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Invited critical review

Toll-like receptors and macrophage activation in atherosclerosis

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ABSTRACT

Atherosclerosis is a multi-factorial inflammatory disease and is the primary initiator of coronary artery and cerebrovascular disease. Initially believed to be exclusively lipid-driven, recent evidence demonstrates that inflammation is a significant driving force of the disease. Cellular components of innate immunity, for example monocytes and macrophages, play a predominant role in atherosclerosis. Toll-like receptors (TLRs) are the most characterised innate immune receptors and recent evidence demonstrates an important role in atherogenesis. Engagement of TLRs results in the transcription of pro-inflammatory cytokines, foam cell formation and activation of adaptive immunity. Recently they have also been implicated in protection from vascular disease. In this review, we detail the role of the innate immune system, specifically macrophages and TLR signalling, in atherosclerosis and acute cardiovascular complications, and thereby identify the potential of TLRs to act as therapeutic targets.

Contents

Abbreviations: TLR, Toll-like receptor; PAMP, Pathogen associated molecular pattern; LDL, Low density lipoprotein; SMC, Smooth muscle cell; VCAM-1, Vascular cell adhesion molecule-1; M-CSF, Macrophage colony stimulating factor; TCFA, Thin-cap fibroatheroma; MMP, Matrix metalloproteinase; LSS, Low shear stress; OSS, Oscillatory shear stress; HSS, High shear stress; PRR, Pattern recognition receptor; DAMP, Damage associated molecular pattern; RLR, RIG-I-like receptor; NLR, NOD-like receptor; CLR, C-type lectin receptor; RIG-I, retinoic acid-inducible gene-I; MDA5, melanoma differentiation-associated gene 5; LGP2, laboratory of genetics and physiology 2; DC, Dendritic cell; cDC, conventional dendritic cell; mDC, myeloid dendritic cell; lDC, lymphoid dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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1. Introduction

Atherosclerosis is the primary initiator of coronary artery and cerebrovascular disease; being responsible for 29% of deaths worldwide [1]. Initially believed to be solely driven by risk factors such as family history, diabetes, hypercholesterolaemia, smoking and hypertension [2], recent evidence has defined inflammation as a fundamental component of its pathogenesis [3].

The innate immune system acts as the first barrier against invading pathogens. Pathogen-associated molecular patterns (PAMPs) activate various families of pattern-recognition receptors (PRRs); the most extensively characterised family being Toll-like receptors (TLRs) [4]. Signalling downstream of TLRs may drive immune responses such as leukocyte recruitment and pro-inflammatory cytokine production exacerbating disease. In inflammatory diseases like atherosclerosis, a bias towards pro-inflammatory stimuli chronically activates innate immunity and dampens anti-inflammatory mechanisms.

In this review, we examine the role of TLRs in the activation of key immune cells such as monocytes and macrophages, atherosclerotic plaque development and its complications, and the potential of these signalling proteins to act as therapeutic targets against atherosclerosis.

2. Atherosclerosis is an inflammatory disease

Atherosclerosis involves interactions between blood-borne mononuclear immune cells exogenous of the vessel wall, and the endogenous cell types such as the endothelium and smooth muscle cells (SMCs). Some pathologists consider intima cushions, characterised by SMC proliferation and proteoglycan deposition, as the precursors of human atherosclerotic lesions. Fatty streaks, which can subsequently develop, are considered to be early atherosclerotic lesions. In conditions of hyperlipidaemia, low density lipoproteins (LDLs) enter the intima, the innermost layer of the vessel wall at sites of permeability, where they may undergo a variety of modifications including oxidation [5]. Pro-inflammatory cytokines and mmLDL upregulate endothelial cell expression of selectins and VCAM-1, facilitating monocyte entry into the intima [6]. OxLDL stimulates endothelial cells and SMCs to secrete monocytic maturation factors such as M-CSF. Monocytes differentiate into macrophages, phagocytose modified lipoproteins – predominantly via the scavenger receptors AI and CD36 [7] – and become foam cells [8]. Fatty streaks (or intimal xanthomas) are primarily composed of foam cells and a few SMCs.

When macrophage foam cells infiltrate the lipid pool and apoptose increasing the free cholesterol content, a lipid necrotic core develops [9]. Macrophages secrete growth factors stimulating an influx of SMCs, collagen synthesis and extracellular matrix deposition, forming a fibrous cap beneath the endothelium [3]. The formation of a fibrous cap maintains plaque integrity and avoids contact of the thrombogenic necrotic core with flowing blood [10] creating a mature fibroatheroma. Microvessels can form around the lipid core as neovascularisation from the adventitia occurs; leakage of these vessels leads to intraplaque haemorrhage [11] (Fig. 1).

Mature fibroatheromas are classified as “stable” or “vulnerable” — the latter are also referred to as thin-cap fibroatheromas (TCFAs) [12]. Stable lesions tend to be smaller with low macrophage and lipid content, high SMC and collagen content, reduced matrix metalloproteinase (MMP) activity and a thick fibrous cap compared to TCFAs [13]. Apoptosis or clearance of SMCs [13,14] and matrix degradation by MMPs [15] may cause thinning of the cap creating a TCFA [12], which is rupture-prone and is considered the highest risk phenotype for major cardiovascular events [15]. TCFAs are characterised by a large necrotic core and numerous macrophages [16]. Fibrous cap rupture exposes the pro-thrombogenic tissue factor-rich necrotic core and material present following intraplaque haemorrhage [6]. The coagulation cascade then forms a deep thrombus which can protrude into the lumen [17]. Endothelial erosion [11] and intraplaque calcified nodules also trigger thrombosis [18]. Plaque rupture is often asymptomatic and its healing leads to discontinuous plaque progression. However if plaque rupture leads to sudden occlusion of blood flow, acute myocardial damage follows.

3. Shear stress modulates atherosclerosis and influences plaque composition

Atherosclerosis displays an uneven distribution throughout the vascular tree suggesting a relationship with local blood flow dynamics [19]. An inverse relationship between wall shear stress and artery wall thickness has been demonstrated in the coronary arteries [20]. Laminar blood flow in a straight vessel imposes high shear stress (HSS) on the endothelium. Curvatures, branches and stenoses in the arterial tree

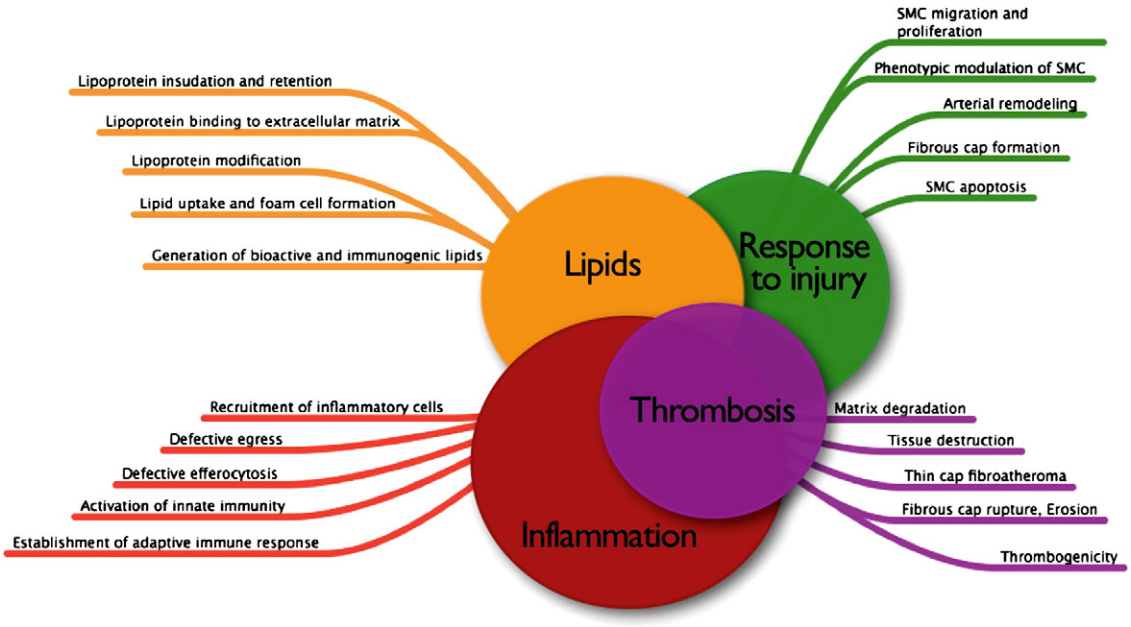

Fig. 1. The multiple features involved with inflammation in atherosclerotic plaque development.

alter blood flow dynamics. In curved vessels, the vessel wall on the inner curvature experiences low shear stress (LSS). Oscillating blood flow occurs at non-planar branch points or downstream of stenoses creating oscillatory shear stress (OSS). These complex blood flow patterns are considered pro-atherogenic [19].

Different patterns of shear stress can affect plaque morphology. OSS promotes stable plaque formation while LSS associates with the development of vulnerable plaques [13]. However LSS and a vulnerable plaque phenotype are insufficient for plaque rupture. Positive remodelling can maintain normal shear stress values [21]. However protrusion of the plaque increases local shear stress patterns [20]. The surface of the lesion's proximal segment is subjected to HSS and high strain while the surface of the distal segment experiences OSS [22]. In humans, the proximal segment has a higher frequency of fibrous cap rupture sites and intraplaque apoptotic material [23], while the distal segment is more abundant in SMCs and collagen [16,24]. It is believed that LSS and OSS promote initial atheroma development, but HSS is thought to encourage plaque rupture in stenosed arteries [23]. Yet the downstream segment is susceptible to thrombosis via endothelial erosion as this region in human carotid plaques displays a higher rate of endothelial cell apoptosis [25].

4. Innate immunity is crucial in atherosclerosis

The innate immune system is the body's first line of defence against invading pathogens. Innate immune cells recognise highly conserved pathogen-associated microbial patterns (PAMPs) via pattern recognition receptors (PRRs), which are germline encoded receptors [26]. Recent evidence has shown that PRRs also recognise endogenous molecules termed damage associated molecular patterns (DAMPS) from damaged cells [26]. PRRs can be broadly divided into two groups; cytosolic receptors which consist of RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs), and transmembrane receptors which consists of C-type lectin receptors (CLRs) and Toll-like receptors (TLRs) [27]. PRRs are expressed on both immune cells and non-immune cells [27] and survey both intracellular and extracellular compartments, with each family controlling specific locations.

4.1. Pattern recognition receptors (PRRs)

The RLR family consists of retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [27,28]. RLRs are essential for the recognition of both dsRNA and ssRNA viruses, therefore playing a role in antiviral innate immune responses. RIG-I has been identified in macrophages in human atherosclerotic lesions [29]. NLRs are composed of C-terminal leucine rich repeats, a central nucleotide binding domain and an N-terminal protein binding motif [27,30]. Only those NLRs which have caspase recruitment domains as their N-terminus, such as NOD1 and NOD2 promote transcription of pro-inflammatory mediators via NFκB [27]. TLRs and NODs act synergistically to promote pro-inflammatory cytokine production as they both recognise bacterial peptidoglycan components [31]. CLRs are unique in that they have a carbohydrate binding domain, which enables recognition of carbohydrates on pathogens. CLR activation can lead to production of pro-inflammatory cytokines or inhibition of TLR-mediated immune complexes [27].

4.1.1. Toll-like Receptors (TLRs)

TLRs are the most extensively studied and characterised PRRs. TLRs are type 1 transmembrane proteins with an ectodomain consisting of leucine rich repeats required to recognise PAMPs, a transmembrane domain which determines cellular localisation, and an intracellular toll-interleukin 1 receptor (TIR) domain needed for downstream signalling. At least 13 TLRs have been discovered thus far, each with a degree of specificity for various endogenous and exogenous ligands [32]. TLRs can be broadly divided into two categories; cell membrane TLRs (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) and nucleic-acid sensing TLRs (TLR3, TLR7, TLR8, TLR9 and murine TLR13), which localise to intracellular vesicles including the endoplasmic reticulum, endosomes and lysosomes [4,33] (Fig. 2).

4.1.2. TLR signalling pathways

Upon recognition of PAMPs by TLRs, there is an upregulation of inflammatory gene transcription. Most TLRs form homodimers with the exception of TLR2 and TLR4. TLR2 forms a heterodimer with TLR1 or TLR6, while TLR4 forms a heterodimer with TLR6. TLR signalling is aided by the recruitment of five adaptor proteins to its TIR domain: myeloid differentiation primary-response protein 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (MAL), TRIF-related adaptor molecule (TRAM) and Sterile-α and armadillo motif-containing protein (SARM) [34]. The TLR signalling pathway is dependent on whether MyD88 or TRIF is recruited to the TIR domain.

4.1.2.1. MyD88-dependent signalling pathway. MyD88 consists of a death domain and a TIR domain. All TLRs, except TLR3, require the MyD88 dependent pathway to initiate downstream signalling. TLR2 and TLR4 utilise TIRAP/MAL as a bridge with MyD88. Following PAMP recognition by TLRs, MyD88 recruits members of the IL-1 receptor-associated kinase (IRAK) family. The IRAKs dissociate from MyD88 and associate with Tumour necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase to generate polyubiquitin chains [35]. Unconjugated K63-linked polyubiquitin chains activate a complex consisting of TAK1 and TAK-1 binding proteins (TAB)1, 2 and 3. This complex then translocates to the cytosol, where TAK1 phosphorylates IKK-β. Subsequently the IKK complex, consisting of IKKα, IKKβ and NEMO, phosphorylates IκBα, an NFκB inhibitory protein. Phosphorylated IκB is degraded by the ubiquitin proteosome system freeing NFκB to translocate into the nucleus and mediate transcription of inflammatory genes. TAK1 also phosphorylates MAPK6 which results in the activation of the MAPKs Erk1, Erk2, p38 and Jnk. Activation of the MAPK pathway results in the formation of activated protein (AP)-1, a transcription factor complex controlling many genes encoding cytokines.

4.1.2.2. TRIF-dependent signalling pathway. TLR3 recognises dsRNA and utilises signalling via TRIF which culminates in IRF3 and NFκB activation [36]. TRIF associates with TRAF3 and TRAF6 via its N-terminal TRAF binding-motifs. TRAF3 activates TBK1 and IKKε which phosphorylate IRF3 enabling its translocation into the nucleus [37,38]. IRF3 mediates the production of proinflammatory cytokines, type 1 IFNs and subsequently IFN-induced genes. The TNFR-associated death domain protein (TRADD) is involved in TRIF dependent signalling. A complex is formed consisting of TRADD, FADD and RIP1. TRADD triggers the ubiquitination of RIP1 which leads to NFκB activation. In addition, TRIF associates with TRAF6 to activate TAK1. This is thought to occur in an ubiquitination dependent manner resulting in phosphorylation of the inhibitory molecule IκBα by IKK-α and IKK-β [36].

TLR4 is unique in that it utilises both the MyD88 and TRIF dependent pathways. It appears that the receptor's cellular localisation determines which pathway occurs [39,40]. First, upon ligand binding membrane bound TLR4 recruits MyD88 which binds to MAL to activate NFκB and MAPK. Secondly, TLR4 is translocated to the endosome via dynamin dependent endocytosis. There TLR4 associates with TRAM to trigger the TRIF dependent pathway resulting in IRF3 activation and late phase activation of NFκB and MAPK [39–41].

4.1.3. Toll-like receptor ligands

TLRs respond to a variety of exogenous (PAMPs) and endogenous (DAMPs) ligands (Fig. 2). Various ligands are present in the atherosclerotic lesion and may elicit responses in the plaque. Heterodimerisation

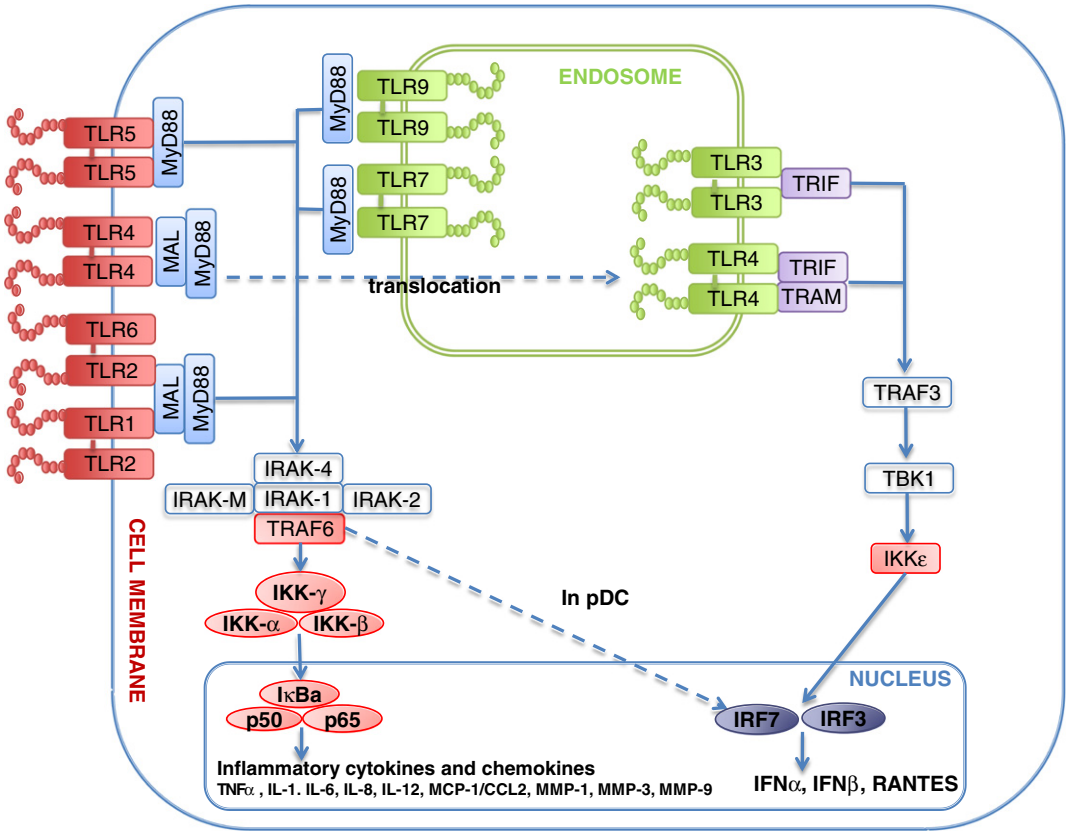

Fig. 2. Toll-like receptor signalling pathways. TLR signalling leads to activation of transcription factors, including interferon regulatory factors (IRFs) and NFκB, which are required for inflammatory gene transcription. With the exception of TLR3, all TLRs recruit MyD88 which triggers downstream signalling, resulting in NFκB activation. TLR3 utilises the TRIF dependent pathway, which results in IFN activation. TLR4 is unique in its requirement of both the MyD88 and TRIF dependent pathways. IRAK: IL-1 receptor-associated kinases; MAL: MyD88-adaptor like; MyD88: Myeloid differentiation protein 88; TLR: Toll-like receptor; TRAF: Tumour necrosis factor receptor-associated factor 6; TRAM: TRIF-related adaptor molecule; TRIF: TIR domain-containing adaptor inducing interferon-β.

of TLR2 with TLR1 or TLR6 increases its specificity enabling discrimination between different ligands [42–45]. Further specificity of TLR action has been reviewed by Piccinini and colleagues where they highlight that homo- or heterodimerisation along with co-receptors and accessory molecules confer specificity to ligand detection [46]. In addition, they identify that DAMPs and PAMPs act in a disparate manner requiring different co-receptors and accessory molecules. Thus complete elucidation of these differing mechanisms could enable targeting of inappropriate, pathogenic inflammation (Fig. 3).

Various PAMPs have been identified in atherosclerotic plaques and exist in the form of bacterial signatures including nucleic acids, peptidoglycans and exogenous heat shock proteins (HSPs). Several DAMPs have also been identified. They include lipoproteins, material from necrotic cells and extracellular matrix proteins. HSPs are released from necrotic cells and trigger proinflammatory cytokine production in a TLR2- and TLR4-dependent manner [47,48]. HSPs have been identified as possible autoantigens involved in (auto)immunological injury in the vessel wall, thought to help initiate atherosclerosis [49,50].

Lipids also act as extracellular ligands. Saturated fatty acids stimulate TLR4 and promote gene expression whilst polyunsaturated fatty acids inhibit TLR4 activation [51]. MmLDL can act in two ways; it can involve macrophages and endothelial cells via a CD14/TLR4/MD-2 mechanism which is MyD88 dependent; or it can induce reactive oxygen species production in macrophages via TLR4 in a MyD88 independent manner [52,53]. Oxidised LDL and β-amyloid peptide stimulate the TLR4/6 heterodimer – which requires the scavenger receptor CD36 – to promote inflammatory gene expression [54].

4.1.4. Expression of TLRs in atherosclerosis

TLRs are expressed by a range of cells including leukocytes, dendritic cells (DCs), and T and B lymphocytes (reviewed in [55]). Edfeldt and colleagues demonstrated TLR1, TLR2 and TLR4 are increased in human atherosclerotic plaques and several TLR-expressing cells are activated [56]. The expression of TLRs on innate immune cells in relation to atherosclerosis will be detailed later.

T lymphocytes (both $CD4^+$ and $CD8^+$) are present in atherosclerotic lesions in both humans and murine models. T-cell clones in human atherosclerotic plaques are immunospecific for self antigens such as oxidised LDL [57]. Differences occur in TLR expression across T lymphocyte subsets and their locations, which reflect their specialised immune functions [55]. B lymphocytes express TLRs both at the mRNA and protein level. Human B cells express TLR1, TLR6, TLR7, TLR9 and TLR10 [58–61]. TLR expression appears to depend on location and maturity of B lymphocytes but discrepancies exist in the literature regarding expression of TLRs on B lymphocytes in humans [62]. Murine expression on B lymphocytes is also very wide ranging but differences exist in comparison to human expression [63–65]. For example, in contrast to humans, TLR expression does not differ between murine naïve B cells and memory B cells [64].

Interestingly, although TLRs are expressed by the aforementioned cell types, in atherogenesis the earliest expression of TLRs occurs in resident vascular cells [55]. There appears to be variation in the expression of TLRs across different vascular beds. TLR3 mRNA is found in the aorta and the temporal and iliac arteries express TLR8 mRNA, whilst the carotid artery expresses both TLR3 and TLR8 mRNA [66]. Human vascular SMCs constitutively express mRNA for TLR1, TLR3,

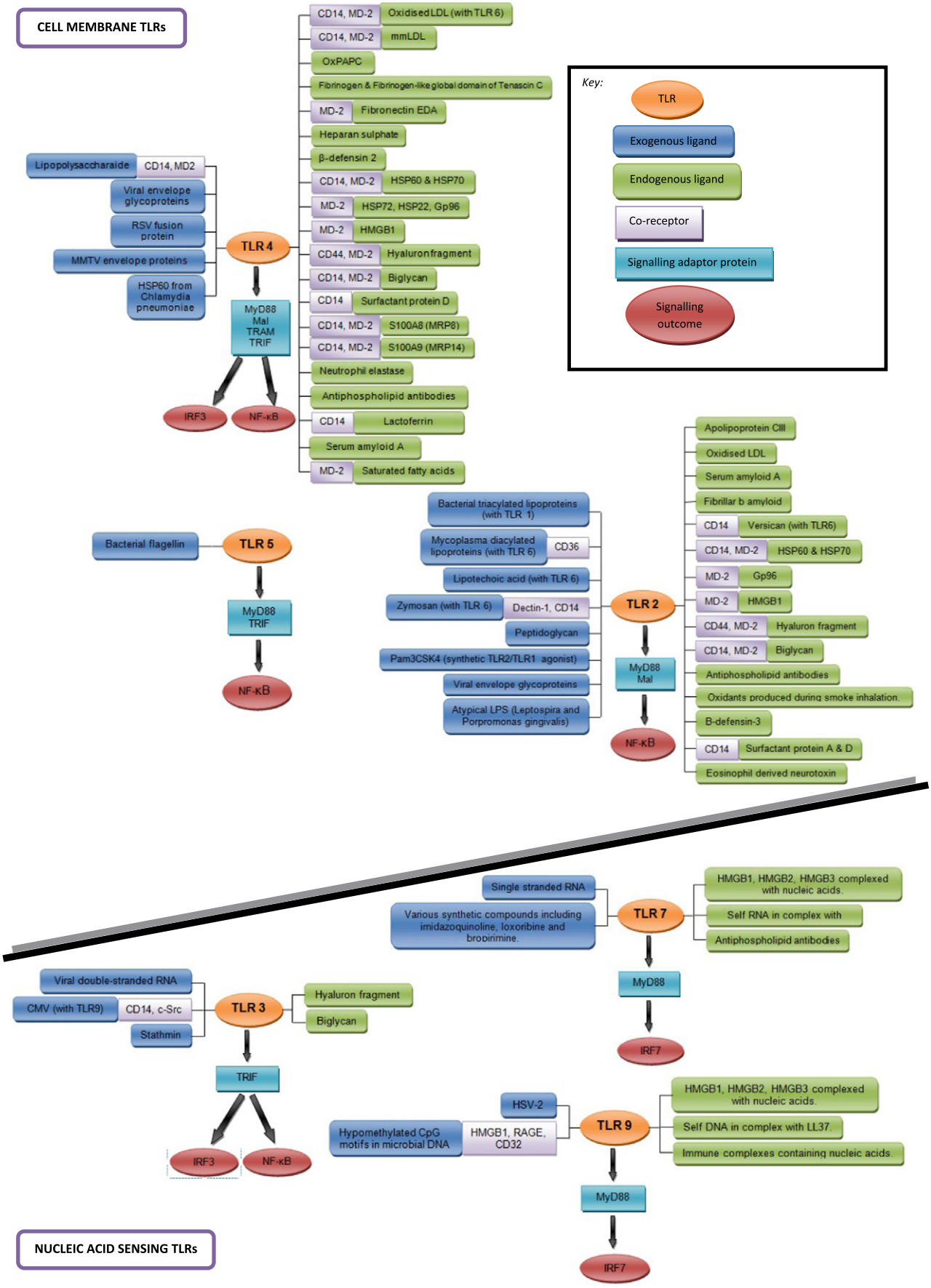

Fig. 3. Exogenous and endogenous ligands of Toll-like receptors.

TLR4 and TLR6 [67]. TLR2 is constitutively expressed by murine aortic SMCs, whereas TLR2 expression can be induced in human coronary artery SMCs by *Chlamydia Pneumonia*, TLR3 and TLR4 ligand stimulation [68].

4.1.5. Role of TLRs in atherosclerosis

TLRs can directly affect atheroma formation as stimulation of macrophages with TLR2, TLR4 and TLR9 ligands promote lipid uptake [69,70]. Recently, experimental evidence using ApoE$^{-/-}$ mice showed that TLR4 and TLR2, albeit to a lesser extent, contribute to early stage intimal foam cell accumulation in the aorta at sites susceptible to lesion formation [71]. TLR4 can stimulate macropinocytosis of lipids in differentiated macrophages [72]. Increased lipid uptake can also be mediated by increased expression of scavenger receptors induced by TLR3, TLR4 and TLR9 [69,73]. TLRs and their ligands can also disrupt cholesterol efflux mechanisms, which can contribute to foam cell formation.

TLR2 expression is increased in endothelial cells at atherosclerosis prone sites such as the inner curvature of the aortic arch in LDLR$^{-/-}$ mice; such expression is associated with early atherosclerosis [74]. However, bone marrow transfer from TLR2$^{-/-}$ mice to LDLR$^{-/-}$ mice had no effect on lesion formation [75]. In contrast, bone marrow transfer prior to Pam_3CSK_4 (synthetic TLR2 agonist) stimulation caused a decrease in lesion development, suggesting that perhaps exogenous agonists induce pro-atherogenic TLR2 signalling via myeloid cells, whereas non-myeloid cells detect endogenous agonists. ApoE$^{-/-}$ mice deficient in TLR4 and TLR2 displayed a 55% decrease in atherosclerotic lesion development, whilst a 65% decrease in macrophage infiltration was seen in ApoE$^{-/-}$ mice deficient in TLR4 [75,76]. In the absence of TLR2 and TLR4, decreased lesion size was mirrored by a decrease in peripheral CCL2 levels; a chemokine that is critical for monocyte recruitment to atherosclerotic plaques [77].

We have investigated the influence of endosomal TLRs on atherosclerosis and arterial injury using both human and murine models of atherosclerosis. Contrary to the initial belief that TLRs are purely pro-atherogenic, we recently reported an unexpected protective role for TLR3 in arterial injury and atherosclerosis [78]. ApoE$^{-/-}$ mice with a TLR3 deficiency have an accelerated onset of atherosclerosis while intraperitoneal administration of Poly(I:C) – a TLR3 agonist – reduces neointimal formation. Moreover, genetic deletion of TLR3 correlates with numerous large breakages of the elastic lamina after placement of an arterial injury-inducing perivascular collar. This data demonstrates for the first time that while cell surface TLRs may promote atherosclerosis, intracellular TLRs protect against hypercholesterolemia and injury-induced lesions. Lesion development in both humans and mice associates with increased expression of TLR3 and TLR3-associated responses, particularly in SMCs which maybe a protective cell type.

It is unknown what endogenous agonists of TLR3 may be involved in protection, as its genetic removal exacerbates atherosclerosis and damage to the elastic lamina. Interestingly stathmin, which is upregulated upon brain injury and participates in microtubule assembly, is described as a candidate TLR3 agonist, linked to the induction of a neuroprotective gene profile [79].

The mechanism of TLR3-induced protection is currently unknown — it may relate to its preference for intracellular signalling via TRIF over MyD88 when compared to other TLRs. IFNβ production – as a result of TLR3 dependent signalling – is linked to reduced inflammasome activation, IL-1 signalling, and IL-10 induction [80]. However, it is uncertain whether the vasculoprotective effect of TLR3 is mediated by IFNβ. Although an arterial injury model shows IFNβ to be protective [81], a more recent report showed a potentially deleterious role in atherosclerosis induced by hyperlipidaemia [82]. Synthetic dsRNA may not be safe as a therapeutic tool, as its administration elicits both pro-inflammatory and anti-inflammatory mediators [78]. A recent study showed that intravenous administration of dsRNA at high doses may lead to endothelial cell apoptosis and increased vascular lesion formation [83]. TLR3 activation in the vasculature can elicit the production of IL-10 [78] and the B7 family members PDL1 and PDL2, which contribute to vascular protection [84]. Blocking the PD1-PDL1 interaction accelerates arterial disease in cardiac allografts [85].

4.2. Activation of innate immune cells by TLRs in atherosclerosis

4.2.1. Mast cells

Mast cells are found in connective tissues and are important for alerting the immune system to local infection. Derived from common myeloid progenitors, they play a prevalent role in allergic reactions, releasing proteins such as histamine, and sustaining inflammation. Mast cells have been observed in coronary and carotid artery plaques; specifically localised at sites of plaque erosion, haemorrhage or rupture [86]. They can secrete pro-inflammatory cytokines and serine proteases, such as chymase, which can activate proteases and MMPs promoting plaque instability, and the conversion of angiotensin I to angiotensin II [87]; promoting vasoconstriction and possibly restricting arterial wall remodelling. Mast cells also contribute to intraplaque haemorrhage, macrophage apoptosis and the recruitment of leukocytes to murine atherosclerotic plaques via the CXCR2/VLA-4 axis [88]. It is thought that substance P causes adventitial mast cell activation and intraplaque haemorrhage via TLR2 upregulation, suggesting that TLR2 plays a role in the vulnerable plaque phenotype [88]. Human and rodent mast cells have been identified to express TLR1-TLR7 and TLR9 [89].

4.2.2. Dendritic cells

Dendritic cells (DCs) are vital for priming innate and adaptive immune responses with their antigen presenting capabilities, while maintaining self tolerance. DCs exist as one of two subsets: conventional (cDCs) or plasmacytoid (pDCs). cDCs can be subdivided into myeloid (mDCs) and lymphoid (lDCs), and are primarily responsible for antigen presentation and priming of naïve T lymphocytes while pDCs secrete large amounts of interferons. Immature DCs encounter pathogens in peripheral tissues activating TLR signalling which triggers DC maturation. DCs then migrate to lymphoid tissues; express MHC and co-stimulatory molecules, and prime naïve T cells in the T-cell zones.

CD11c^{+} DCs accumulate at vascular regions predisposed to atherosclerosis correlating with increased expression of VCAM-1 [90]. Mature DCs are observed more frequently in advanced lesions. High expression of HLA-DR (MHC-II) and interactions with T cells are particularly evident in rupture-prone regions of the plaque [91]. A deficiency in the fractalkine receptor CX_3CR1 in the aorta impairs the accumulation of dendritic cells in the intima [92], suggesting they may differentiate from Ly-6C^{lo} monocytes which normally express high levels of CX_3CR1. OxLDL acts as an antigen upregulating DC expression of HLA-DR and its co-stimulatory molecules, in parallel with an increase in T cell proliferation [93]. DCs express scavenger receptors – namely LOX-1, CD36 and CD205 – which mediate their uptake of oxLDL activating the NFκB pathway, and maturation into DCs with a pro-inflammatory cytokine profile [94]. DCs may become activated by oxLDL in the plaque, migrate to secondary lymphoid organs and prime the clonal expansion of oxLDL-specific T cells.

cDCs express TLR2-8 while pDCs express TLR7 and TLR9. In contrast to mDCs, pDCs strongly express TLR7 and TLR9 mRNA but only weakly express TLR2 and TLR4 mRNA [95]. However, both subtypes respond to stimulation with the TLR7 ligand R848; but in response, pDCs express IFN-α whilst mDCs express IL-12 [96]. TLR7 and TLR9 induce the production of type 1 IFNs and NFκB dependent cytokines via the MyD88 dependent pathway. pDCs constitutively express IRF7 which binds to MyD88 and forms a complex with IRAK1, IRAK4, TRAF3, TRAF6 and IKKα [28]. Phosphorylated IRF7 translocates into the nucleus where it facilitates the production of type 1 IFNs. In contrast, cDCs mediate the activation of IRF1 resulting in IFN-β gene

expression [97,98]. Severe hypercholesterolaemia inhibits the TLR-induced production of pro-inflammatory cytokines by DCs, by inhibiting the nuclear translocation of NFκB; subsequently Th1 responses are diminished while Th2 responses are enhanced [99]. These studies collectively suggest pro-inflammatory Th1 responses are predominant in moderate hypercholesterolaemia while a shift to Th2 responses occurs in severe hypercholesterolaemia [100].

4.2.3. Monocytes

Monocytes are derived from macrophage-DC precursors in the bone marrow. They constitute 5–10% of peripheral blood leukocytes [101] and can circulate for several days before entering tissues via the leukocyte adhesion cascade and differentiating into macrophages. A deficiency in adhesion molecules such as P-selectin, ICAM-1 and VCAM-1, or blockage of their interactions with their respective ligands can reduce monocyte recruitment and atherosclerotic lesion size [102,103].

Mature murine monocytes have been classified into two main subsets according to their expression levels of Ly-6C, the chemokine receptors CCR2 and CX_3CR1. Ly-$6C^{high}CCR2^{high}CX_3CR1^{low}$ monocytes – termed inflammatory or classical monocytes – typically home to inflamed sites, with the ability to respond to MCP-1 [104], and secrete pro-inflammatory cytokines. They represent 90% of the monocytes that accumulate in murine atherosclerotic lesions. Hypercholesterolaemia increases the proliferation and differentiation of Ly-$6C^{high}$ monocytes and macrophage foam cell formation, while impairing conversion to resident subsets [105].

Ly-$6C^{low}CCR2^{low}CX_3CR1^{high}$ monocytes – termed resident or non-classical monocytes – patrol the endothelium under homeostatic conditions, relying on LFA-1 and CX_3CR1 for crawling and extravasation [106]; with the ability to rapidly invade infected sites and initiate an immune response. CX_3CR1 seems important for their survival as the anti-apoptotic gene Bcl-2 is not expressed in CX_3CR1-deficient resident monocytes [107]. Enforcing the survival of monocytes in CX_3CR1-deficient mice restores atherogenesis [108] while inhibiting Fractalkine (CX_3CL1) stabilises LSS-induced plaques [109]. CX_3CR1-deficient mice have reduced lesions and macrophage accumulation while retaining the characteristics of stable plaques [110]. The number of circulating Ly-$6C^{lo}$ monocytes correlates with lesion size [111]. This could be explained by the capability of resident monocytes to become loaded with cholesterol by binding to circulating oxLDL in both humans and mice [112,113]. Therefore Ly-$6C^{lo}$ monocytes may be a significant source of lipids in early atherosclerotic plaques [104]. Resident monocytes are reduced in plaques but are less inflammatory and may promote stable plaque formation in the latter stages of atherosclerosis [114].

Inflammatory and resident monocytes exist approximately at a 1:1 ratio in the blood of mice [115]. One study showed Ly-$6C^{lo}$ monocytes are a more mature form of monocytes [116] while other studies show little conversion of Ly-$6C^{hi}$ to Ly-$6C^{lo}$ monocytes [117]. A mouse model of myocardial infarction demonstrated that Ly-$6C^{hi}$ monocytes dominate the early stages phagocytosing damaged tissue while Ly-$6C^{lo}$ monocytes promote healing and angiogenesis in the latter stages [117]. Inflammatory monocytes use the chemokine receptors CX_3CR1, CCR2 and CCR5 to enter plaques while resident monocytes use CCR5 only [118]. Ly-$6C^{hi}$ monocytes seem dependent on CCR2 for their mobilisation from bone marrow and tissue infiltration [111,119]. Additive inhibition of CCL2, CX_3CR1 and CCR5 virtually abolishes atherosclerosis and monocyte accumulation, while exhibiting a halt in bone marrow and blood monocytosis [111].

In humans, CX_3CR1 is expressed at low levels on classical $CD14^{hi}CD16^{-}$ monocytes while nonclassical $CD14^{low}CD16^{+}$ monocytes (normally excluded from inflamed tissues) express high amounts of CX_3CR1 [115]. CD14 is a co-receptor for TLR4 enabling lipopolysaccharide detection while CD16 (or FCγRIII) belongs to the Fcγ receptor family that binds antibodies during immune responses such as antibody-dependent cell-mediated cytotoxicity. Approximately 90% of circulating monocytes in humans belong to the classical subset [104]. The amount of pro-inflammatory $CD14^{+}CD16^{+}$ monocytes and serum TNF-α levels is elevated in patients with coronary artery disease [120], and this monocyte subset negatively correlates with fibrous cap thickness [121]. While CCR2 and CX_3CR1 are similarly expressed between human and mouse monocyte subsets, there are differences in the expression of other genes between the two species, such as the macrophage scavenger receptor [122], which can make the determination of human monocytic behaviour difficult when based on murine studies.

Studies of monocyte subsets in mouse models of the peritoneal infection *Leishmania monocytogenes* and myocardial infarction suggest Ly-$6C^{hi}$ monocytes differentiate into classical M1-type macrophages and dendritic cells with a pro-inflammatory transcriptional programme, while Ly-$6C^{lo}$ monocytes differentiate into M2 macrophages of an alternatively activated phenotype exhibiting tissue repair activity [106,117]. However *in vitro* studies suggest monocytes are very plastic. GM-CSF differentiates monocytes into dendritic cells [123] while M-CSF promotes macrophage development regardless of the monocyte subset present. Another transcriptome-level study showed an upregulation of M1 macrophage-associated genes following GM-CSF-driven differentiation of human monocytes while M2 macrophage-associated genes are activated in response to M-CSF-driven differentiation [124].

4.2.4. Macrophages

Macrophages are differentiated cells within tissues derived from monocytes or directly from myeloid progenitor cells during haemopoiesis [125]. Macrophages recognise PAMPs via various cell surface receptors e.g. TLRs and scavenger receptors. Inflammatory stimuli increase the expression of certain macrophage receptors like PRRs augmenting their immune behaviour relating to clearance of bacteria and their cytokine equilibrium [126] (Fig. 4).

Gordon et al. proposed a paradigm of macrophage activation to include 4 stages: differentiation by growth factors such as GM-CSF and M-CSF, priming by IFN-γ or IL-4 and IL-13, activation into a functional phenotype from a TLR stimulus for example, and finally deactivation to enable repair functions by anti-inflammatory mediators like IL-10 and TGF-β [127]. The differentiation of monocytes into macrophages is essential for the development of atherosclerotic lesions; for example M-CSF-deficient mice are resistant to the development of atherosclerosis [128].

Macrophages are extremely plastic and capable of modifying their behaviour upon microenvironmental cues; as a result a dichotomy is believed to exist in parallel with the Th1 and Th2 lymphocyte subsets. In response to bacterial motifs (e.g. LPS) and IFN-γ, macrophages undergo "classic activation"; a type of priming that mirrors Th1 lymphocytes [129] and can aid the resolution of infection and tumour resistance. Adaptive immunity, such as IFN-γ production by Th1 cells, maintains the classical phenotype strengthening defence against intracellular microorganisms [130]. LPS or IFN-γ primed macrophages, also termed M1, are considered IL-12^{high}IL-10^{low} [131]. They are integral to Th1 responses killing microorganisms and tumour cells while secreting pro-inflammatory cytokines. Th1 responses may drive atherosclerosis as inactivation of the transcription factor T-bet – which promotes T cell development – reduces MHC-II expression by macrophages in atherosclerosis [132].

Alternative macrophage activation (or M2) was first identified upon exposure to IL-4 [127]. IL-4 and IL-13 increase expression of the decoy IL-1RII and the IL-1R antagonist (IL-1Ra) inhibiting IL-1. M2 macrophages fine tune Th2 responses and adaptive immunity; clear cell debris via their scavenger receptors; promote tolerance, tissue remodelling and repair [131]. Some intracellular pathogens are able to exploit mannose and scavenger receptors and induce an alternatively activated phenotype enabling their survival and replication

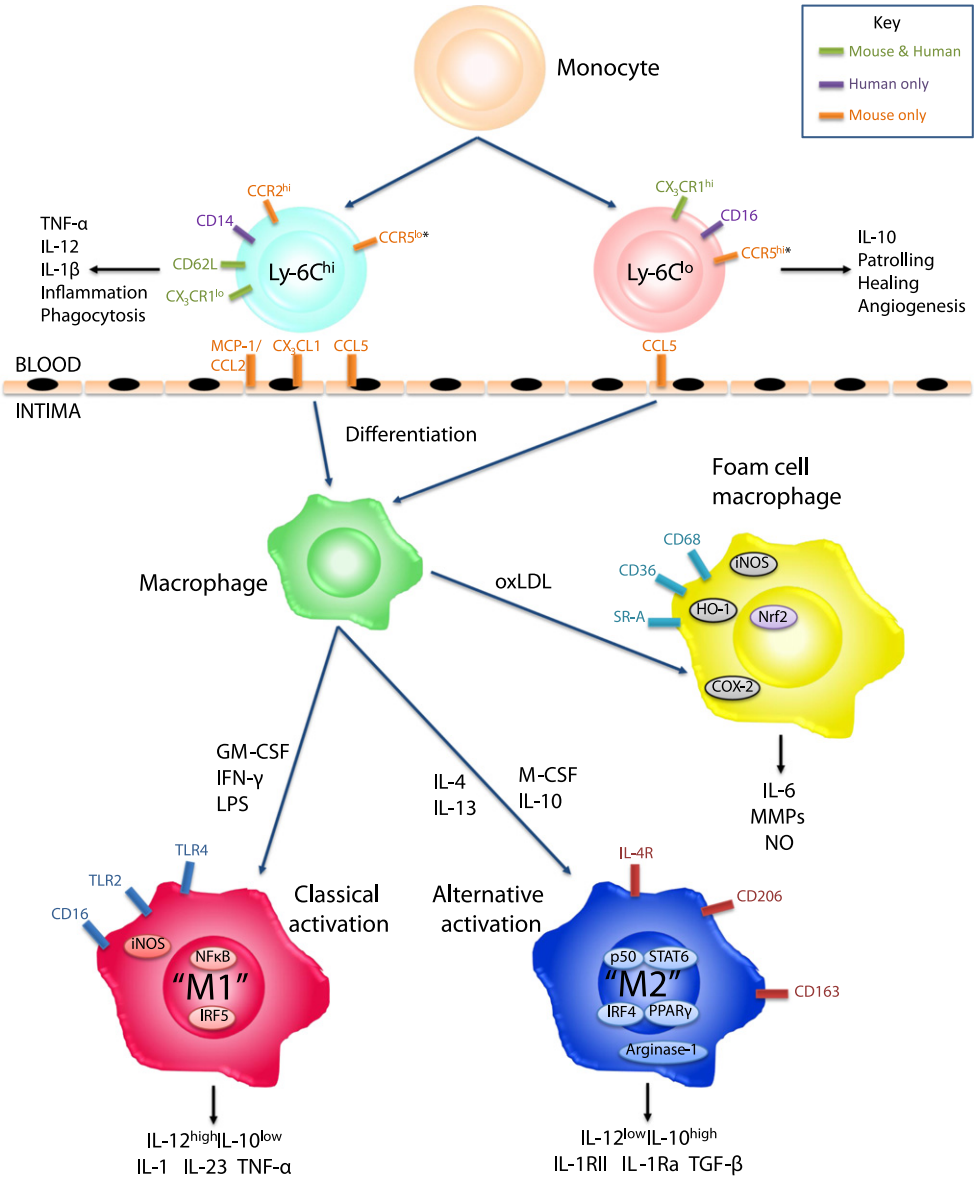

Fig. 4. Activities identified in monocyte and macrophage subsets that could affect atherosclerosis and plaque vulnerability. Monocytes can become one of the classical Ly-6C^{hi} or the resident Ly-6C^{lo} subsets. Ly-6C^{hi} monocytes display pro-inflammatory activity. They are the more abundant of the two subsets in murine plaques and could promote plaque vulnerability. Ly-6C^{lo} monocytes are associated with homeostatic activity but could be pro-atherogenic in the early stages while promoting plaque stability in the later stages. Following differentiation, macrophages can be either classically activated (M1) or alternatively activated (M2). Activation of transcription factors such as NFκB and IRF5, possibly following Toll-like receptor stimulation, may promote pro-inflammatory activity contributing to a vulnerable plaque phenotype. Transcription factors such as IRF4 and PPARγ can skew macrophages towards the M2 subset promoting anti-inflammatory activity; such characteristics may encourage plaque stability. Priming of macrophages by oxLDL can develop a foam cell macrophage which displays features of M1 and M2 activation while producing matrix metalloproteinases (MMPs) associated with plaque rupture. *Refers to CCR5 expression levels observed in ApoE$^{-/-}$ mice.

within the host macrophage [133]. M2 polarising signals like IL-4 and IL-10 are able to inhibit pro-inflammatory chemokine expression by inhibiting STAT1 and NFκB [134]. The p50 subunit of NFκB regulates M2-associated gene expression while inhibiting M1 polarisation and IFN-β production [135]. Murine M2 macrophages express increased levels of arginase-1; an enzyme responsible for synthesising proline, a key component of collagen, and polyamine precursors important for cell growth [136]. *In vitro* studies suggest regulatory T lymphocytes and M2 macrophages drive each other's differentiation reciprocally [137,138].

M2 macrophages are heterogeneous and Mantovani et al. proposed that they exist as one of three subtypes generated by different inflammatory and opsonic signals: M2a macrophages induced by IL-4 or IL-13, M2b by exposure to immune complexes, IL-1R or TLR agonists, or M2c activated by glucocorticoid hormones or IL-10 [131]. M2a and M2c macrophages display increased activity of the arginase pathway demonstrated by expression of arginase-1 by murine macrophages. While M2a and M2c macrophages are considered IL-12^{low}IL-10^{high}, M2b macrophages express high levels of both cytokines while promoting Th2 cell differentiation and regulating inflammation [139].

The widely used M1-M2 nomenclature describing macrophages could be premature; macrophages may instead exist as a continuum. A recent review by Mosser and Edwards suggested a spectrum of macrophage activation which can be categorised according to three different functions: host defence, wound healing and immune regulation [130]. The macrophage's phenotype could also be dependent on which tissue it is localised to [140].

Lipids are known to affect macrophage activation patterns [127]. Macrophages residing in the adipose tissue of lean mice express markers of M2 macrophages while adipose tissue macrophages

from obese mice upregulate M1 macrophage markers and contribute to insulin resistance; an effect which is abolished by the knockout of CCR2 or expression of IL-10 [141], and is also observed in humans [141]. $CD11c^{+}$ macrophages from adipose tissue of obese mice have increased expression of several M1 and M2 genes [142]. As previously mentioned the CD36 and SR-A scavenger receptors enable lipid uptake by macrophages and are deemed proatherogenic [143]. A recent study demonstrated that blockage of oxLDL with an Fc-CD68 fusion protein increased the collagen content of plaques, reduced plaque extension, spontaneous ruptures, and infiltration by T lymphocytes and macrophages in $ApoE^{-/-}$ mice [144], thereby possibly stabilising plaques. As CD68 is an oxLDL binding receptor, it may cooperate with other scavenger receptors to facilitate lipid uptake.

Foam cells display decreased expression of arginase-1, while increasing production of MMP12 and nitric oxide. These patterns were particularly evident close to the lipid core [145] and maybe advancing atherosclerosis. $MMP14^{high}TIMP3^{low}$ foam cells – which are stimulated by pro-inflammatory cytokines – proliferate and invade very readily but undergo apoptosis amid LPS or starvation challenge [146]. These characteristics may aid formation of the lipid necrotic core where these cells were most frequently observed. MMP upregulation and TIMP downregulation may be responsible for collagen degradation in the core and fibrous cap of advanced plaques. Oxidised phospholipids within atherosclerotic plaques can downregulate M1 and M2 gene expression, and are proposed to induce a Mox macrophage phenotype associated with Nrf2-mediated expression of redox-regulatory genes, such as haem oxygenase-1 [142]. It has been hypothesised that foam cells, derived from recently recruited macrophages, may possess an immature phenotype matching neither M1 nor M2 macrophages, while mature foam cells display features of classical and alternative activation [147].

Macrophage heterogeneity may play an important role in lesion development and outcome [148]. Interestingly, M2 macrophages accumulate first in murine atherosclerotic lesions, while lesion progression correlates with the predominance of M1 over M2 macrophages [136]. It appears inflammatory cytokine-induced M1 macrophages are associated with disease progression so agonists of the PPARγ receptor, which induces arginase-1 expression enhancing the M2 phenotype, could stabilise plaques. Both IFN-γ and TLR4 have already been implicated in M1 polarisation [149], and their genetic deletion reduces atherosclerosis development [76]. C-reactive protein (CRP), an acute-phase protein widely regarded as a marker of systemic inflammation and cardiovascular risk, was recently demonstrated to induce monocytic differentiation and the conversion of M2 macrophages into M1 macrophages via NFκB, upon binding to the CD32 and CD64 Fcγ receptors [150]. Haemoglobin-haptoglobin complexes present in plaques following intraplaque haemorrhage promote the differentiation of monocytes into atheroprotective M2-like macrophages, identified by their expression of CD163 [151].

4.2.5. Are TLRs critical for macrophage polarisation and activation in atherosclerosis?

As described earlier, CD14 is an important marker to discriminate between the monocyte subsets in humans. CD14 is also a co-receptor for TLR4, which activates intracellular signalling cascades such as MyD88-dependent signalling, and NFκB and is already implicated in M1 macrophage polarisation [149]. Hence TLRs could be pivotal in the polarisation of macrophages. Short-term culture of mixed cell populations from human carotid endarterectomies implicated a role for NFκB in the regulation of pro-inflammatory genes such as TF, IL-6, IL-8, TNF-α and several MMPs [152]. NFκB has been implemented in the transcription of the M1 markers iNOS and COX-2 [153,154]. M1 macrophages primed by IFN-γ upregulate the expression of TLR4 and components of the MyD88 signalling pathway. Detection of LPS by TLR4 in synergy with CD14, stimulates NFκB-dependent transcription of inflammatory chemokines e.g. CXCL8 (IL-8) and CCL2 (MCP-1) [155].

TLR2 is more highly expressed on human $CD16^{+}$ monocytes being further enhanced by M-CSF and IL-10 [156]. Necrotic cells but not apoptotic cells can activate NFκB inducing the expression of pro-inflammatory and tissue repair genes by macrophages in a TLR2-dependant manner [157]. A deficiency in IKKβ – an inhibitor of NFκB – increases expression of pro-inflammatory cytokines and the M1 marker iNOS while diminishing the M2 markers arginase-1 and IL-4R [158]. But mycobacterial infection can induce expression of arginase-1 by classically activated macrophages in a TLR-dependent manner [159]. A previous review suggested exposure of monocytes to TLR agonists may prime them into M2b macrophages [131], which share some characteristics with M1 macrophages. However the binding of immune complexes to M2b macrophage Fcγ receptors inhibits TLR4 and type I interferon signalling [160].

One set of transcription factors that is activated by TLR signalling is the interferon-regulatory factor (IRF) family. During helminth infection, IRF4 was shown to skew macrophage polarisation towards the M2 subset [161]. The knockout of another family member IRF5 in mice causes defective pro-inflammatory cytokine and type I interferon production [162]. IRF4 inhibits IRF5 from binding to MyD88, while in a model of Epstein-Barr viral infection, IRF4 can bind directly to the promoter of IRF5 [163]. Thus IRF4 acts as a negative feedback mechanism mitigating inflammatory cytokine production [164].

4.3. TLRs as therapeutic targets

With increasing evidence of TLRs playing significant roles in atherosclerosis, they may provide a potential alternative for therapeutic targets in atherosclerosis. It was previously thought that antagonists of TLRs, namely TLR2 and TLR4, are the main pathway for treatment in atherosclerosis. We have recently shown the possibility of promoting TLRs in combating atherosclerosis [78]. However, obstacles still exist with regards to translational medicine due to differences between human and murine immune systems, including the cellular expression patterns of TLRs. In addition, it is also essential to identify the optimal timing of intervention. Albeit, given the contribution of TLRs in mediating atherosclerosis, therapeutic targeting of TLRs by drugs could have massive clinical implications in combating atherosclerosis, which is proving to be a major threat to human health globally.

5. Conclusions

Recent evidence suggests a prominent role for innate immune responses in the pathogenesis of atherosclerosis. Upon recognition of PAMPs by TLRs, there is an upregulation of inflammatory gene transcription. Various PAMPs and DAMPs are present in the atherosclerotic lesion and may elicit responses in the plaque. Many TLRs – most notably TLR1, TLR2 and TLR4 – are upregulated in atherosclerotic vessels and contribute to lesion formation, while TLR3 appears to be atheroprotective. Innate immune cells play a significant role in atherogenesis and in shaping the plaque's phenotype; those displaying a pro-inflammatory phenotype, especially the abundant macrophages, are associated with plaque vulnerability. Such pro-inflammatory activity triggered by TLR stimulation maybe a key factor in promoting the development of high-risk atherosclerotic lesions such as TCFAs. Lipids, growth factors and cytokines have been shown to contribute to the polarisation of macrophages towards a particular phenotype; classically activated M1 macrophages could promote plaque vulnerability while alternatively activated M2 macrophages may promote plaque stability. As TLRs and their downstream transcription factors have demonstrated a critical role in determining the inflammatory activity of macrophages, this axis serves as a potentially strong therapeutic target and could curtail lesion progression or promote plaque stability in the advanced stages.

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