} and M^{IL-10}, but not to the same extent as SCARB1. It is likely that post-transcriptional regulation accounts for differences in all of these cholesterol transporters since protein changes were more pronounced and not always in the same direction to that of the mRNA levels. These findings suggest that SCARB1, in combination with MRC1, offers the potential as an additional marker for the M^{IL-4} phenotype given its level of upregulation. Increased SCARB1 in M^{IL-4} compared to $M^{IFN\gamma+LPS}$ likely accounts for their higher cholesterol efflux capacity.

A pro-inflammatory and oxidised atherosclerotic plaque environment is associated with later stage disease, plaque instability and vulnerability to rupture [31]. Infection is a risk factor for myocardial infarction[55]while LPS has been detected within human plaques[56]. $M^{IFN\gamma+LPS}$ and M^{oxPAPC} phenotypes can arise due to differences in sterile inflammatory stimuli where the effect of oxidised phospholipids on macrophage lipid metabolism is less well understood, particularly in human macrophages. Oxidised phospholipids, derived from oxidation of lipoproteins and apoptotic cell membranes, induce TLR2-mediated acute inflammatory responses in macrophages [57]. Some reports of M^{oxPAPC} polarised mouse macrophages have shown that the phagocytic activity of these macrophages is impaired [57]. Interestingly, in this study we found that M^{oxPAPC} showed effective lipid

uptake and processing, similar to the levels observed in M^{IL-4} and M^{IL-10} macrophages. At the same time, M^{oxPAPC} showed low foam cell formation, which is likely accounted for by their high capacity to efflux cholesterol. M^{oxPAPC} showed lower expression of *ABCG1* and *SCARB1* compared to M^{IL-4} and M^{IL-10} suggesting that efflux may be coupled to other transporters or they can process lipids more efficiently due to other regulatory factors. In contrast, $M^{IFN\gamma+LPS}$ showed lower acLDL uptake compared to other phenotypes, accompanied by low foam cell formation.

Atherosclerosis associated macrophage polarisation dramatically affects the cell's capacity to handle lipid. Whole transcriptome sequencing of differentially polarised macrophages revealed distinct phenotypes where differences in key lipid handling gene and protein levels have a lasting effect on downstream lipid handling, as observed by differences in LDL uptake, cellular cholesterol levels and the capacity to efflux excess cellular cholesterol. Functional differences account for low foam cell formation in differentially polarised inflammatory macrophages: $M^{IFN\gamma+LPS}$ exhibit low lipid uptake while increased lipid uptake in M^{oxPAPC} is matched by increased lipid efflux activity. All of these processes influence the formation of lipid–laden macrophage foam cells, underlying the progression of atherosclerosis and vulnerability to cardiovascular disease.

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Author contributions:
Conception and design: HLW, SCW, EKT. Performed experiments: KB, EH, JL, FS. Experimental analysis: KB, EH, BL, IS. Interpretation: KB, EH, HLW, EKT, SCW. Wrote manuscript: HLW, KB. Edited manuscript: EH, BL, JL, EKT, SCW.

Conflict of Interest.
No conflicts of interest.

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Figure Legends

Figure 1: Global analyses of human macrophage phenotype transcriptomes.
Human monocyte-derived macrophages from 8 donors were polarised with IFNγ+LPS, IL–4, IL–10, oxPAPC or CXCL4 and examined by RNA–seq for global and specific pathway changes in their transcriptomes. (**A**) Principal component analysis (PCA) and (**B**) numbers of differentially expressed genes ($|\log_2 FC| > \log_2(1.5)$ and FDR < 0.05 (Benjamini and Hochberg method)) among all conditions. (**C**) Principal component analysis (PCA) and (**D**) numbers of differentially expressed genes excluding M^{CXCL4}. PC – principal component, DEG – differentially expressed gene.

Figure 2: Foam cell formation capacity by macrophage phenotypes.
(**A**) Representative Oil–Red–O staining of control and acLDL (25μg/ml for 24 h) loaded human monocyte-derived macrophages displaying presence of foam cells. Red arrow indicates positive Oil-Red-O staining where % control – % acLDL–loaded Area $_{ORO}$ > Area $_{haematoxylin}$, scale bars = 100 μm. (**B**) Quantification of foam cell formation capacity of human monocyte-derived macrophages, unpolarised (M^{un}), or polarised with IFNγ+LPS, IL–4, IL–10 or oxPAPC, determined using Image J polygonal seletion and area measurements, where foam cells were considered positive for oil-red-O staining greater than the area of the nucleus. Δ% foam cells = control % foam cells – acLDL % foam cells; mean ± SEM, n = 5 separate donors, matched/repeated measures one–way ANOVA with Dunnett's post–hoc test, ** $p \leq 0.01$ compared to M^{un}.

Figure 3: LDL uptake by macrophage phenotypes.
(**A**) Flow cytometry quantification of AlexaFluor–488–acLDL internalisation by human monocyte-derived macrophages, (M^{un}), or polarised with IFNγ+LPS, IL–4, IL–10 or oxPAPC, n = 8. (**B**) Differentially expressed mRNAs in the 'Binding and uptake of ligands by scavenger receptors' pathway using reactome.org analysis (annotations retrieved from reactome.org on 16 October 2018) in M^x compared to unpolarised (M^{un}) measured using RNA–seq; n = 8, thresholds for differential gene expression were $|\log_2 FC| > \log_2(1.5)$ and FDR < 0.05 (Benjamini and Hochberg method). RNA–seq. (**C**) *MSR1* and *CD36* mRNA expression among MDM phenotypes relative to unpolarised (M^{un}); n = 8 donors, dotted lines = $|\log_2(1.5)|$, *** FDR ≤ 0.001. (**D**) Flow cytometry quantification of MSR1 and CD36 cell surface protein levels by MDM phenotypes, n = 5. (**A** and **D**) Mean ± SEM, matched/repeated measures one–way ANOVA with Dunnett's post-hoc test, * $p < 0.05$, *** $p \leq 0.001$, compared to M^{un}. MFI – geometric mean fluorescence intensity.

Figure 4: Internal lipid content and lipoprotein processing in macrophage phenotypes.
Colorimetric quantification of change in (**A**) total cholesterol, (**B**) cholesterol ester and (**C**) free cholesterol; mean ± SEM, n = 4 donors, matched/repeated measures one–way ANOVA with Dunnett's post–hoc test, by human monocyte-derived macrophages polarised with IFNγ+LPS, IL–4, IL–10 or oxPAPC compared to unpolarised (M^{un}). (**D**) Differentially expressed mRNAs in the 'LDL clearance' pathway (annotations retrieved from reactome.org on 16 October 2018) of M^x compared to M^{un} measured using RNA–seq; n = 8, thresholds for differential gene expression were $|\log_2 FC| > \log_2(1.5)$ and FDR < 0.05 (Benjamini and Hochberg method). (**E**) RNA–seq quantification of *SOAT1*, *NCEH1*, *LIPA* mRNA expression among MDM phenotypes compared to M^{un}, n = 8, dotted lines = $|\log_2(1.5)|$, * FDR < 0.05, ** FDR ≤ 0.01, *** FDR ≤ 0.001.

Figure 5: Cholesterol efflux by MDM phenotypes.

Fluorescence quantification of MDM cholesterol efflux for total cholesterol (**A**) via both lipid acceptors, HDL (**B**) and Apo–AI (**C**) separately; mean ± SEM, n = 4 donors, matched/repeated measures one–way ANOVA with Dunnett's post–hoc test, ** $p \leq 0.01$, *** $p \leq 0.001$ by human monocyte-derived macrophages polarised with IFNγ+LPS, IL–4, IL–10 or oxPAPC compared to unpolarised (M^{un}). (**D**) Differentially expressed genes in the 'HDL assembly' and 'HDL remodelling' pathways (annotations retrieved from reactome.org on 16 October 2018) in M^{x} compared to M^{un} measured using RNA–seq; n = 8 donors, thresholds for differential gene expression were $|\log_2 FC| > \log_2(1.5)$ and FDR < 0.05 (Benjamini and Hochberg method). (**E**) RNA–seq quantification of *ABCA1*, *ABCG1*, *SCARB1* mRNA expression among MDM phenotypes compared to M^{un}, n = 8 donors, dotted lines = $|\log_2(1.5)|$, ** FDR ≤ 0.01, *** FDR ≤ 0.001. (**F**) Flow cytometry quantification of MDM phenotype for ABCA1 (n = 5), ABCG1 (n = 6) and SCARB1 (n = 4 donors) cell surface protein levels; mean ± SEM, matched/repeated measures one–way ANOVA with Dunnett's post–hoc test, * $p < 0.05$, *** $p \leq 0.001$, compared to M^{un}.

Response to Reviews.
Ref.: Ms. No. ATH-D-19-00697R1

Macrophage polarisation associated with atherosclerosis differentially affects their capacity to handle lipid

Atherosclerosis

Please find responses to the reviewers' comments as outlined in red text below.

"Dear Dr. Wilson,

Thank you for submitting your paper to Atherosclerosis. It has been reviewed and the referees' reports are appended below.
We are pleased to tell you that the manuscript has been found potentially acceptable for publication. However, there are some minor points, as detailed by the reviewers, that need to be addressed before we can proceed further, and we would ask you to revise your paper addressing each issue raised. ALL CHANGES TO THE TEXT ARE TO BE SHOWN IN RED (not as tracked changes).
When you resubmit, you should also include a point-by-point response to each critique; if you revise to a satisfactory standard, your paper will be accepted for publication.

Editors' comments:

The manuscript has been evaluated by the Editors and the two original Reviewers. While the revised version has improved, there are still mostly textual changes that need to be addressed before the paper can be accepted. "

Reviewers' comments:

Reviewer #1: This manuscript provides a wealth of data using the state-of-art methodologies. Validation data provided, including good functional assays. Informative Graphical Abstract.

Introduction.
1. I am not sure I understand the statement Macrophages regulate lipoproteins within their own environment.
We have also noted the change requested by reviewer 2, to the start of this second paragraph of the introduction. We have therefore altered the second sentence according to reviewer 2's request (see below), and kept the first sentence as it was, since reviewer 2 implied this first sentence should remain.

Similarly: The accumulation of free and esterified cholesterol droplets……..
We have changed the sentence "The cytosolic accumulation of free and esterified cholesterol droplets in macrophages results in the generation of foam cells, underlying atherosclerotic plaque formation [9]."
Now reads: "The cytosolic accumulation of esterified cholesterol and acylglyceride droplets in macrophages results in the generation of foam cells, driving atherosclerotic plaque formation"

2. Reference missing to original work for the statement: In arterial atherosclerotic plaques, pro-inflammatory macrophages……..
We have now cited Stöger et al., 2012, after this sentence.
3. I would encourage the authors to cite original work more to back up their statements rather than review articles.
We have now cited additional original articles in our manuscript, including Murray and Teitelbaum, 1992; Stöger et al., 2012,; Hashizume and Mihara, 2012 to support specific statements. Analysis of our reference list shows 70% of the articles we now cite are original, 30% are review articles. We have chosen to cite review articles where they support the statements most clearly and provide a comprehensive assimilation of the literature.
4. Page 4, macrophage phenotypes occur change to develop? No references supporting your next statement. This whole paragraph would benefit from better citation of the literature.
We have now cited Johnston et al., 2018; Zhang et al., 2020, as examples in the literature to support the statement "the majority of our current understanding of macrophage polarisation and its impact on atherosclerosis derives from murine models of atherosclerosis and analysis of mouse bone marrow derived macrophages" in this section on page 4.
Results. I have been able to read further through the results but found it really hard towards the end. I think it all comes back to needing a better Introduction so that the results can be extracted more quickly.
We reconfigured the introduction and results comprehensively in our last revision to support this reviewer's recommendations. Since the recommendations for a better introduction are not specific we consider we are this reviewer may not wish us to make further changes without such specifications. We are open to editorial guidance on this specific point.

1). Please be careful with the use of the word expression-you are measuring CD163 RNA levels. Same for rest of sentence. Also later on you refer to protein expression rather than levels.
In the sentence on page 5 "M^{IL-10} showed upregulation of *CD163..*" we have changed the wording expression to RNA. Throughout the manuscript we have italicised the gene names when referring to their RNA and kept the names in regular font when referring to protein, as per scientific convention.
We have changed any wording of "protein expression" to "protein levels" consistently in the manuscript, and have added mRNA where appropriate (to also comply with the suggestion in point 7 below).
2). Next, paragraph-the word transcription not correct here-you are looking at a transcriptome profile.
Page 5, we have changed "CXCL4 macrophages showed remarkably similar transcription to…" to "CXCL4 macrophages showed a remarkably similar transcriptome profile…"
3). Up-regulated at higher magnitudes than what? The beginning of the sentence not referenced.
Page 5, sentence "In addition, previously reported M^{CXCL4} markers S100A8 (S100 calcium binding protein A8) and MMP7 (matrix metallopeptidase 7) showed expression changes in our study that were highly variable among donors and up–

regulated at higher magnitudes in most other phenotypes, especially $M^{IFN\gamma+LPS}$ (Supplementary Tables 2 and 3)."
Is now changed to:
"*S100A8* (S100 calcium binding protein A8) and *MMP7* (matrix metallopeptidase 7) have previously been reported as M^{CXCL4} markers [Erbel et al., 2015]; our RNA-seq data showed their expression in M^{CXCL4} was highly variable among donors and was up–regulated in most other phenotypes, especially $M^{IFN\gamma+LPS}$ (Supplementary Tables 2 and 3)."

4). P5. Our transcriptome analysis of the differently polarised-otherwise making an assumption that is not proven? Also differences in RNA levels of …….
We have changed (page 5/6):
"Our transcriptome analyses of macrophage phenotypes revealed key differences in atherosclerosis and lipid handling genes (Supplementary Table 5)."
To
"Our transcriptome analyses of macrophage phenotypes revealed differences in lipid handling and other associated pathway enrichment for differentially expressed genes (Supplementary Table 5)."

5). Data in Fig 2A and B good-but the sentence in the text confusing to read. Fig legend A, I think I would say that red arrow indicate first-think sentence is round the wrong way.
Legend to Fig 2A is changed as suggested.

In B not clear what n=5 means.
"n = 5 separate donors" is now written in the legend to Figure 2.

I think the text-describing Figure 2A and B and Fig 3A would be better under one Section and in one Figure.
Thank you for this suggestion: we suggest that Figures 2 and 3A and associated text (main text and figure legends) should probably remain as they are to maintain the narrative as per our previous response to reviewer 1. In our last revision we altered the introduction, and ordered the results with the following narrative structure: foam cell formation which is then broken down mechanistically to determine lipid accumulation, processing and efflux. Since this was a response to reviewer 1, and since reviewer 2 accepted our revision on this basis, we suggest we do keep the figures are currently presented.

I think you have too much Introduction in the Results-I think after your first sentence in paragraph 2 of the Introduction, you could Introduce how acLDL is internalised by saying For Example…. I think most of the two paragraphs coming after the Subheading Cellular free cholesterol content in polarised macrophages on acLDL loading should be in the Introduction.
We have moved the following text from the results to the second paragraph of the introduction as advised:
"In the case of lipoprotein, cellular lipases such as LIPA break down the lipoprotein particles that have been taken up… for subsequent storage in lipid droplets."

Please be careful, the uptake of LDL and modified LDL are quite different animals.
We agree that LDL and modified LDL behave differently. We have checked the manuscript and ensured we only refer to acLDL where this is what we have measured experimentally, or "LDL" when we are making more generalised and discussion-related statements.

6). If you removed the Introduction in the Results to the Introduction,
In our last revision we altered the start of the results section considerably in response to both reviewers' recommendations. The start of the results section now comprises a brief paragraph that serves to introduce the rationale. This rationale is included in the final section of the introduction. We consider that the narrative in the results section will be less clear if we remove the introduction to the first section of the results.
you could say more in the Subheading Cellular free-cholesterol content…….just not very informative.
We have re-worded the "cellular free-cholesterol content…" results subheading to improve informativity.
7). Fig 3C etc are you looking at mRNA or Total RNA?
We are looking at mRNA. We have corrected figure legends to make this clear in the descriptions. We have adjusted some wording in the figure legends to make the information more accurate and informative as shown in red text.
Description of Figure 3B really muddled me at first-overall down-regulation of what in what.
We have re-worded this results section (page 6), along with the legend to Fig. 3B, to make this description clearer. This was a down-regulation of a pathway identified using Ingenuity Pathway Analysis, which we now describe.
A similar expression profile-I think here you are trying to say RNA levels translated into similar reduction in protein levels.
We have re-worded the last sentence in this same paragraph (page 6, Reduced LDL uptake section) to make the description clearer.
8). I would argue that it is agreement with rather consistent with. Very vague in lipoprotein particle breakdown.
We have re-worded the last sentence in this same paragraph (page 6, Reduced LDL uptake section) to state "In agreement with…"
9). The last section can be reduced-the first two sentences perhaps in the Introduction, and then start with the third.
We have made these changes as suggested.

Reviewer 2:
The sentence "Macrophages regulate lipoproteins within their local environment [7]. Uptake of lipoproteins occurs via receptor-mediated endocytosis, pinocytosis, phagocytosis and scavenger receptor mediated mechanisms [4]" should read Macrophages regulate lipoproteins within their local environment [7]. Uptake of lipoproteins occurs via receptor-mediated endocytosis, such as scavenger receptor, pinocytosis and phagocytosis [4].
We have made these exact changes to the second paragraph of the introduction, as suggested here.

Editorial Office comments:

-Atherosclerosis applies formatting guidelines to all accepted papers, with the aim of improving their readability.
Manuscripts that do not conform to the format guidelines of the Atherosclerosis Journal will be returned to the authors for reformatting.
When revising your manuscript, please follow carefully the recommendations of our Atherosclerosis Style Guide to be downloaded from the following link (http://cdn.elsevier.com/promis_misc/Atherosclerosis_style_guide_checklist.docx).

- Make sure to apply the formatting requirements to all figures and tables where necessary (e.g. style of p values, gene and protein nomenclature).

- Make sure to use uniform lettering and sizing of your original artwork, including letters to indicate panels, throughout all figures.

- Make sure to submit high resolution versions of each figure. We can improve these if considered necessary by the journal.

- A graphical abstract is required at revision. Supplied

Statement of Originality.

The authors declare that the work described in our manuscript entitled "**Macrophage polarisation associated with atherosclerosis differentially affects their capacity to handle lipid**", for submission as a research article to *Atherosclerosis,* has not been published previously.

The article is not under consideration for publication elsewhere, publication of the article is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If the article is accepted, it will not be published elsewhere by the authors, including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

Figure(s)

Figure 1

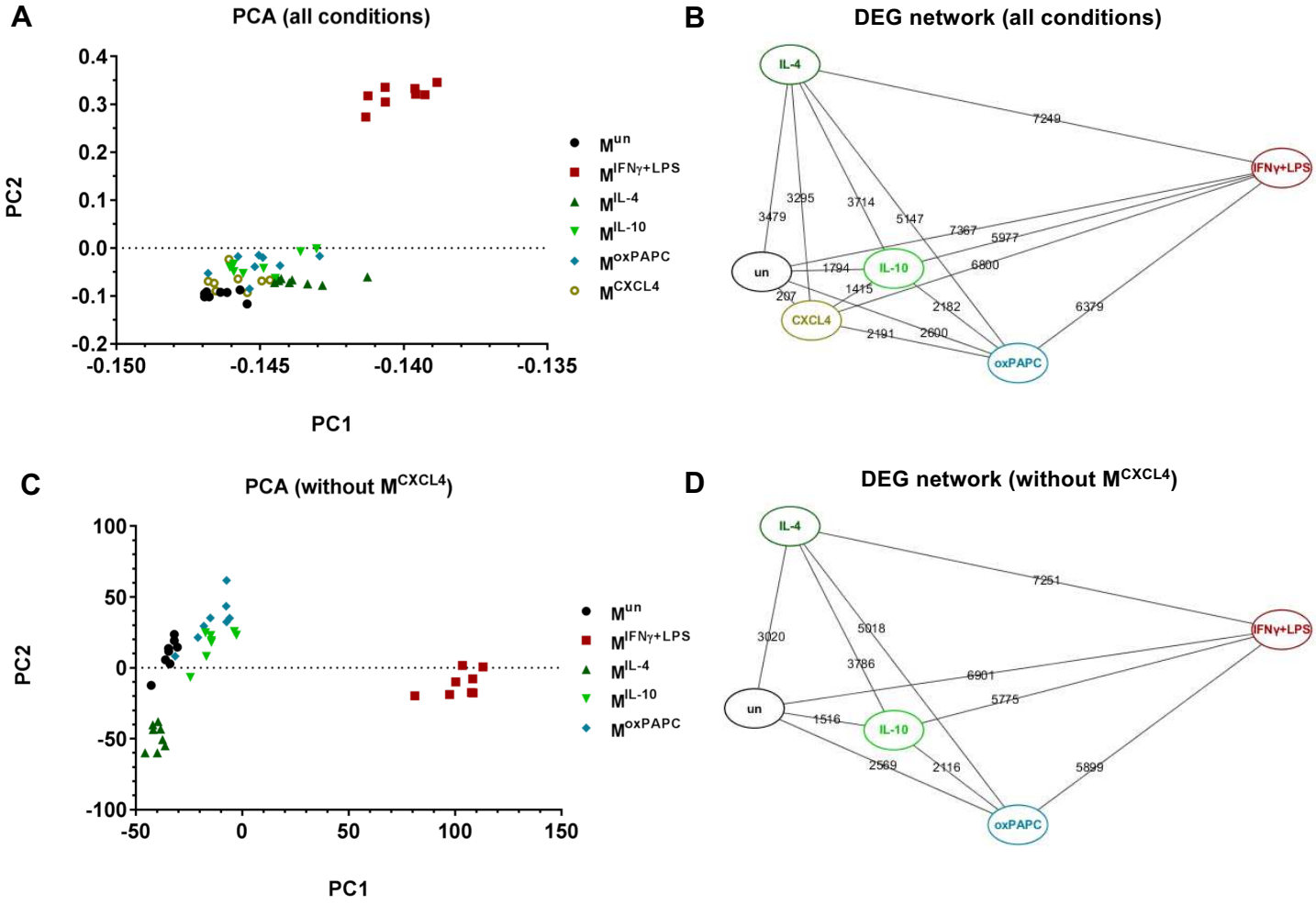


Figure 2

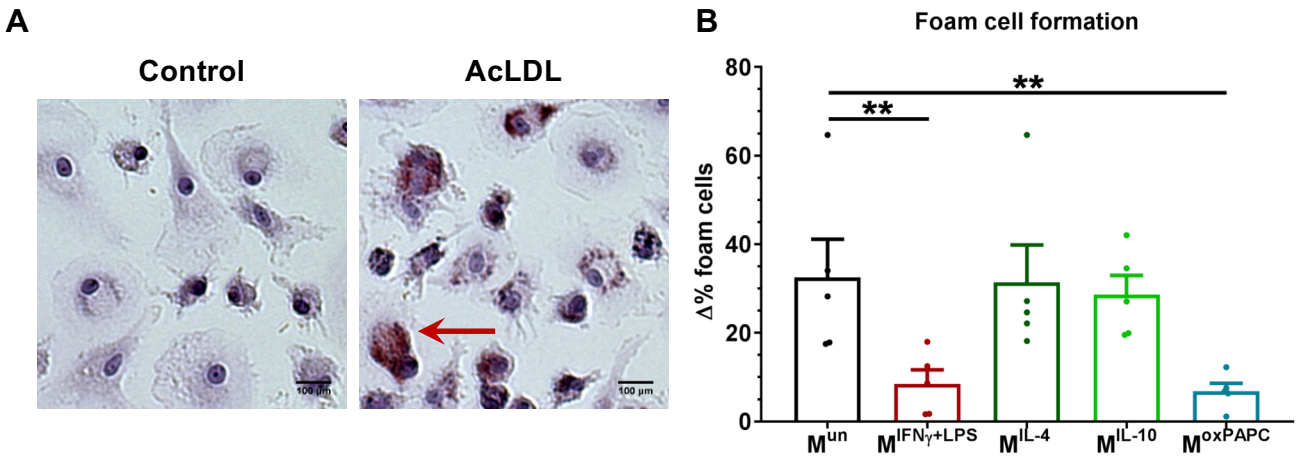

A
Control
AcLDL
100 µm
100 µm
B
Foam cell formation
Δ% foam cells
80
60
40
20
0
**
**
M un
M IFNγ+LPS
M IL-4
M IL-10
M oxPAPC

Figure 3

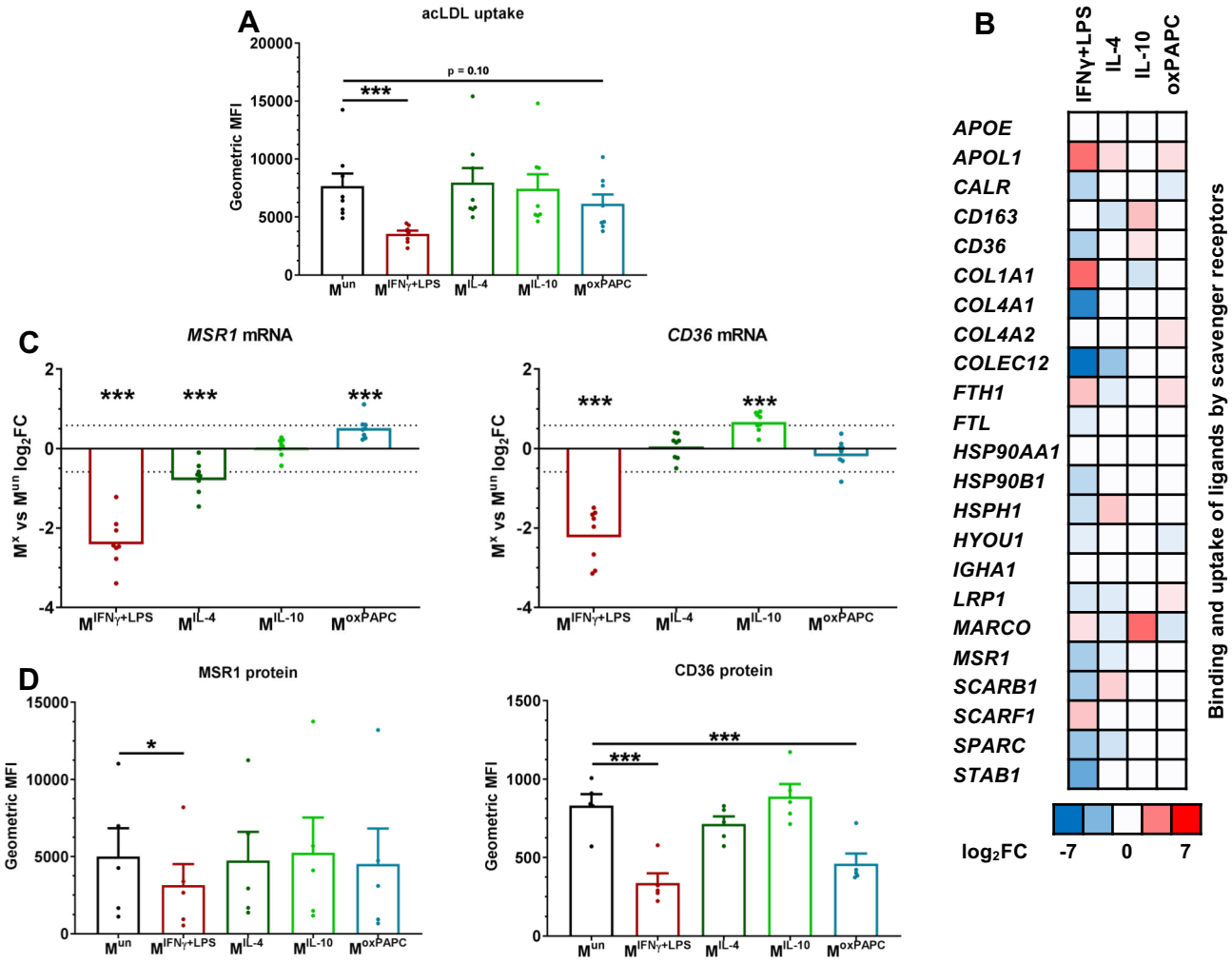

A
acLDL uptake
Geometric MFI
p = 0.10
***
B
IFNγ+LPS
IL-4
IL-10
oxPAPC
APOE
APOL1
CALR
CD163
CD36
COL1A1
COL4A1
COL4A2
COLEC12
FTH1
FTL
HSP90AA1
HSP90B1
HSPH1
HYOU1
IGHA1
LRP1
MARCO
MSR1
SCARB1
SCARF1
SPARC
STAB1
log2FC -7 0 7
Binding and uptake of ligands by scavenger receptors
C
MSR1 mRNA
CD36 mRNA
Mx vs Mun log2FC
D
MSR1 protein
CD36 protein
Geometric MFI
Mun
MIFNγ+LPS
MIL-4
MIL-10
MoxPAPC

Figure 4

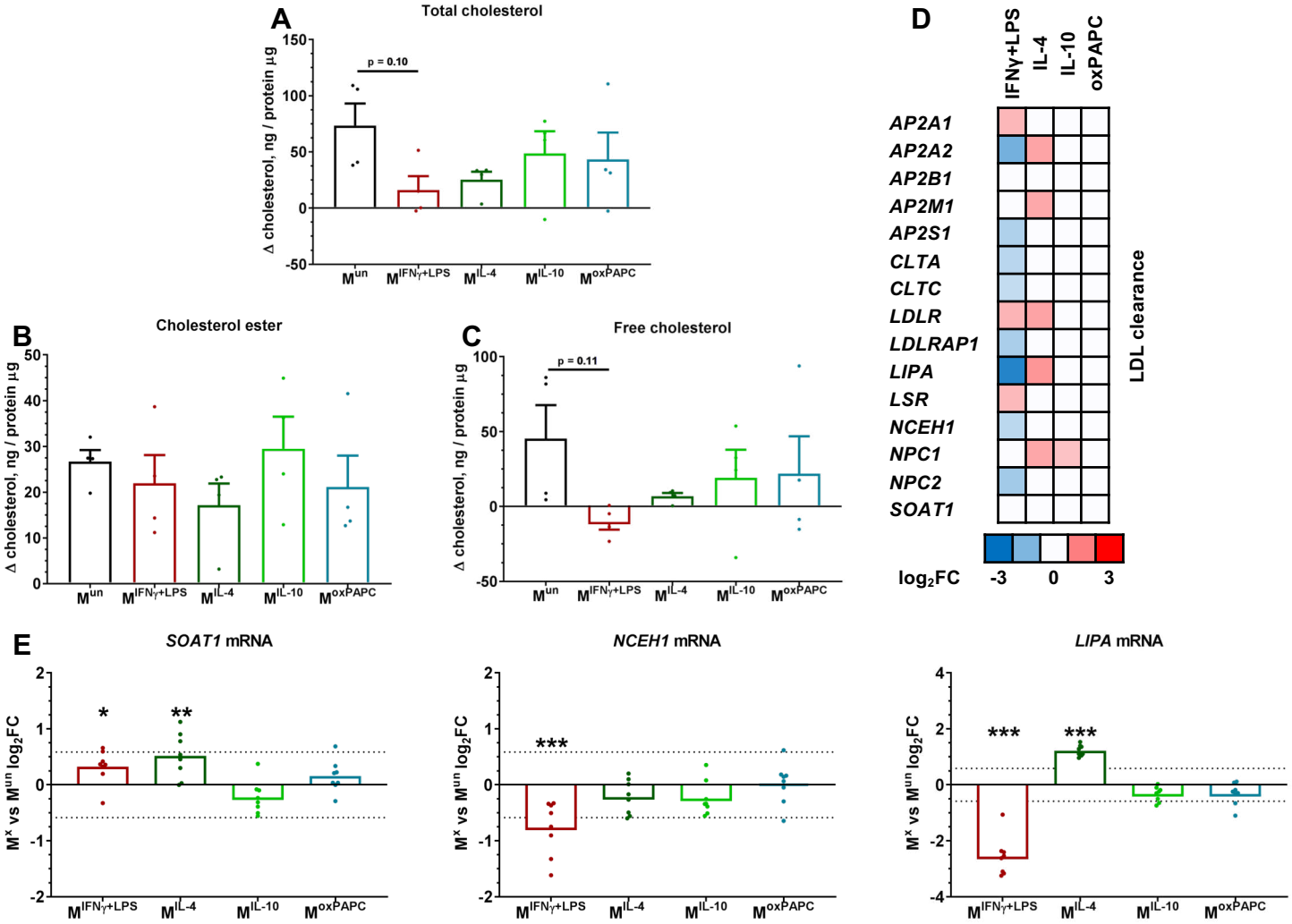
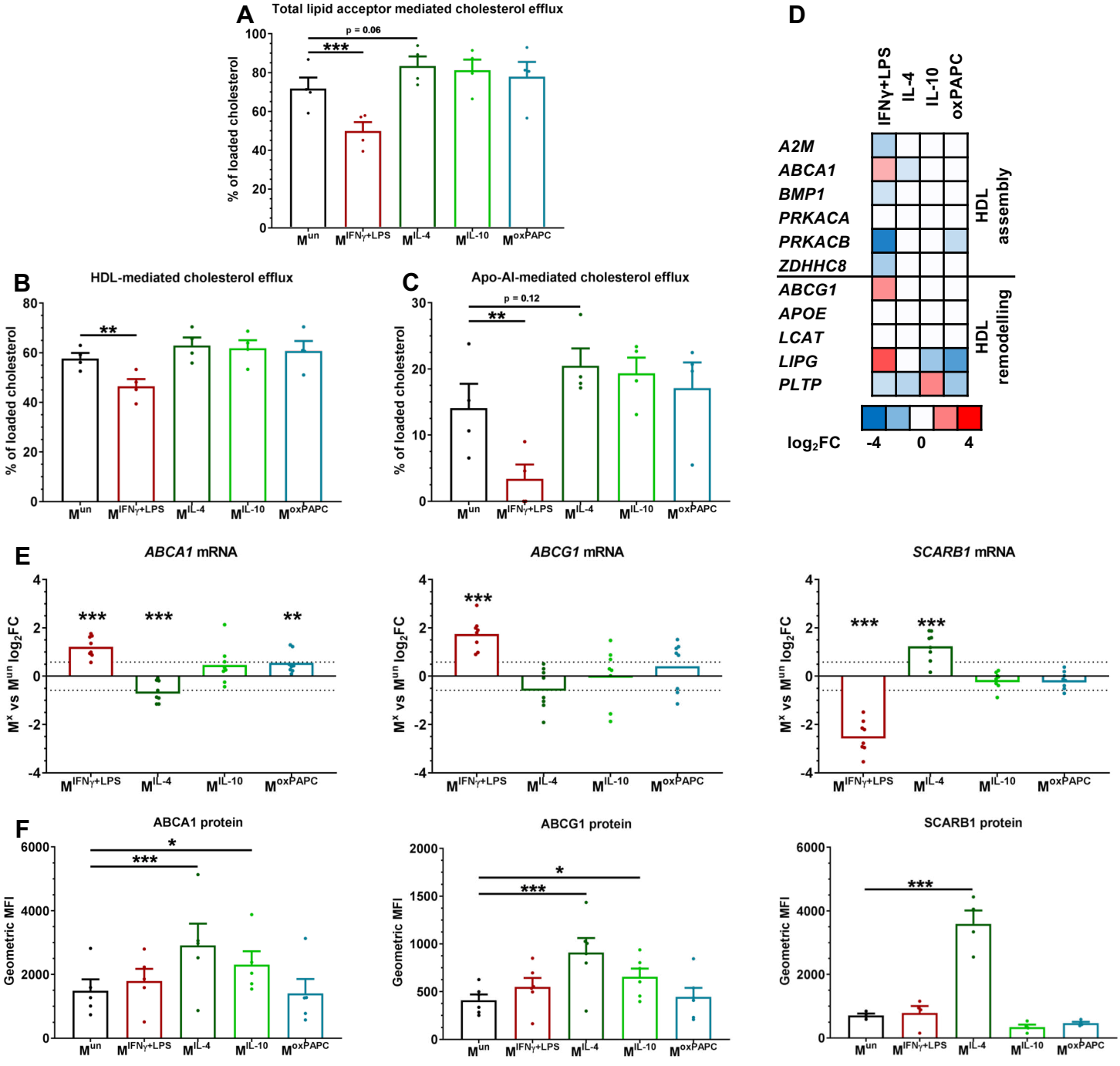

A
Total cholesterol
Δ cholesterol, ng / protein µg
p = 0.10
B
Cholesterol ester
C
Free cholesterol
p = 0.11
D
IFNγ+LPS
IL-4
IL-10
oxPAPC
AP2A1
AP2A2
AP2B1
AP2M1
AP2S1
CLTA
CLTC
LDLR
LDLRAP1
LIPA
LSR
NCEH1
NPC1
NPC2
SOAT1
LDL clearance
log2FC
-3
0
3
E
SOAT1 mRNA
NCEH1 mRNA
LIPA mRNA
Mx vs Mun log2FC

Figure 5


A
Total lipid acceptor mediated cholesterol efflux
% of loaded cholesterol
p = 0.06
***
B
HDL-mediated cholesterol efflux
**
C
Apo-AI-mediated cholesterol efflux
p = 0.12
**
D
IFNγ+LPS
IL-4
IL-10
oxPAPC
A2M
ABCA1
BMP1
PRKACA
PRKACB
ZDHHC8
ABCG1
APOE
LCAT
LIPG
PLTP
HDL assembly
HDL remodelling
log2FC
-4
0
4
E
ABCA1 mRNA
ABCG1 mRNA
SCARB1 mRNA
Mx vs Mun log2FC
F
ABCA1 protein
ABCG1 protein
SCARB1 protein
Geometric MFI
Mun
MIFNγ+LPS
MIL-4
MIL-10
MoxPAPC

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Author contributions:

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