Inflammation induced foam cell formation in chronic inflammatory disease

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ABSTRACT

Atherosclerosis is the leading cause of cardiovascular disease and is both a metabolic and inflammatory disease. Two models describe early events initiating atherosclerotic plaque formation whereby foam cells form in response to hyperlipidaemia or inflammation-associated stimuli. While these models are inextricably linked and not mutually exclusive, identifying the unique contribution of each in different disease settings remains an important question. Circulating monocytes are key mediators of atherogenesis in both models as precursors to lipid laden foam cells formed in response to either excess lipid deposition in arteries, signalling via pattern associated molecular patterns or a combination of the two. In this review we assess the role of monocytes in each model and discuss how key steps in atherogenesis may be targeted to enhance clinical outcomes in patients with chronic inflammatory disease.
INTRODUCTION

Atherosclerotic plaque formation is typically associated with a state of hyperlipidaemia characterised by high levels of circulating lipid-containing oxidised low density lipoproteins (oxLDL) that promote fatty streak formation. Fatty streaks form mainly in medium to large arteries and may progress to form plaques encased by a fibrous cap that may be degraded resulting in rupture, coagulation and thrombosis contributing to myocardial infarction and stroke. Lipid-laden macrophages, known as foam cells, are thought to form from migrated monocytes responding to oxidation of lipoprotein deposited within the intima.\textsuperscript{1,2} In this scenario, the progression of asymptomatic fatty streaks to atherosclerotic plaques depends upon lipid levels in circulation which determine the extent of deposition of lipid into arteries and the inflammatory milieu which promotes endothelial activation and lipid oxidation.\textsuperscript{3} For convenience we will term this the lipid-centric model of foam cell formation. However, a growing body of literature indicates that soluble and cellular immune factors associated with chronic inflammation can promote atherogenesis independent of hyperlipidaemia. This is evident by the increased risk of atherosclerosis in a number of inflammatory conditions such as sepsis (3 fold),\textsuperscript{4} human immunodeficiency virus (HIV) infection (3 fold),\textsuperscript{5,6} healthy ageing,\textsuperscript{7} systemic lupus erythematosus (SLE)\textsuperscript{8} and rheumatoid arthritis (RA) (2 fold)\textsuperscript{9} which is independent of traditional cardiovascular risk factors including lipid levels. Further, emerging data suggest that the role of monocytes/macrophages in atherosclerosis is not simply that of a passive acceptor of oxidised lipid. Monocytes, circulating blood precursors of tissue macrophages and myeloid-derived dendritic cells (mDCs), influence plaque development following recruitment into the intima and differentiation to foam cells. While the mechanisms governing inflammation-derived foam cell formation by monocytes remains ill-defined, recent evidence suggests that recognition of inflammatory pattern associated
molecular patterns (PAMPs) by pattern recognition receptors (PRRs) may play a key role. For the purposes of this review we will term this the inflammation-centric model.

In this review we discuss the role of inflammatory components in monocyte/macrophage derived foam cell and atherosclerotic plaque formation through PRRs and the implication of this for cardiovascular risk in the setting of inflammatory diseases.

ROLE OF MONOCYTES IN THE LIPID-CENTRIC MODEL OF ATHEROGENESIS

As macrophage precursors recruited to sites of lipid deposition, monocytes play a major role in changes to fatty streak composition that can lead to atherosclerotic plaques (Figure 1A). High levels of circulating lipid accumulate in areas of oscillatory blood flow around the aortic arch and bifurcations and induce endothelial activation, upregulating expression of adhesion molecules (i.e. VCAM-1 and P-selectin) and monocyte chemotactic proteins (eg CCL-2) that facilitate monocyte recruitment. Monocytes are activated by oxLDL, which may be produced from LDL by activated endothelial cells or interactions with free radicals and metal ions. In response, monocytes upregulate adhesion molecules and chemokine receptors (i.e. CD11b, CCR2, CX3CR1) which increase recruitment. The differential expression of these receptors has a key influence on the role of individual monocyte subsets in atherogenesis.

Three subsets of monocytes have been defined in human circulation which are characterised by expression of LPS co-receptor CD14 and FcγRIIIA (CD16). The majority of monocytes are classical monocytes (CD14++CD16−,~80%), while intermediate (CD14++CD16+,~8%) and non-classical (CD16++CD14+,~5%) monocytes make up minor populations in healthy individuals. Studies of the role of individual monocyte subsets in atherogenesis have mostly been conducted using mouse models, in particular “Western diet” fed hyperlipidaemic apoE deficient (apoE(−/−)) mice that readily form atherosclerotic plaques. In contrast to humans, mouse monocytes are usually characterised as having only two subsets roughly equivalent to
classical (Gr1+/Ly6C+) and non-classical (Gr1-/Ly6C–) monocyte subsets as determined by gene expression analysis. In apoE(-/-) mice, Gr1+ monocytes expressing high levels of CCR2 and low levels of CX3CR1 take on an ‘inflammatory’ role and migrate into fatty streaks to form foam cells,² while the Gr- subset expressing high levels of CX3CR1 preferentially ‘patrols’ the endothelium. Similarly, human non-classical monocytes have been identified to be ‘patrolling’, while intermediate and classical subsets play an inflammatory role.¹¹ Ly-6C-hi(Gr-1+) monocytes are expanded in apoE(-/-) mice fed a high fat diet suggesting hyperlipidaemia promotes higher circulating levels of ‘inflammatory’ monocytes.² While the role of individual human monocyte subsets in early atherogenesis have not been confirmed due to the lack of appropriate models of foam cell formation, cross-sectional studies showed that numbers of intermediate monocytes independently predicted cardiovascular events in individuals undergoing elective coronary angiography¹² and the percentage of CD45RA+ (highly expressed on CD16+ subsets) monocytes correlates with serum LDL-C levels.¹³

Following adhesion, monocytes extravasate to the neointima where exposure to macrophage colony stimulating factor stimulates their differentiation to macrophages with increased surface and intracellular expression of scavenger receptor A (SR-A) and B (CD36) that facilitate ingestion of either acetylated LDL or native/oxidised LDL, HDL and anionic phospholipids, respectively (Figure 1A).¹⁴ Other scavenger receptors (e.g. SR-BI and BII) as well as Toll-like receptors (TLRs) also contribute to lipid uptake. Alternatively, monocytes may differentiate to mDCs that can be activated by bacterial ligands via TLRs, potentially contributing to lipid accumulation. However, the ability of DCs to take up lipid and the role of TLRs in this process remains controversial.¹⁵

Oxidised LDL and other lipid species are hydrolysed in lysosomes within macrophages to liberate free cholesterol (FC) that is either exported via cholesterol efflux transporters such as ATP-binding cassette transporter 1 (ABCA1) and ABCG1 to apolipoproteins acceptors in the
vasculature for delivery to, and further metabolism in, the liver or stored in lipid droplets following esterification to cholesterol ester (CE) by Acyl-CoA:cholesterol acyltransferase-1 (ACAT1). Monocytes and possibly macrophages or DCs may also egress from plaque to physically remove accumulated lipid.16

Cells which fail to egress from the plaque can ingest large quantities of lipid mainly via SR-A and CD36 and potentially become resident foam cells although CD36(−/−)SR-A(−/−)apoE(−/−) mice form foam cells in the absence of these receptors,17 indicating that pathways independent of scavenger receptors contribute to foam cell formation. Importantly, the factors governing the tendency of macrophages to remain in the intima and form foam cells are incompletely understood, although recent evidence suggests differentiation into classically activated (lipopolysaccharide (LPS) stimulated) proinflammatory M1 or alternatively activated (IL-4 stimulated) anti-inflammatory M2 macrophages may be an important determinant, as M2 macrophages are more likely to form foam cells in vitro under inflammatory conditions.18 These findings highlight the plasticity of macrophages to alter phenotype in different immunological environments. It is of note that M1/M2 macrophages generated in vitro may show different immune characteristics to their in vivo counterparts highlighting the requirement of further studies to identify the role of M1/M2 macrophages in physiologically relevant models of atherosclerosis.

Under conditions where cholesterol influx exceeds cholesterol efflux, lipid droplets accumulate and necrotic and apoptotic pathways are ultimately activated in the foam cell resulting in cell death and the subsequent release of FC, CE and inflammatory cytokines that act on endothelial and immune cells in a positive feedback loop to induce further monocyte migration into the intima.
INFLUENCE OF INFLAMMATION ON ATEROGENESIS

Whilst there is a clear mechanistic link between hyperlipidaemia and atherogenesis, inflammatory factors can promote foam cell formation and potentiate plaque development independent of hyperlipidaemia (Figure 1B). Markers of inflammation (IL-6, TNF receptor, high sensitivity C-reactive protein (hs-CRP)) and viral infection/autoimmune disorders (type I interferons (IFN-α, IFN-β)) are independently associated with adverse cardiac outcomes in people with atherosclerosis. While these markers have not been clinically validated, they may provide insight into mechanisms of atherogenesis. As such, risk estimates that incorporate the impact of inflammation (plasma hs-CRP levels) on atherogenic risk in addition to traditional risk factors (e.g. Reynolds risk score) have been shown to better predict cardiac outcomes in healthy individuals than risk scores which rely on traditional risk factors alone (i.e. smoking status, blood pressure, HDL/LDL etc).

In contrast to lipid–mediated atherogenesis where accumulated oxLDL promotes initial monocyte recruitment, under inflammatory conditions proinflammatory cytokines (i.e. IL-6 and TNF) and potentially even pathogen/endothelial interactions independently promote monocyte recruitment to the endothelium. Bacterial products may also directly influence atherogenesis since mouse J774 macrophages stimulated in vitro with bacteria commonly identified in atherosclerotic plaques form foam cells via TLR dependent mechanisms independent of hyperlipidaemia. Human monocytes are particularly efficient at responding to PAMPs and express 10 different TLRs responsible for identifying non-host PAMPs such as LPS (TLR-4) and lipoprotein (TLR-2) components of bacterial cell walls as well as viral nucleic acids (TLR-7/8) and endogenous ligands. Through conserved myeloid differentiation factor-88 (MyD88) dependent (MyD88/NF-κB) or independent (TRIF/IRFs) signalling pathways, TLRs induce production of both proinflammatory (IL-1β, IL-6, TNF) and anti-inflammatory cytokines (IL-10) as well as type I interferons (IFNα and β).
Following extravasation, monocyte/macrophage TLR responses to PAMPs present in the neointima enhance uptake of LDL increasing foam cell formation in part by upregulating LDL receptor (LDLR) expression (Figure 1B, discussed further below). Evidence that loss of SR-A and CD36 does not abolish foam cell formation in hyperlipidaemic mice is consistent with this, suggesting that other lipid-independent pathways are involved. These lipid independent processes promote further monocyte recruitment and foam cell formation by mechanisms described in the lipid centric model, therefore linking inflammatory processes to lipid oxidation and uptake.

While fatty streaks are mainly composed of lipid deposits and monocytes/macrophages, other immune cells accumulate as the plaque matures. High serum cholesterol levels are associated with macrophage activation resulting in IL-1β, IL-6, TNF and IFN-γ production that contribute to localised inflammation and monocyte recruitment. Furthermore murine macrophages incubated in vitro with oxLDL or FC produced following the blocking of macrophage cholesterol efflux have increased production of IL-6 and TNF in response to LPS, suggesting plaque resident macrophages exposed to lipid may have a heightened inflammatory response to stimuli therefore indicating crosstalk between the lipid- and inflammation-centric models. Proinflammatory cytokines produced by foam cells within the plaque may also contribute to localised inflammation: Their inflammatory nature is supported by in vitro studies showing that human monocyte-derived M2 macrophages, which normally have an anti-inflammatory phenotype, ingest high levels of oxLDL and produce proinflammatory factors (IL-6, IL-8, MCP-1) following foam cell formation, thus taking on a more M1-like proinflammatory phenotype. In contrast, other studies show that peritoneal foam cells produced in LDLR knockout mice (ldlr(-/-)) fed a western-type diet accumulate desmosterol. This sterol activates liver X receptor (LXR) pathways and suppresses TLR4-dependent activation of NF-κB that may dampen the proinflammatory response of these...
cells to inflammatory stimuli in the vessel wall. Further work, especially human studies, are required to fully characterise the inflammatory nature of foam cells and whether differences exist between foam cells in mature plaques and in fatty streaks.

Taken together, the above evidence suggests that inflammatory mechanisms acting on monocytes/macrophages promote atherosclerosis via proinflammatory foam cell formation which may be independent of hyperlipidaemia.

**ATHEROSCLEROSIS IS INCREASED IN INFLAMMATORY CONDITIONS**

Atherosclerosis is a leading cause of morbidity and mortality in many acute and chronic inflammatory conditions such as sepsis/endotoxaemia, HIV, RA, SLE and ageing, however the precise causal relationships between atherosclerosis and these inflammatory states are unclear (Table 1). Despite the efficacy of combination antiretroviral therapy (cART) which lowers the viral load of HIV+ individuals to levels undetectable by standard clinical assays (<50 copies of viral RNA/mL) and reduces progression to acquired immune deficiency syndrome (AIDS), HIV-infected individuals have an estimated 1.75 relative risk of acute myocardial infarction compared to matched HIV-uninfected individuals, which is independent of traditional risk factors such as dyslipidaemia.\(^{28}\) While the HIV accessory protein Nef may increase CVD risk by impairing macrophage cholesterol efflux,\(^{29}\) virologically-suppressed HIV+ individuals still experience 3-fold higher risk of atherosclerosis independent of traditional risk factors\(^{28}\) suggesting a role for factors other than HIV-encoded proteins. Early cART regimes containing protease inhibitors were associated with dyslipidaemia and increased CVD risk, however current cART regimens are less likely to induce dyslipidaemia (except those containing some nucleoside reverse transcriptase inhibitors such as Abacavir) and despite the reduction of HIV-associated dyslipidaemia in virologically suppressed individuals,\(^{30}\) increased CVD risk persists. CVD is a leading cause of mortality in older individuals but while older age is associated with increased risk factors
for atherosclerosis including hyperlipidaemia, age itself is a significant independent risk factor for atherosclerosis. Ageing is associated with chronic low level inflammation, increased carotid intima-media thickness (cIMT) and muscular changes in the heart as well as elevated levels of plasma markers/mediators of inflammation (IL-6, TNF, hs-CRP and LPS) which are associated with atherogenesis.

Autoimmune disorders such as RA and SLE are also independently associated with increased risk of CVD. Chronic inflammation within the synovium of RA patients results in the recruitment of multiple cell types including monocytes/macrophages, further potentiating localised inflammation via the production of IL-1β, IL-6, IL-8 and TNF that results in joint destruction and functional impairment. This also leads, however, to systemic increases in inflammatory cytokines which are associated with a 2-fold increase in atherosclerosis. Similarly, duration of SLE disease is an independent predictor of atherosclerosis and patients < 40 years old have 5.6 fold higher prevalence of atherosclerotic plaque to controls. SLE disease is associated with high plasma levels of proinflammatory cytokines and IFN as well as hyperlipidaemia which is thought to drive atherogenesis. SLE patients are also prone to infection and have high levels of circulating LPS and autoimmune complexes that may promote foam cell formation via TLR mediated mechanisms, although further studies are required to identify the role of TLRs in atherosclerosis in SLE patients.

Taken together atherogenic risk is increased in chronic inflammatory disease settings independent of traditional risk factors such as hyperlipidaemia, suggesting that atherogenesis may be driven by inflammatory mechanisms. While the causes of chronic inflammation differ, signals elicited by PAMPs are a common feature of these conditions.
**Increased presence of PAMPs in chronic inflammatory disease**

HIV+ individuals have increased plasma levels of PAMPS such as LPS due to increased mucosal permeability in the gut.\(^{35}\) These PAMPs may exacerbate the role played by monocytes in atherogenesis as predicted by the inflammation centric model described above as plasma sCD14 levels were associated with increased cIMT in HIV+ individuals\(^{36}\) (Figure 1). High systemic levels of bacteria and bacterial products which occur during sepsis in HIV- individuals also lead to widespread immune activation and inflammation and is an independent risk factor for atherosclerosis.\(^{4}\) While LPS levels found in the plasma of HIV+ individuals are not as high as those observed in sepsis patients (≥300 pg/mL vs 40-60 pg/mL for virologically suppressed HIV+ individuals), chronically elevated plasma levels of LPS above 50 pg/mL (referred to as chronic endotoxaemia) are associated with a 3-fold increased risk of atherosclerosis.\(^{4}\) This finding is significant, as our laboratory has found that elderly individuals show similar levels of LPS to younger, HIV+ individuals.\(^{37}\) This increase in the elderly is possibly caused by increased permeability between tight junctions of epithelial cells in the gut. SLE patients also have high plasma LPS levels that are thought to dysregulate the transcriptome of monocytes.\(^{34}\) Furthermore, studies in patients with chronic kidney disorders requiring dialysis, who are continuously exposed to bacterial products, show increased cardiovascular risk associated with increased LPS levels in the blood suggesting that bacterial components may enhance atherogenesis in this setting.\(^{38}\)

The presence of bacterial cell wall components such as lipoprotein and LPS is commonly caused by respiratory infections (*Chlamydia pneumoniae*), bacterial contamination of dialysis tubing (*Escherichia coli*) and periodontitis (*Porphyromonas gingivalis*) triggering generalised immune activation and inflammation via TLR signalling. Furthermore, patients with concurrent vascular disease and chronic periodontitis show higher bacterial load and bacterial diversity than matched donors with vascular disease as determined by 16S rDNA analysis of
vascular biopsies. Interestingly a recent longitudinal study has shown that improvement in oral health is associated with a lower rate of progression of cIMT in otherwise healthy individuals further suggesting a role for bacterial PAMPs on atherogenesis.

Viral PAMPs from cytomegalovirus (CMV) reactivation, hepatitis C infection and residual HIV replication in cART-treated HIV-infected individuals may be associated with atherogenesis. Recent findings show that CMV seropositive HIV-infected individuals have a 50% increased risk of non-AIDS comorbidities compared to CMV seronegative HIV+ individuals, and that CMV seropositivity is associated with increased cardiovascular events. CMV mRNA has also been detected within atherosclerotic plaques and is associated with increased systemic inflammation. Furthermore in vivo studies in MCMV-infected apoE(-/-) mice identified that CMV-induced atherosclerosis occurred without changes in circulating cholesterol, HDL or triglycerides but was associated with increased IFN\(\gamma\) levels, consistent with inflammation-driven pathogenesis. CMV infection is widespread in the elderly (approximately 90.8% of people \(\geq 80\) years old are seropositive in the United States). CMV is associated with increased CVD-related mortality especially in those individuals with increased inflammation as measured by plasma hsCRP. Endogenous proteins found within arthritic joints such as heat shock protein-60 (hsp-60), fibrinogen and fibronectin, act as the major source of TLR activation in RA patients although the influence of these proteins on TLR-mediated atherogenesis is unknown.

Immune activation and inflammation in chronic inflammatory diseases influence atherogenesis independent of hyperlipidaemia via both increased levels of PAMPS as well as increased circulating cytokines that can influence the atherogenic potential of monocytes/macrophages.
MONOCYTE ACTIVATION IN INFLAMMATORY DISEASE

Many chronic inflammatory diseases are associated with altered monocyte phenotype and function, which may alter the potential of these cells to influence atherogenesis (Table 1). Inflammatory diseases are associated with increased plasma markers (sCD14, sCD163) and drivers (LPS) of monocyte activation that are used as surrogate markers of these processes, although few human studies have evaluated the contribution of monocyte activation in inflammatory disease on atherogenesis. While individual chronic inflammatory conditions may activate monocytes via different mechanisms, stimulation of monocytes via TLRs and other PRRs is a common factor. TLR-2 expression is elevated on circulating monocytes from RA, SLE and HIV-infected patients suggesting that these cells may be primed in their responses to bacterial PAMPs. This is also true of healthy, older individuals who show age-related increases in systemic inflammation termed “inflamm-ageing”. In addition to responding to inflammatory PAMPs, monocytes/macrophages also produce IL-1β, IL-6 and TNF via TLR-4 recognition of oxLDL. Murine studies have shown that oxLDL acts synergistically with LPS to increase proinflammatory cytokine production and foam cell formation in peritoneal macrophages from C3H/NeN mice in a TLR-4 dependent manner. This shows that monocytes bridge the lipid-centric and inflammatory models of atherosclerosis. Monocytes from HIV+ individuals, RA and SLE patients and the elderly show increased response to LPS, which may exacerbate the interaction between bacterial PAMPs and oxLDL. Furthermore HIV infection, RA, ageing and sepsis are all associated with an increased proportion of intermediate and non-classical monocyte subsets which are significant producers of proinflammatory cytokines. This is supported by epidemiological studies showing that increases in the proportion of intermediate monocytes independently predict cardiac events in HIV+ individuals and people with chronic kidney disease and that cellular changes to monocytes including increased surface expression of
tissue factor on intermediate monocytes is associated with increased cIMT in HIV+ individuals. In addition to a shift towards CD16+ subsets, monocytes from HIV+ individuals share phenotypic characteristics to those of HIV- individuals with acute coronary syndrome (elevated surface CD11b and tissue factor expression) and produce more proinflammatory cytokines in response to LPS. As CD16-expressing monocytes have higher surface expression of CD11b and CX3CR1 compared to classical monocytes, this may result in enhanced monocyte adhesion to endothelial cells, however further human studies are required to evaluate the significance of this mechanism to atherosclerosis.

Thus the activation of monocytes by PAMPS and the expansion of proinflammatory CD16 expressing monocyte subsets in the setting of inflammatory disease may promote atherosclerosis by promoting recruitment of pro-atherogenic monocytes into blood vessel walls. Furthermore activation of monocytes via inflammatory signalling pathways may also enhance lipid uptake promoting monocyte differentiation to foam cells.

**TLR ACTIVATION IN ATHEROSCLEROSIS**

Atherosclerotic tissue from patients with coronary artery disease has been shown to have 3-fold increased mRNA expression and protein levels of TLR-1/2 and -4 compared to tissue obtained from healthy control arteries with expression in plaques largely restricted to macrophages and endothelial cells. Furthermore, circulating monocytes and DCs from patients with acute coronary artery syndrome also have increased surface expression of TLR-2 and -4. TLRs recognizing both bacterial (TLR-2, -4, -5) and viral and endogenous ligands (TLR-7/8, -9) have been identified to influence atherogenic risk in both mouse and human studies. Evidence summarised below shows that TLR signalling can drive foam cell formation from macrophages independent of hyperlipidaemia, suggesting that monocytes with increased TLR expression may form foam cells more readily.
Bacterial driven atherogenesis

As mentioned above, certain bacterial infections such as *C. pneumoniae* and *E. coli* are associated with increased risk of atherosclerosis and foam cell formation and the titre of IgA antibodies to *C. pneumoniae* has been associated with carotid and femoral atherosclerosis.65 The relationship between bacterial infection and atherosclerosis has been demonstrated in animal models: rabbits fed a diet containing modest levels of cholesterol and infected with *C. pneumoniae* experienced accelerated atherosclerosis as measured by cIMT in comparison to uninfected controls fed a similar diet.66 Similarly inoculation of *C. pneumoniae* into the carotid artery of ldlr(-/-) mice enhanced atherosclerosis and caused a 2-fold increase in cIMT.67 DNA analyses of resected mature plaques from individuals undergoing coronary atherectomy have identified a wide variety of bacteria, suggesting that bacterial products may mediate localised inflammation that drives plaque development.68 These bacteria may also act by invading phagocytic cells localised within plaque, as it has been possible to culture viable bacteria from such tissue.69

The potential importance of bacterial PAMPs such as LPS in promoting foam cell formation is increased since TLR-4 mRNA expression is increased in surgically extracted atherosclerotic plaque62 and on circulating monocytes from individuals with acute coronary syndrome in comparison to controls.70 A role for TLR-4 in promoting atherosclerosis is supported by studies showing that polymorphisms that reduce TLR-4 activity are associated with decreased cardiovascular risk in humans.71 Animal models suggest that the role mechanism of TLR-mediated atherogenesis is independent of hyperlipidaemia as inhibition of TLR-4 signalling in LPS stimulated apoE(-/-) mice using NF-κB inhibitors reduced LPS-induced plaque size by 50%, without altering plasma levels of cholesterol or related lipids.72 Furthermore TLR-4 knockout in the apoE(-/-) mouse results in a 75% decrease in intimal lipid accumulation73 and reduced levels of MCP-1,74 a soluble marker of immune activation
associated with atherosclerosis, without effects on serum cholesterol and triglyceride levels. Infection of apoE(-/-) mice with *C. pneumoniae* increased diet-induced atherosclerosis in a MyD88 dependent manner that was abrogated by deletion of either TLR-2 or TLR-4 showing the need for multiple TLR pathways in infection scenarios. In this model, atherosclerosis was enhanced in apoE(-/-)LXRA(-/-) mice suggesting that LXRA modulates the effects of TLR ligands. One potential mechanism may be increased expression of macrophage cholesterol exporters which are positively regulated by LXRA (see below). *In vitro* studies show that incubation of RAW264.7 murine macrophages with purified *C. pneumoniae* LPS causes foam cell formation; the pathways leading to foam cell formation in these isolated cell systems need to be assessed *in vivo*.

While the lipid centric atherogenesis model assumes that formation of foam cells occurs via accumulation of exogenous modified LDL via scavenger receptors, Funk and colleagues first observed *in vitro* that incubation of RAW 264.7 cells with LPS resulted in foam cell formation in a dose dependent manner that was independent of the addition of exogenous LDL. Interestingly, foam cells formed under these conditions contain accumulated triglyceride-containing lipid droplets rather than esterified cholesterol, suggesting that these cells may differ biochemically from foam cells generated by incubation with modified cholesterol-containing lipoprotein particles. Lipoprotein from gram positive bacteria, recognised by TLR-2, also promotes foam cell formation independent of hyperlipidaemia. Infection of RAW 264.7 cells with *C. pneumoniae* promotes foam cell formation in a TLR-2 dependent manner and foam cell formation is impaired in peritoneal macrophages from TLR-2(-/-) C57BL/6 mice incubated with Pam3-cys or infected with *C. pneumoniae*. 

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Taken together, evidence is accumulating that bacterial infection and/or the presence of bacterial products may promote foam cell formation via TLRs expressed on endothelial cells and macrophages via mechanisms that do not require hyperlipidaemia.

**Virus driven atherogenesis**

While most research into the role of TLR-stimulated atherosclerosis has focused on bacterial products, viruses and endogenous proteins also influence atherogenesis. Individuals infected with HIV, CMV and hepatitis C also have increased CVD risk. Antigen presenting cells, particularly plasmacytoid DCs (pDCs), produce type 1 IFN in response to viral ligands, which induces antiviral response in surrounding tissues and activating monocytes and macrophages. While the role of pDCs in atherogenesis is not well characterised, IFN-α mRNA gene expression is strongly associated with TNF expression in surgically extracted human carotid plaque tissue suggesting activation of pDCs activate macrophages to produce TNF in plaque. Furthermore, IFN-α produced by pDCs primes macrophage responses to other PAMPs by up regulating TLR-4 expression and priming responses to bacterial PAMPs although further studies are required to conclusively identify the contribution of pDCs to IFN-α production *in vivo*. Similar studies in SLE patients also showed that IFN inducible gene expression correlated with SR-A mRNA expression suggesting that IFN-α may promote lipid uptake in plaque macrophages.

Monocytes, particularly non-classical and intermediate subsets, respond to viral PAMPs such as single stranded (ss) RNA via TLR-7/8 and produce type I IFN and proinflammatory cytokines. A recent study evaluating the role of HIV in foam cell formation found that HIV RNA is recognised by TLR-8 and promotes foam cell formation in human monocyte-derived macrophages (MDM) via the production of TNF. Conversely, TLR recognition of ssRNA may be potentially protective against atherosclerosis by repressing macrophage
proinflammatory cytokine production; silencing of TLR-7 in apoE(-/-) mice accelerated atherogenesis and plaque vulnerability. In contrast, macrophages from human plaques are reported to show decreased response to TLR-2 and TLR-4 ligands following TLR-7 stimulation. The role of TLR-7/8 in atherogenesis requires further investigation.

Despite transducing common signalling pathways, TLR signalling may play different roles in promoting or protecting against atherogenesis. Further work is required to delineate the complex interactions between these pathways.

**POTENTIAL MECHANISMS OF TLR DRIVEN FOAM CELL FORMATION**

Despite many studies showing that TLR ligands stimulate foam cell formation by monocytes/macrophages, the mechanism driving increased lipid accumulation and storage for many of these molecules is unknown. TLR signalling may promote foam cell formation in three ways; 1) by upregulating lipid uptake, 2) by increasing lipid biosynthesis, and 3) by down-regulating the processing and export of lipid following ingestion (Figure 2).

**Enhanced lipoprotein uptake via LDL receptor**

While foam cells form following the ingestion of modified LDL and other phospholipids via scavenger receptors, lipoprotein and TLRs expressed on monocytes/macrophages also promote foam cell formation following uptake of unmodified LDL. In vitro incubation of human monocytic THP-1 cells with LPS stimulates uptake of unmodified, native LDL via LDLR resulting in cells morphologically similar to foam cells. Whether increased LPS levels in HIV+ individuals and the elderly specifically promotes foam cell formation in these individuals via this mechanism is yet to be determined. LDLR transcription is regulated by sterol regulatory element binding protein 2 (SREBP2) which is increased by activation of TLR-4 via crosstalk between MyD88/IKK pathways and consequently, LPS enhances
LDLR gene and protein expression and LDL uptake in THP-1 cells *in vitro*. LPS stimulation of macrophages *in vitro* also increases minimally modified LDL uptake by macropinocytosis.\(^8^7\) Thus LPS may increase receptor and non-receptor-mediated uptake of unmodified LDL and stimulate foam cell formation independent of exogenous modified lipoproteins or scavenger receptors.

**Biosynthesis of lipids by macrophages**

TLR signalling promotes *de novo* lipid synthesis by macrophages. Monocytes synthesize cholesterol in the endoplasmic reticulum, and this biosynthesis is essential to allow differentiation to macrophages. The rate determining step of cholesterol synthesis is catalysed by HMG-CoA reductase, the primary target of the statin class of cholesterol-lowering drugs. Inflammatory factors such as LPS, TNF and IL-1\(\beta\) induce HMG-CoA reductase gene expression in THP-1 macrophages by activating NF-\(\kappa\)B and SREBP2 transcription factors.\(^8^6\) Treatment of monocytes from RA patients with simvastatin *ex vivo* decreased TLR-2 responses to *Staphylococcus aureus* peptidoglycan via inhibition of RhoA indicating that statins also inhibit TLR-mediated cytokine production via their anti-inflammatory properties.\(^8^8\) Newly synthesised or internalised, unesterified cholesterol either traffics to cholesterol efflux receptors for export (discussed below) or is esterified by ACAT-1 and stored as CE in lipid droplets. TNF increases ACAT-1 expression in human monocytes resulting in accumulation of CE and foam cell formation.\(^8^9\) This ability is not shared by other proinflammatory cytokines such as IL-1\(\beta\), IFN\(\gamma\) or IL-6, suggesting a unique property of TNF in both promoting cholesterol biosynthesis and esterification. Together these findings show that cross talk between TLR signalling pathways and their products skew cholesterol metabolism within monocytes/macrophages towards lipid synthesis and foam cell formation.
**Impaired cholesterol efflux**

Monocytes/macrophages metabolise FC to facilitate export to either lipoprotein acceptors (apoA1) or HDL via the cholesterol efflux regulatory proteins ABCA1 and ABCG1, respectively (Figure 2). The transcription of these proteins is upregulated by the LXR pathway that, if dysregulated, can promote foam cell accumulation via decreased cholesterol efflux.90

PAMP-mediated TLR signalling impairs cholesterol efflux in mouse macrophages by down-regulating ABCA1 mRNA expression via NF-κB-dependent mechanisms.91 However it is likely that both MyD88 dependent and independent mechanisms92 through transcription factor IRF-393 are responsible for ABCA1 down-regulation in this setting. In contrast one study using THP-1 cells stimulated with LPS in vitro has shown an increase of ABCA1 mRNA expression via the LXR pathway,94 indicating that further studies, especially in human cells, are required to elucidate the impact of TLR-mediated pathways on cholesterol efflux. Whether viral PAMPs also result in decreased ABCA1 by inhibiting LXR pathways is currently unknown, however as TLR-7/8 results in increased signalling via IRF-3 this may be possible.

As mentioned above, LXRα modulates the pro-atherogenic effects of C. pneumoniae infection.75 This was associated with inhibition of TLR-mediated NF-κB and IRF-3 activation when cells were stimulated with LXR agonists. Thus, the LXR and TLR signalling pathways may interact to mediate cross-talk between lipid and inflammatory mediated foam cell formation. LXR down-regulates proinflammatory cytokine production in LPS-stimulated monocytes and reduces TNF effects on cholesterol efflux.95 ABCA1 can also act as an exporter of LPS from within macrophages and thereby decrease LPS induced tolerance.96 There is therefore a complicated interrelationship between TLR and LXR signalling with
cholesterol efflux and foam cell formation on the one hand and cytokine secretion and inflammatory pathways on the other.

These *in vitro* studies are supported by *in vivo* data which showed that acute endotoxaemia in C57BL/6 chow-fed mice reduced cholesterol efflux from macrophages and altered hepatic gene expression involved in biliary transport of cholesterol. In this study, it was further shown that incubation of human MDM with LPS decreased cholesterol efflux and expression of proteins involved in cholesterol efflux and metabolism including ABCA1. These findings are further supported by studies of patients with Tangier’s disease that show decreased ABCA1 expression and cholesterol efflux in macrophages resulting in foam cell formation and associated with increased cIMT. Interestingly monocytes from HIV+ individuals with high viral load have been reported to have increased ABCA1 mRNA expression in comparison to HIV- controls and virologically suppressed HIV+ individuals however how this affects the atherogenic properties of such monocytes remains to be determined.

In summary TLR signalling influences foam cell formation by targeting cholesterol influx, metabolism and efflux in monocytes/macrophages resulting in accumulation and retention of cholesterol within the cell. TLR signalling potentially affects cholesterol trafficking and metabolism pathways after monocytes migrate into the subendothelial neointima and differentiate into macrophages, however chronic exposure of peripheral monocytes to PAMPs may also alter cholesterol efflux pathways and predispose monocytes to form foam cells prior to their trans-endothelial migration.
REGULATING TLR INDUCED FOAM CELL FORMATION

**TLR modulators**

The influence of multiple TLRs, and the conserved nature of their downstream signalling pathways, suggests that TLR pathway modulators may provide a potential therapeutic option for treating inflammation-driven atherogenesis. Chloroquine, an anti-malarial drug which inhibits TLR activation by disrupting endosomal acidification, reduced plaque lesion size induced by western-type diet in apoE(-/-) mice. Treatment of hyperlipidaemic apoE(-/-) mice with synthetic TLR-7 and -9 antagonists reduced foam cell formation by up to 65% whilst the TLR-4 antagonist RS-LPS also reduced foam cell formation and atherosclerotic plaques in diabetic apoE(-/-) mice. While inhibition of TLR-4 and TLR-7 signalling is protective in mouse models, the effectiveness of chloroquine or RS-LPS in human atherosclerosis is currently unknown. Human trials evaluating chloroquine are underway with the Atheroma Reduction with Chloroquine in the Metabolic Syndrome (ARCH-MS) nearing completion (late-2015). The results of this trial will provide insight into whether directly targeting TLRs *in vivo* influences inflammatory atherogenesis in high risk individuals.

**MicroRNAs**

Several microRNA species (miRNAs) have been linked to the regulation of TLR signalling genes implicated in foam cell formation. miRNAs act by binding in a sequence-specific manner to the 3’ untranslated region of mRNA resulting in stabilisation or degradation of specific targets, thereby acting as either a positive or a negative regulator of gene expression respectively. Expression of miRNA-155, a positive regulator of TLR-4 signal transduction that upregulates proinflammatory cytokine production, is increased following TLR-4 stimulation. miRNA-155 expression has recently been shown to be 2 fold higher in human atherosclerotic plaque compared to healthy tissue, and over-expression of miRNA-155 in
C57BL/6 mouse peritoneal macrophages is associated with decreased cholesterol efflux. Knock out of miRNA-155 in apoE(-/-) mice also decreases CCL2 expression on monocytes, reduces their recruitment to the endothelium, reduces proinflammatory cytokine production in macrophages and reduces the size of atherogenic lesions via repression of NF-κB. These observations suggest that miRNA-155 may play a key role in atherogenesis at multiple levels by regulating monocyte recruitment and differentiation into foam cells. In support of these observations, it has been recently shown that antagomiR-155, an inhibitor of miRNA-155, decreases oxLDL induced foam cell formation in macrophages from apoE(-/-) mice. Moreover this study also identified that miRNA-155 expression is increased in CD14+ monocytes from patients with coronary heart disease suggesting a role in response to injury. While miRNA-155 appears to regulate lipid-driven atherosclerosis in atherosclerosis—prone mice fed a high fat diet, it is possible that it could also affect PAMP-driven atherosclerosis via similar mechanisms. The use of strategies to silence miRNAs should be treated with caution, however, as they regulate many genes. Stimulation of THP-1 cells with oxLDL increased miRNA-155 expression, however silencing of miRNA-155 in vitro was associated with enhanced lipid uptake and increased TNF production, suggesting that the mechanism of miRNA-155 regulation of TLR-4 signalling and atherogenesis is complex and remains to be fully defined.

In contrast to miRNA-155, miRNA-146a is a negative regulator of TLR-4 signalling reducing endothelial cell activation by repressing NF-κB-dependent pathways and may therefore act in a negative feedback loop to reduce LPS-induced inflammation. However, longitudinal intervention studies evaluating miRNA-146a expression in PBMC from patients with coronary artery disease before and after treatment with statins and angiotensin-converting-enzyme inhibitors (ACEIs) identified that patients with higher baseline levels of miRNA-146a have worse disease outcomes. Significantly, miRNA-146a levels positively correlated
with TLR-4 mRNA levels that were independent predictors of cardiac events. Twelve month treatment with statins and ACEIs lowered miRNA-146a and TLR-4 levels, suggesting altering miRNA-146a levels may also contribute to the anti-inflammatory effect of these therapies. This finding indicates that targeting miRNA-146a may be an effective treatment option for inflammation-induced CVD.

Limited data suggest that miRNAs may play an important role in the regulation of TLR mediated foam cell formation, however further work is required to elucidate these effects and identify which miRNAs may be potential therapeutic targets for regulating foam cell formation.

CONCLUDING REMARKS

While hyperlipidaemia stimulates atherogenesis by providing more lipid for foam cell formation and an inflammatory milieu to increase lipid oxidation, the observation that inflammatory stimuli such as PAMPs can either synergistically or independently drive foam cell formation from monocytes/macrophages raises new questions regarding the mechanisms of plaque formation in chronic inflammatory diseases. Inflammatory mechanisms may act as both a precursor to mechanisms described in the lipid centric model, while also promoting atherogenesis via both oxidative and non-oxidative cholesterol uptake and decreased cholesterol efflux. Therapeutically targeting interactions between TLRs and foam cell formation may reduce adverse cardiovascular outcomes in individuals with chronic inflammatory diseases. Some crucial questions remain to be answered surrounding the roles of individual monocytes subsets in promoting atherosclerotic plaque formation and which subset(s) are best able to reverse the development of plaques from fatty streaks. Development of more physiological models of fatty streak progression will provide us with a better
understanding of how inflammation may prime monocytes for heightened foam cell formation in chronic inflammatory disease.
AUTHORSHIP

T.A.A wrote the manuscript with intellectual input and revision by A.C.H and A.J.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.
Figure 1: Traditional and alternate inflammatory mechanisms of early atherogenesis

Monocyte recruitment and foam cell formation at sites of deposited lipid (fatty streak). A) Circulating monocytes attach to endothelial cells following upregulation of adhesion molecules at sites of deposited lipid. Cells transmigrate into the intima where they differentiate into macrophages, up regulating scavenger receptors (SR-A/CD36) and LDLR which bind and subsequently internalise oxLDL to form foam cells. Metabolised LDL cholesterol is transported to HDL via the cholesterol efflux transporters ABCA1 and transported to the liver for excretion. B) In settings of chronic inflammation, monocytes with increased expression of adhesion molecules (i.e. CD11b, CCR2 and CX3CR1) are recruited to endothelial cells independent of accumulated lipid potentially due to the influence of circulating PAMPs and proinflammatory cytokines (i.e. IL-6, TNF). Cells transmigrate into the intima where they differentiate into macrophages and signals derived from native and oxLDL receptors and TLRs synergise to form foam cells. Cholesterol efflux and cell egress is impaired resulting in excess lipid accumulation which triggers necrotic pathways leading to cell death and release of lipid bodies. LDL accumulates in the intima triggering traditional monocyte recruitment and atherogenesis described in A.
**Figure 2: Mechanisms of TLR induced foam cell formation**

Signal transduction via multiple TLRs (pink) up regulate foam cell formation by influencing cholesterol influx (green), synthesis (yellow) or efflux mechanisms (purple) in monocytes/macrophages. TLR signalling enhances uptake of oxLDL by increasing surface expression of LDLR, CD36 and SR-A and native LDL by macropinocytosis (green). Free cholesterol, liberated from oxLDL, promotes further cholesterol synthesis, esterification to CE for storage in lipid droplets or is transported to cholesterol efflux transporters. TNF up regulates HMG-CoA reductase and ACAT-1 expression promoting cholesterol accumulation (yellow). TLR signal transduction through NF-κB and interferon regulatory factor-3 (IRF-3) down regulates liver X receptor (LXR) pathways that regulate cholesterol efflux transporters (ABCA1) impairing cholesterol efflux to HDL and apolipoproteins (purple). Modulators of TLR signalling may be exploited to impair TLR-mediated foam cell formation by targeting TLR receptors (Chloroquine, RS-LPS) or signalling pathways (miR146a).
Table 1. Inflammatory disease risk factors associated with monocyte derived atherosclerosis

<table>
<thead>
<tr>
<th>Disease</th>
<th>↑Risk of atherosclerosis</th>
<th>PAMPS</th>
<th>Monocytes (%)</th>
<th>TLR expression on monocytes</th>
<th>Markers of inflammation</th>
<th>Correlation with atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepsis</td>
<td>↑ 3 x⁴</td>
<td>LPS, lipoprotein HIV, LPS, Lipoprotein, CMV</td>
<td>↑CD16+³⁷</td>
<td>↑ TLR-2, TLR-4</td>
<td>↑ IL-6, TNF, sCD14</td>
<td>TF</td>
</tr>
<tr>
<td>HIV</td>
<td>↑ 3 x⁵,⁶</td>
<td>LPS, HIV, LPS, Lipoprotein, CMV</td>
<td>↑CD16+³⁷</td>
<td>↑ TLR-2</td>
<td>↑ IL-6, TNF, hs-CRP, IFN, CD14, neopterin</td>
<td>Intermediate monocytes (%),⁵⁹ TF,⁶¹ hs-CRP</td>
</tr>
<tr>
<td>Ageing</td>
<td>↑ 1.05⁶ *</td>
<td>LPS, CMV Lipoprotein</td>
<td>↑CD16+⁵⁴</td>
<td>↑ TLR-2, TLR-4</td>
<td>↑ IL-6, TNF, hsCRP, d-dimer</td>
<td>TNF, TNFR</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>↑ 2 x⁹</td>
<td>LPS, CMV Lipoprotein</td>
<td>↑CD14++CD16+⁵⁶</td>
<td>↑ TLR-2, TLR-4, TLR-4</td>
<td>↑ hsCRP, sCD14, IL-6, TNF</td>
<td>hsCRP, IL-6, TNF⁹</td>
</tr>
<tr>
<td>SLE</td>
<td>↑ 4.8⁵ †</td>
<td>LPS</td>
<td>↑CD16++CD14+⁵³</td>
<td>↑ TLR-2, TLR-4, TLR-4</td>
<td>↑ IFN-α, IL-6, TNF</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

LPS – lipopolysaccharide, HIV – Human immunodeficiency virus, CMV – Cytomegalovirus, hs-CRP – high sensitivity C reactive protein, sCD14 – soluble CD14, SLE – Systemic lupus erythematosus

*hazard ratio

† odds ratio
REFERENCES


Figure 1 Traditional and alternate inflammatory mechanisms of early atherogenesis. Monocyte recruitment and foam cell formation at sites of deposited lipid (fatty streak). (a) Circulating monocytes attach to endothelial cells following the upregulation of adhesion molecules at sites of deposited lipid. Cells transmigrate into the intima, where they differentiate into macrophages, upregulating scavenger receptors (SR-A/CD36) and LDLR, which bind and subsequently internalise oxLDL to form foam cells. Metabolised LDL-cholesterol is transported to lipoprotein acceptors (e.g. HDL) via cholesterol efflux transporters (e.g. ABCA1) and transported to the liver for excretion. (b) In settings of chronic inflammation, monocytes with increased expression of adhesion molecules (i.e. CD11b, CCR2 and CX3CR1) are recruited to endothelial cells independent of accumulated lipid potentially because of the influence of circulating PAMPs and proinflammatory cytokines (i.e. IL-6, TNF). Cells transmigrate into the intima where they differentiate into macrophages and signals derived from native and oxLDL receptors and TLRs synergise to form foam cells. Cholesterol efflux and cell egress is impaired resulting in excess lipid accumulation, which triggers necrotic pathways leading to cell death and release of lipid bodies. LDL accumulates in the intima triggering traditional monocyte recruitment and atherogenesis described in (a).
Figure 2 Mechanisms of TLR-induced foam cell formation. Signal transduction via multiple TLRs (pink) upregulate foam cell formation by influencing cholesterol influx (green), synthesis (yellow) or efflux mechanisms (purple) in monocytes/macrophages. TLR signalling enhances uptake of oxLDL by increasing surface expression of LDLR, CD36 and SR-A and native LDL by macropinocytosis (green). FC, liberated from oxLDL, promotes further cholesterol synthesis, esterification to CE for storage in lipid droplets or is transported to cholesterol efflux transporters. TNF upregulates HMG-CoA reductase and ACAT-1 expression promoting cholesterol accumulation (yellow). TLR signal transduction through NF-κB and IRF-3 downregulates LXR pathways that regulate cholesterol efflux transporters (e.g. ABCA1) impairing cholesterol efflux to HDL and apolipoproteins (purple). Modulators of TLR signalling may be exploited to impair TLR-mediated foam cell formation by targeting TLR receptors (chloroquine, RS-LPS) or signalling pathways (miR146a).