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Innate Immune Receptors in Atherosclerosis

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1. Introduction

The inflammatory response is an important process, aiming to restore tissue homeostasis following tissue injury or infection. Acute inflammation is a tightly controlled process. If an inflammatory stimulus persists or if normal immune function is perturbed, inflammation may become chronic. Atherosclerosis is a chronic inflammatory disorder involving components of both the innate and adaptive immune systems (Ross, 1999). The innate immune system provides the first line of defence against invading pathogens. Innate immune detection of pathogens relies on a set of pattern recognition receptors (PRRs) that recognise and respond to conserved pathogen-associated molecular patterns (PAMPs). Growing evidence supports roles for PRRs in the initiation and progression of atherosclerosis. In this chapter, the agonists, signalling pathways, expression and functions of PRRs, in particular in reference to atherosclerosis, will be discussed. The potential therapeutic benefit of targeting PRRs for treatment of atherosclerosis will also be explored.

2. Pattern recognition receptors

PAMPs are recognised by an expanding number of PRRs, which currently includes at least 50 members. PRRs can be categorised into one of three families: Toll-like receptors (TLRs), Retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), and Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Both extracellular and intracellular compartments are patrolled by PRRs with each family of receptors specialising in surveying a given location. TLRs are transmembrane PRRs either located in the cellular membrane (interacting with the extracellular space) or within intracellular vesicles such as endosomes or lysosomes. Cytosolic PRRs include RLRs and NLRs that detect intracellular PAMPs. While cytosolic PRRs are universally expressed in the majority of cells in the body (Takeuchi & Akira, 2010), TLR expression is more restricted. TLR, NLR and RLR ligation by an agonist stimulates downstream signalling cascades activating 2 major types of transcription factor: the nuclear factor kB (NFκB), and interferon response factors (IRFs).

2.1 Toll-like receptors (TLRs)

The TLR family contains at least 13 different members in mammals. Following ligand binding, TLRs dimerise, with most receptors (with the exception of TLR2 and TLR4) forming homodimers. Components of the bacterial cell wall including bacterial lipoproteins, endotoxin and flagellin are sensed by TLR2, TLR4 and TLR5 respectively. TLR3, TLR7, TLR8

and TLR9 are not located on the cell surface and are instead located on the membranes of endoplasmic reticulum (ER), endosomes and lysosomes, where they detect nucleic acids derived from bacteria or viruses. The TLR family share their cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain – essential for signal transduction - with their larger parent family which includes interleukin-1-receptor (IL-1Rs). The extracellular regions of TLRs contain tandemly arranged leucine rich repeats (LRR) creating a horseshoe-shaped solenoid structure (Liu et al., 2008a). TLRs are connected to their downstream signalling cascades via five TLR adaptor molecules that are recruited to and homophilically interact with the TIR domain: myeloid differentiation protein 88 (MyD88), Toll–interleukin-1 receptor domain-containing adaptor inducing interferon-β (TRIF), TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (MAL), TRIF related adaptor molecule (TRAM) and sterile alpha and HEAT/Armadillo motif (SARM).

2.2 RIG-I-like receptors (RLRs)

Double stranded RNA (dsRNA) in the cytoplasm can be sensed in both immune and non-immune cells via RLRs. The RLR family includes retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RLRs possess a central RNA helicase domain with the ATPase binding motif DExD/H. The C-terminal regulatory domain is responsible for binding to dsRNAs. RIG-I and MDA5 have two N-terminal caspase activation and recruitment domains (CARDs), which allows homophilic interactions between activated RIG-I or MDA5 and the adaptor protein mitochondrial antiviral signaling (MAVS, also known as IPS-1, VISA, and Cardif), which is found in the outer mitochondrial membrane (Takeuchi & Akira, 2010).

2.3 NOD-like receptors (NLRs)

NLRs belong to a large family of soluble proteins that are present in the cytoplasm and detect intracellular ligands. There are 23 NLR genes in humans and 34 in mice. Three distinct subfamilies of NLRs exist: NODs, NLRPs (or NALPs) and IL-1β-converting enzyme (ICE)-protease activating factor (IPAF). NLRs are composed of the following domains: a C-terminal ligand-sensing leucine-rich repeat (LRR) domain, a central nucleotide-binding and oligomerization (NACHT) domain (responsible for oligomerization), and an N-terminal effector pyrin domain (PYD), caspase recruitment domain family (CARD) or baculoviral IAP repeat (BIR) mediating homophilic interactions in downstream signalling. The physiological function of most NLRs is still not understood.

3. PRR agonists in atherosclerosis

A vast and diverse array of ligands including viruses, lipids and extracellular matrix components are collectively recognised by PRRs (Lundberg & Hansson, 2010). Each individual PRR exhibits specificity in the repertoire of ligands that it recognises and responds to. In a process known as 'sterile inflammation', activation of PRRs can occur in the absence of exogenous stimuli (Rifkin et al., 2005). The PRR agonists in this context are generated as a result of tissue damage and inflammation and are known as 'damage-associated molecular patterns' (DAMPs). Thus, PRR ligands encompass both exogenous PAMPs and endogenous DAMPs. Increasing evidence suggests that different co-receptors

and accessory molecules and thus different mechanisms of action are used by TLRs in response to ligation by PAMPs and DAMPs (reviewed in (Piccinini & Midwood, 2010)). Exogenous PRR ligands, such as viruses and bacteria, and endogenous PRR ligands, including extracellular matrix components, modified lipids and heat shock proteins are PRR ligands that may be relevant in the context of atherosclerosis.

3.1 Exogenous PRR agonists

Exogenous agonists are the best defined PRR ligands and include components of bacteria and viruses. TLR2 recognises a diverse array of PAMPs using heterodimerisation with TLR1 or TLR6. TLR2 is key in the recognition of Gram-positive bacteria (Underhill et al., 1999a; Underhill et al., 1999b). Lipoteichoic acid, is a ligand of TLR2/TLR6 heterodimers as are peptidoglycan and zymosan (Gantner et al., 2003; Ozinsky et al., 2000; Schroder et al., 2003). Using CD36 as a co-receptor, TLR2/TLR6 heterodimers also recognise mycoplasma diacylated lipoproteins peptide (Brightbill et al., 1999; Hoebe et al., 2005; Takeuchi et al., 2001). Triacylated lipoproteins are ligands for TLR1/TLR2 heterodimers (Jin et al., 2007; Takeuchi et al., 2002). Endotoxin (lipopolysaccharide), a component of the outer membrane of Gram-negative bacteria, is an agonist for the TLR4 signalling complex (Shimazu et al., 1999; Wright et al., 1990). Compared to TLR2 and TLR4, other TLRs have a relatively limited repertoire of TLR ligands. TLR3 senses viral double-stranded RNA and some small interfering RNAs. A synthetic dsRNA analogue Poly(I:C) is commonly used as a TLR3 activator (Takeuchi & Akira, 2010). Bacterial flagellin is recognised by TLR5 and TLR9 detects unmethylated CpG DNA, typically of bacterial origin (O'Neill & Bowie, 2007). TLR7 is the main sensor of ssRNA derived from RNA viruses including human immunodeficiency virus and influenza A. More recently, TLR7 on myeloid dendritic cells has also been shown to be capable of sensing bacterial RNA (Mancuso et al., 2009).

Genomic RNA of dsRNA viruses and dsRNA generated as the replication intermediate of ssRNA viruses in the cytosol are ligands for RLRs. Short dsRNAs with 5′ triphosphate ends are sensed by RIG-I whereas MDA5 recognises longer dsRNAs (Kato et al., 2006). NOD1 and NOD2 sense peptidoglycan. NOD1 recognises a peptidoglycan motif: dipeptide γ-d-glutamyl-meso-dia-minopimelic acid (iE-DAP) and NOD2 recognises muramyl dipeptide (Chamaillard et al., 2003; Girardin et al., 2003a; Girardin et al., 2003b; Inohara et al., 2003). Whole pathogens including bacteria with pore-forming toxins and viruses including influenza virus are activators of the NLRP3 inflammasome (reviewed in (Schroder & Tschopp, 2010)).

Numerous exogenous PAMPS may be ligands for PRRs in atherosclerosis. *Chlamydia pneumonia, porphyromonas gingivalis* and cytomegalovirus are exogenous PRR ligands found in atherosclerotic plaques (Chiu et al., 1997; Kuo et al., 1993). These bacterial and viral infectious agents have been associated with an increased risk of atherosclerosis development (Kalayoglu et al., 2002; Kiechl et al., 2001; Scannapieco et al., 2003) and are recognised by TLR2 and TLR4 (Burns et al., 2006; Compton et al., 2003; Naiki et al., 2008). The failure to detect active viral replication within atherosclerotic plaques (Kol et al., 1995; Zhou et al., 1999) suggests PRR activation by infectious agents and not viral replication itself is the link between infectious disease and cardiovascular risk. Exogenous heat shock proteins (HSPs), nucleic acids (Lehtiniemi et al., 2005; Ott et al., 2006) and peptidoglycan

(Laman et al., 2002) are also present in atherosclerotic lesions and thus may activate PRRs in atherogenesis.

3.2 Endogenous PRR agonists

Many endogenous PRR ligands are present in atherosclerotic lesions and thus PRR activation in atherosclerosis could result from a combination of exogenous and endogenous ligand sensing. Indeed, work by Curtiss and colleagues supports a role for endogenous TLR ligands in atherogenesis (Mullick et al., 2005). Extracellular matrix (ECM) is degraded during tissue injury and remodelling leading to the generation of ECM components, which can function as PRR ligands. Fibrinogen can activate TLR4 signalling as can the fibronectin alternatively spliced exon encoding type III extra domain A (EDA) and tenascin C (Midwood & Orend, 2009; Okamura et al., 2001; Smiley et al., 2001). Hyaluronan, a large glycosaminoglycan component of the ECM, and biglycan activate TLR2 and TLR4 signalling (Schaefer et al., 2005; Scheibner et al., 2006; Taylor et al., 2004). Hyaluronan can induce IL-1 release by macrophages in a NLRP3-dependent manner (Yamasaki et al., 2009) while biglycan also activates the NLRP3 inflammasome (Babelova et al., 2009). The large ECM proteoglycan versican is a TLR2/6 ligand associated with cytokine production in tumor-infiltrating macrophages (Kim et al., 2009). These ECM components may be generated during injury and remodeling of the vessel wall and thus may activate PRRs in atherosclerosis.

Lipids, key components of atherosclerotic plaques, are TLR ligands. Minimally modified low-density lipoproteins induce cytokine and reactive oxygen species generation via TLR4 signalling complexes (Miller et al., 2003). In association with CD36, TLR4/TLR6 hetero-dimers sense oxidized LDL leading to increased chemokine expression (Stewart et al., 2010). Saturated fatty acids elicit TLR4 activation whereas polyunsaturated fatty acids inhibit TLR4 activation (Lee et al., 2003). However, the ability of saturated fatty acids to directly induce TLR signaling has been questioned (Erridge & Samani, 2009). TLR2 can also sense ApoCIII, a component of very-low-density lipoprotein (VLDL) (Kawakami et al., 2008).

HSPs are present in murine atherosclerotic lesions and are ligands for TLR2 and TLR4 (Asea et al., 2002; Kanwar et al., 2001). However, some studies using low-endotoxin preparations have disputed the role of HSPs as ligands for TLRs (Bausinger et al., 2002). The nuclear protein high-mobility group box-1 (HMGB-1) is expressed in human atherosclerotic smooth muscle cells (Porto et al., 2006). HMGB1 binds DNA and is a ligand for TLR2, TLR4, TLR9 and other nucleic acid sensors (Park et al., 2004; Yanai et al., 2009). mRNA from necrotic cells, which may be present in atherosclerotic plaques, is a TLR3 agonist (Kariko et al., 2004).

The NLRP3 inflammasome can be activated by many factors including extracellular ATP, potassium efflux and reactive oxygen species. In addition, intracellular crystals such as monosodium urate crystals and cholesterol crystals can activate the NLRP3 inflammasome (Duewell et al., 2010; Martinon et al., 2006).

4. Signalling of pattern recognition receptors

4.1 Toll-like receptor signalling

TLR signalling is composed of two distinct signalling pathways depending on whether the adaptor molecule MyD88 is used following ligation and activation (Figure 1).

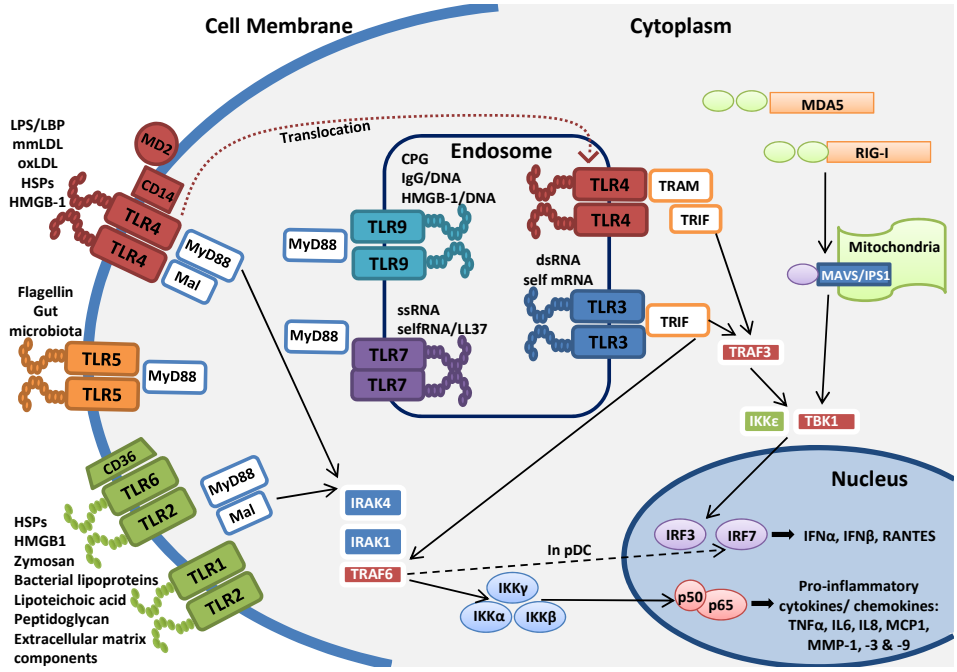

Fig. 1. **Toll-like receptor & RIG-I-like receptor signalling. MyD88-dependent signalling:** All TLRs, except TLR3, recruit the adaptor protein MyD88. MyD88 recruits IRAK1 and IRAK4 to its death domain, which then recruit TRAF6. IRAK1 with TRAF6 subsequently activate a complex consisting of TAK1, which activates the canonical IκB kinase (IKK) complex and the mitogen-activated protein kinase pathway (not shown). NFκB (p50/p65) is then activated to transcribe multiple proinflammatory cytokine genes. The production of type I IFNs by TLR7 and TLR9 depends on MyD88 in plasmacytoid dendritic cells (pDCs). **TRIF-dependent signalling:** TLR3 and endosomal TLR4 (via TRAM), utilise TRIF to interact with the non-canonical IKKs, TBK-1 and IKKε via TRAF3, which phosphorylate IRF3 to induce expression of IFNs. **RIG-I-like receptor signalling:** RIG-I and MDA5 interact with IPS-1 to activate TBK1 enabling the phosphorylation of IRF3 and IRF7. These transcription factors then homo- or heterodimerize, translocate into the nucleus and activate the transcription of type I IFN genes.

4.1.1 MyD88-dependent signalling pathway

All TLRs with the exception of TLR3 use MyD88 to initiate downstream signalling. TLR2 and TLR4 require TIRAP/MAL as a bridge between the TLR and MyD88 (Kagan & Medzhitov, 2006; Yamamoto et al., 2002). Following PAMP recognition by TLRs, MyD88 recruits members of the IL-1 receptor-associated kinase (IRAK) family, including IRAK4, IRAK1, IRAK2 and IRAKM. IRAK4 is activated first, followed by IRAK1 and IRAK2, with all being essential for robust activation of NFκB and MAPK (Kawagoe et al., 2008). The IRAKs then dissociate from MyD88 and associate with TRAF6, an E3 ubiquitin ligase, leading to the activation of a complex consisting of transforming growth factor-β-activated kinase 1 (TAK1), TAK1 binding protein (TAB)1, TAB2 and TAB3. This complex translocates

into the cytosol where TAK1 phosphorylates IKKβ. Subsequently, the IKK complex, consisting of IKKα, IKKβ and NFκB essential modulator (NEMO), phosphorylates IκBα, an NFκB inhibitory protein. Phosphorylated IκBα is degraded by the ubiquitin proteosome system, freeing NFκB to translocate to the nucleus and mediate transcription of inflammatory genes. TAK1 also phosphorylates MAPK6 activating the MAP kinases Erk1, Erk2, p38 and Jnk. Activation of the MAPK pathway triggers the formation of activated protein (AP)-1, a transcription factor complex controlling genes encoding many cytokines (Johnson & Lapadat, 2002).

TLR7 and TLR9 induce the production of type 1 IFNs and NFκB-dependent cytokines via the MyD88 dependent pathway. Plasmacytoid dendritic cells (pDCs) constitutively express Interferon Regulatory Factor (IRF)7 which binds to MyD88 forming a complex with IRAK1, IRAK4, TRAF3, TRAF6 and IKKα (Kawai & Akira, 2008). Phosphorylated IRF7 then translocates to the nucleus and facilitates the production of type 1 IFNs (Kawai & Akira, 2010). In contrast, conventional dendritic cells (cDCs) mediate the activation of IRF1 resulting in IFN-β gene expression (Negishi et al., 2006; Schmitz et al., 2007).

MyD88 is critical for the downstream inflammatory effects following ligation of many TLRs. MyD88 knockout ($MyD88^{-/-}$) mice do not respond to peptidoglycan and lipoprotein stimulation of TLR2 (Takeuchi et al., 2002; Takeuchi et al., 2000), Imidazoquinoline stimulation of TLR7 (Hemmi et al., 2002) or stimulation of TLR9 by CpG DNA motifs (Häcker et al., 2000; Schnare et al., 2000). Similarly, $MyD88^{-/-}$ mice have an abolished response to TLR4 stimulation by LPS (Kawai et al., 1999) or TLR5 stimulation by bacterial flagellin (Hayashi et al., 2001).

4.1.2 TRIF-dependent signalling pathway

TLR3 utilises signalling via TRIF to elicit responses (Alexopoulou et al., 2001). TRIF associates with TRAF3 and TRAF6 via its N-terminal TRAF binding-motifs (Takeuchi & Akira, 2010). TRAF3 activates 2 noncanonical IKK-related kinases, TBK1 and IKKε which phosphorylate IRF3 enabling its nuclear translocation (Häcker & Karin, 2006; Oganesyan et al., 2006). IRF3 mediates the production of proinflammatory cytokines, type 1 IFNs and increased expression of IFN-induced genes including Adar1, Ifit3 and IRF7 (Tenoever et al., 2007). TRIF also interacts with RIP1 and RIP3 (Takeuchi & Akira, 2010). The TNFR-associated death domain protein (TRADD) is involved in TRIF dependent signalling (Ermolaeva et al., 2008). A complex is formed consisting of TRADD, FADD and RIP1. TRADD triggers the ubiquitination of RIP1 activating NF-κB. Following stimulation by Poly(I:C), a synthetic dsRNA analogue, FADD activates caspase-8 and caspase-10 (Takahashi et al., 2006). These cleaved caspases activate NFκB (Takahashi et al., 2006). In addition, TRIF associates with TRAF6 to activate TAK1. This is thought to occur in an ubiquitination-dependent mechanism similar to the MyD88-dependent pathway resulting in phosphorylation of the inhibitory molecule IκBα by IKKα and IKKβ (Alexopoulou et al., 2001). SARM is a an inhibitor of TRIF-mediated signalling in humans (Carty et al., 2006).

TLR4 is unique in that it can utilise both the MyD88 and TRIF dependent pathways with the sequential activation of 4 adaptor molecules. It appears that the receptor's cellular localisation determines which pathway is triggered (Kagan et al., 2008; Tanimura et al., 2008). Upon ligand binding, membrane bound TLR4 recruits MyD88 which binds to MAL to activate NFκB and MAPK (Kagan & Medzhitov, 2006). Secondly, TLR4 translocates 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 (Kagan et al., 2008; Rowe et al., 2006; Tanimura et al., 2008).

4.2 RIG-I-like receptor (RLR) signalling

RLR signalling activates NFκB, MAPK, and IRFs to induce type I IFNs. LGP2 may regulate the functions of RIG-I and MDA5 as LGP2-deficient mice have elevated levels of type I IFNs. Overexpression of IPS-1 (also called MAVS) activates the promoters of NFκB and type I IFNs inhibiting viral replication. The induction of IFNβ by IPS-1 requires TBK1 and IKKi (Kawai et al., 2005). IPS-1 has a C-terminal transmembrane domain required for mitochondrial targeting (Seth et al., 2005), and deleting this region of IPS-1 prevents IRF3 and NF-κB activation. TRAF3 directly binds both IPS-1 and TBK1/IKKi enabling type I IFN induction in response to ssRNA viral infection. TBK1 is broadly expressed in many tissues while IKKi expression is stimulated upon pro-inflammatory signals such as TNF-α and IFN-γ. It has been suggested that TBK1 aids the initiation of signalling following viral infection while IKKi regulates the immune response in the later stages of viral infection (Kawai & Akira, 2007). IKKi can phosphorylate STAT1 and IRF3 to regulate antiviral gene expression. IPS-1 also interacts with RIP-1 and FADD (Kawai et al., 2005) forming a complex with caspase-10 and caspase-8. The detection of poly I:C triggers the cleavage of these caspases (Takahashi et al., 2006) activating their death effector domain to activate NF-κB.

4.3 NOD-like receptor (NLR) signalling

NODs activate MAPKs and NFκB via the serine–threonine kinase RICK and consequently activate TAK1 kinase. NLRs activate the release of the IL-1 family of inflammatory cytokines through the formation of large cytoplasmic complexes known as 'inflammasomes', which include caspase-1. Inflammasomes are characterised into three main complexes —the NLRP3/NALP3 inflammasome, the NLRP1/NALP1 inflammasome and the IPAF/NLRC4 inflammasome. The NLRP3 inflammasome is currently the most studied and consists of the NLRP3 scaffold, the apoptosis-associated speck-like protein-containing CARD (ASC) adaptor, and caspase-1. ASC links the NLR and caspase; normally caspase 1 and 11 (Wang et al., 1998). Upon activation, caspase-1 cleaves the precursor cytokines into their bioactive form, most notably activating IL-1β and IL-18.

4.4 Integration of pattern recognition signalling

The pattern recognition system involves numerous interactions between components of different pathways. NOD stimulation, TLR activation and proinflammatory cytokine stimulation can act as priming signals leading to NFκB activation, pro-IL1β synthesis, and the activation of inflammasomal components such as caspase-11 and NLRP3 (Mariathasan & Monack, 2007). A second signal then activates caspase-1 in the inflammasome complex. Such second signals include activation by ATP of the P2X7 purinergic receptor with potassium efflux, PAMPs and DAMPs such as oxidative stress, large particles and ultraviolet light (Wang et al., 1998).

The interaction of IPS-1 with NLR proteins can modulate the activation of NFκB and IRF3 signalling. NLRX1/NOD5 may interact with IPS-1 and inhibit its binding to RIG-I and the production of type I IFNs and pro-inflammatory cytokines. RIG-I can also directly activate the inflammasome. Finally, NOD2 can translocate into mitochondria, and signal via IPS-1, inducing type I IFN secretion via IRF3 during viral infection (reviewed in (Ting et al.,2010)).

5. Expression of PRRs in health and atherosclerotic disease

TLRs are expressed by both leukocyte subsets and resident tissue cells (reviewed in (Cole et al., 2010)). In contrast to veins, which are relatively atherosclerosis-resistant, the arterial system is more predisposed to atherosclerotic lesion formation. This is mirrored by the sensitivity of venous and arterial cells to TLR agonists with arterial cells responding to a broader range of TLR agonists than venous cells (Erridge et al., 2008). Different arterial beds exhibit heterogeneity in their TLR mRNA expression. Carotid arteries and the aorta share a similar pattern of TLR expression with high expression of TLRs 1 through 6 and minimal to no expression of TLRs 7, 8 and 9. Iliac arteries display the broadest expression of TLRs expressing all but TLR3 whereas mesenteric and subclavian arteries express a narrower range of TLRs. TLR2 and TLR4 are the only TLR described to be ubiquitously expressed in normal human arteries (Pryshchep et al., 2008). During human atherogenesis, TLR expression (in particular expression of TLR1, TLR2 and TLR4) is increased in diseased vessels compared to healthy vessels (Edfeldt et al., 2002). Increased expression of TLR2 and TLR4 are found both in macrophages and in resident cells including adventitial fibroblasts, endothelial cells and smooth muscle cells from human atherosclerotic vessels (Edfeldt et al., 2002; Otsui et al., 2007; Vink et al., 2002; Xu et al., 2001). Similar to human atherosclerotic tissue, expression of TLR2 and TLR4 is increased in murine models of the disease (Mullick et al., 2008; Xu et al., 2001).

In early atherosclerotic lesions, endothelial cells are the first cells to display TLR expression. In LDLR-/- mice endothelial cells at atherosclerosis-prone regions of the vasculature, such as the inner curve of the aortic arch, display increased TLR2 expression, which is also associated with areas of monocyte recruitment (Mullick et al., 2008). Whether endothelial TLR2 expression is a cause or effect of monocyte recruitment is unknown. Smooth muscle cells (SMC) also respond to PAMPs and express TLRs. TLR-1, -3, -4 and -6 are constitutively expressed at the mRNA level by cultured human vascular smooth muscle cells and TLR2, TLR3 and TLR4 stimulation induces SMC production of cytokines and chemokines such as IL6 and MCP1 (Stoll et al., 2004; Yang et al., 2005a; Yang et al., 2005b; Yang et al., 2006). Recently, atheroma-derived SMC have been shown to exhibit a specific increase in TLR3 expression and TLR3-dependent functional responses compared to control aortic SMC (Cole et al., 2011).

Although all leukocyte populations express TLRs, TLR expression on monocytes/ macrophages and dendritic cells is the best characterised. Monocytes, which constitute 5-10% of circulating blood leukocytes in both mouse and man, are key players at all stages of atherogenesis. Constant recruitment of monocytes into atherosclerotic plaques occurs and their recruitment is proportional to plaque size (Swirski et al., 2006). Human blood monocytes highly express TLR2 and TLR4 mRNA and respond to stimulation with their respective TLR ligands by secreting pro-inflammatory cytokines including TNFα and IL6 (Kadowaki et al., 2001; Visintin et al., 2001). TLR4 expression on peripheral blood monocytes appears to correlate with disease activity with monocytes from patients with acute coronary syndromes expressing more TLR4 than monocytes from patients with stable angina (Methe et al., 2005; Shiraki et al., 2006). Similarly, TLR2 expression is also increased on circulating blood monocytes from patients with atherosclerotic disease (Kuwahata et al., 2009; Mizoguchi et al., 2007). Circulating monocytes in ApoE-/- mice with advanced atherosclerosis also exhibit increased expression of TLR2 and TLR4 (Schoneveld et al., 2008). Subsets of monocytes and macrophages with differing characteristics have been described.

The balance of these subsets in disease may determine the outcome for the patient. In both humans and mice, two major subsets of monocytes; 'inflammatory' and 'resident', have been described (Gordon & Taylor, 2005), which can be distinguished on the basis of size, granularity and expression pattern of adhesion molecules and chemokine receptors. Macrophages can also be divided into subsets and can be broadly defined as M1 'classically activated' or M2 'alternatively activated' (Gordon & Taylor, 2005). In terms of TLR responses, differing levels of LPS responsiveness has been described in two subsets of CD14+ peripheral blood monocytes (Moreno-Altamirano et al., 2007), type I interferon production following TLR2 stimulation has been shown to occur specifically in murine inflammatory monocytes from bone marrow and spleen (Barbalat et al., 2009) and M2 macrophages have been shown to exhibit 12-fold higher expression of TLR5 than M1 macrophages (Martinez et al., 2006). Despite these few studies, as yet, the differential expression of TLRs on monocyte and macrophage subsets has not been examined in detail.

The role of dendritic cells (DC) in atherosclerosis is unknown however in normal arteries dendritic cells form networks in the intima, which is described as being part of a 'vascular-associated lymphoid tissue' (Bobryshev & Lord, 1995; Millonig et al., 2001; Wick et al., 1997). Their location in the healthy vessel wall, particularly in regions prone to atherosclerotic lesion development such as branch-points, suggests a role in atherosclerosis development (Lord & Bobryshev, 1999; Millonig et al., 2001). DCs can be broadly classified as either myeloid (mDC) or plasmacytoid (pDC) with both subsets being present in atherosclerotic plaques (Erbel et al., 2007; Niessner et al., 2006). mDCs express TLRs 2-8 at the mRNA level and secrete cytokines and upregulate costimulatory molecule expression in response to TLR-2, -3 and -4 activation (Jarrossay et al., 2001; Matsumoto et al., 2003). On the other hand, pDCs strongly express TLR7 and TLR9 mRNA and are activated, mature and secrete cytokines following exposure to the TLR9 ligand CpG (Hornung et al., 2002; Jarrossay et al., 2001; Kadowaki et al., 2001; Matsumoto et al., 2003). Both mDCs and pDCs express and respond to TLR7 ligation with R848 albeit with different functional outcomes: mDCs express IL12 while pDCs express IFNα (Ito et al., 2002).

Although less is known regarding the expression of RLR and NLRs in atherosclerosis, increasing evidence supports a similar trend to that seen for TLRs. Intimal macrophages in aortic atherosclerotic lesions highly express RIG-I (Imaizumi et al., 2007). In healthy human coronary artery ring cultures, IFNγ treatment augmented the expression of the RNA sensors TLR3, MDA5 and RIG-I (Ahmad et al., 2010).

6. Functional consequences of PRR activation in atherosclerosis

6.1 The role of the IL1/TLR superfamily in atherosclerotic lesion development

The use of mice deficient in IL1/TLR superfamily molecules has revealed key roles for these signalling pathways in atherosclerotic lesion development. Deletion of MyD88 in ApoE-/- mice inhibits atherosclerotic lesion formation by 60% and also results in a 75% reduction in macrophage recruitment (Bjorkbacka et al., 2004; Michelsen et al., 2004). In addition, following carotid ligation, an 89% reduction in lesion formation is observed in ApoE-/- mice bred with an IRAK4 kinase-inactive knock-in mouse (Rekhter et al., 2008). MyD88 and IRAK4 are part of both the TLR and interleukin receptor (IL1R and IL18R) signalling pathways. ApoE-/-IL18-/- double knockout mice exhibit smaller lesions with a more stable phenotype compared to ApoE-/- (Elhage et al., 2003). Similarly, IL1β deficiency in ApoE-/- mice leads to a 30% reduction in lesion size and a reduction in pro-inflammatory mediators

such as VCAM-1 and MCP-1 (Kirii et al., 2003). Overexpression of the endogenous IL1 inhibitor, IL1 receptor antagonist (IL1RA), attenuates lesion production (Merhi-Soussi et al., 2005) whereas IL1RA deletion in ApoE-/- augments lesion development at early timepoints (Isoda et al., 2004).

TLR2 and TLR4 have been the most extensively studied in animal models of atherosclerosis. A missense mutation in the TLR4 gene causing resistance to endotoxin has been identified in C3H/HeJ mice (Poltorak et al., 1998; Qureshi et al., 1999). These mice are resistant to diet-induced atherosclerosis (Nishina et al., 1993). However, no effect on lesion development was observed when bone marrow from C3H/HeJ mice was transplanted into ApoE-/- mice (Shi et al., 2000) suggesting resident vascular cell TLR4 signalling may be more important than TLR4 on hematopoietic cells. Interestingly, a similar observation has been made for TLR2, with TLR2-/- bone marrow transfer into LDLR-/- mice having no effect on lesion development. However, a role for hematopoietic cells in recognition of exogenous TLR2 ligands was revealed when LDLR-/- mice were transplanted with TLR2-/- bone marrow prior to stimulation with a synthetic TLR2 ligand as this led to reduced lesion development (Mullick et al., 2005). In vascular injury models, deficiency of TLR2 or TLR4 leads to reduced neointima formation and activation of TLR2 and TLR4 with agonists augments neointima formation (Schoneveld et al., 2005; Vink et al., 2002). Furthermore, genetic deletion of either TLR2 or TLR4 in atherosclerosis-prone mice confers marked protection from atherosclerotic lesion development attenuating plaque formation by 30-69% (TLR2-deletion) and 55% (TLR4-deletion) (Liu et al., 2008b; Michelsen et al., 2004; Mullick et al., 2005). Lesional macrophage content is also significantly reduced in these TLR deficient animals (Liu et al., 2008b; Michelsen et al., 2004). Administration of a TLR2 agonist to LDLR-/- mice promotes lesion development (Mullick et al., 2005). A rabbit hypercholesterolemia model has revealed that the expression of TLR2 and TLR4 may have a synergistic effect on lesion development (Shinohara et al., 2007).

A protective role for TLR3 in arterial injury and early atherosclerosis has been described, challenging the prevailing view that TLRs are purely detrimental in atherogenesis. TLR3 activation using the synthetic ligand Poly(I:C) led to attenuated neointima formation in C57BL/6 but not TLR3-/- mice following carotid injury (Cole et al., 2011). Furthermore, TLR3 was shown to mediate protection against medial damage even in the absence of exogenous TLR3 stimulation suggesting that following injury an endogenous TLR3 ligand is released which maintains the integrity of the vessel wall. In addition, ApoE-/-TLR3-/- mice exhibited increased lesion formation compared to ApoE-/- at an early but not later timepoint (Cole et al., 2011). The mechanisms of the protective effects of TLR3 remain to be explored as does the identification of endogenous TLR3 ligands in atherosclerosis. mRNA from necrotic cells and stathmin, a microtubule regulatory protein have both been identified as potential endogenous TLR3 ligands (Bsibsi et al., 2010; Kariko et al., 2004). A recent study showed that intravenous administration of poly(I:C) induces endothelial dysfunction and increased atherosclerotic lesion development (Zimmer et al., 2011). Together the studies of Cole *et al.,* and Zimmer and colleagues suggest a complex role for dsRNA sensing in atherosclerosis.

Evidence from human polymorphism and atheroma-cell culture studies also support roles for TLRs in atherosclerosis. Asp299Gly and Thr399Ile are two single-nucleotide TLR4 polymorphisms that are associated with reduced responses to inhaled LPS (Arbour et al., 2000). Despite individuals who carry these polymorphisms having lower circulating levels of proinflammatory cytokines and adhesion molecules (Cook et al., 2004), no definitive effect of

these polymorphisms on cardiovascular disease has been identified (reviewed in (Frantz et al., 2007)). The TLR2 polymorphism Arg753Gln has been found, in a relatively small study, to be associated with restenosis and an increased risk of developing mycobacterial disease (Hamann et al., 2005). In human atherosclerosis, TLR2 and MyD88 have been shown to play a predominant role in NFκB activation, the production of proinflammatory cytokines including MCP-1 and IL6 and the generation of the matrix degrading enzymes MMP-1, -2, -3 and -9 (Monaco et al., 2009). This finding suggests that TLR2 signalling may promote plaque vulnerability and rupture. The same study found that TLR4 and its adaptor protein TRAM were not rate-limiting for cytokine production in human atherosclerosis but may have a role in MMP-1 and -3 production (Monaco et al., 2009).

6.2 Involvement of TLRs in lipid-associated signalling

Foam cells are a hallmark feature of atherosclerotic lesions. TLR2, TLR4 and TLR9 ligation on macrophages promotes lipid uptake and foam cell formation (Funk et al., 1993; Kim et al., 2009; Lee et al., 2008; Oiknine & Aviram, 1992). Whilst TLR4-dependent fluid phase uptake (macropinocytosis) of lipids occurs in differentiated macrophages (Choi et al., 2009), TLRs also promote macrophage lipid uptake indirectly. In response to TLR3, TLR4 and TLR9 activation, macrophage expression of the scavenger receptors SRA, macrophage receptor with collagenous structure (MARCO) and lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) is increased (Doyle et al., 2004; Lee et al., 2008). Similarly TLR2, TLR3 and TLR4 ligation induces expression of fatty acid binding proteins such as aP2 and Mal1 in murine but not human macrophages (Feingold et al., 2010; Kazemi et al., 2005). Lipid-X receptors (LXRs) regulate expression of genes including ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1), which are involved in cholesterol efflux. Activation of TLR3 and TLR4, via signalling pathways involving IRF3, leads to attenuated expression of ABCA1 and ABCG1 through inhibition of LXR transcriptional activity (Castrillo et al., 2003). Furthermore, in a recent study, low-grade endotoxemia *in vivo* inhibited reverse cholesterol transport in mice and also impaired cholesterol efflux in *ex vivo* cultured human macrophages (McGillicuddy et al., 2009). Thus TLR signalling can both promote lipid uptake and disrupt cholesterol efflux therefore promoting foam cell formation and atherosclerotic lesion development.

6.3 The role of NLR and RIG-I in atherosclerosis

The role of NLR in the development of atherosclerosis is emerging. The NLRP3 inflammasome, is the best characterised NLR thus far in atherosclerosis. Neointima formation is reduced in ASC-/- mice compared to control mice following wire-injury of the femoral artery (Yajima et al., 2008). In addition, neointima in ASC-/- mice exhibited attenuated IL1β and IL18 expression. BMT experiments revealed hematopoietic cell ASC expression is important for neointima formation in this model (Yajima et al., 2008). Cholesterol crystals, previously thought to be present only in advanced atherosclerotic lesions have been shown to be present in lesions of ApoE-/- mice as soon as 2 weeks after the initiation of high-fat feeding (Duewell et al., 2010). Cholesterol crystals can activate the NLRP3 inflammasome in human and murine macrophages leading to caspase-1 cleavage and IL1β release suggesting that these crystals may be endogenous danger signals in atherosclerosis (Duewell et al., 2010; Rajamaki et al., 2010). Bone marrow transfer of hematopoietic cells from mice lacking NLRP3, ASC or IL1α/β into LDLR-/- mice leads to attenuated lesion formation and reduced serum IL18 levels compared to mice receiving

bone marrow cells from wild-type mice (Duewell et al., 2010). However more recently, Menu *et al* have crossed ApoE-/- mice with NLRP3-/-, ASC-/- and caspase1-/- mice to create double knockout mice (Menu et al., 2011). Suprisingly, deletion of these 3 key components of the NLRP3 inflammasome in ApoE-/- mice did not greatly affect atherosclerotic lesion development, macrophage recruitment nor lesion stability suggesting that these molecules do not affect atherosclerosis (Menu et al., 2011). The use of different murine models may explain the differences between these findings and those of Duewell and colleagues. The role of other NLRs and RIG-I in atherosclerosis remains to be examined.

7. The therapeutic potential of PRRs in treating atherosclerosis

Atherosclerosis, the leading cause of coronary artery and cerebrovascular disease, which together comprise the leading causes of death worldwide (Lopez et al., 2006), are a significant social and economic burden. Thus, there is a pressing need to identify new molecular targets and develop novel therapeutics for the treatment of atherosclerosis. Since PRRs are key players at all stages of atherosclerotic lesion development, targeting PRRs is an exciting prospect for the treatment of cardiovascular disease.

TLR2 and TLR4 are the best-characterised PRRs in atherosclerosis. Both receptors have been ascribed pro-atherogenic roles and thus inhibition of these receptors is currently the most appealing prospect for generation of PRR therapeutics. Reduction of protein expression of TLR2 and TLR4 in murine and human cells has been achieved using angiotensin II blockade, statin and insulin treatment (Ahn et al., 2007; Foldes et al., 2008; Ghanim et al., 2008). Whether such reductions translate to inhibition of functional responses and whether this inhibition is achievable in patients with cardiovascular disease remains to be determined. TLR2 blockade can inhibit cytokine, chemokine and MMP production in human atherosclerosis, while disruption of TLR4 signalling had little effect on the same outcomes (Monaco et al., 2009). Blockade of TLR2 is also beneficial in a murine model of myocardial ischemia/reperfusion injury (Arslan et al., 2010). TLR4 antagonists such as Eritoran are in development for immune disorders (reviewed in (Hennessy et al., 2010)) and may be beneficial in the treatment of cardiovascular disease. However, caution is needed when extrapolating murine data into human targets and therapeutics. Deletion of IL-1, TLR-2 and TLR-4 is equally effective in murine models of atherosclerosis, however only TLR-2 has a predominant role in human disease (Monaco et al., 2009). In addition to developing antagonists of pro-atherogenic PRRs, it is important to consider generating PRR agonists to target athero-protective PRRs such as TLR3.

Before the most effective PRR therapeutics can be generated, it will be important to discern the precise pattern of PRR expression and the consequence of PRR signalling on all cell types in the vessel wall in both health and disease. As lesions develop, the composition of atherosclerotic plaques change and thus different PRRs and cell types may need to be targeted at different time-points during disease progression.

8. Concluding remarks

Evidence supporting a key role for PRRs in the initiation and development of atherosclerosis is growing and yet the full contribution of PRR activation and signalling to atherogenesis is only just emerging. Prominent pro-atherogenic roles have been assigned to TLR2 and TLR4 as these receptors have been shown to promote foam cell formation, macrophage

recruitment and cytokine/MMP production – all key components of atherosclerotic plaque development. However, recent data has shown TLR3 to be atheroprotective. Furthermore, the roles and functions of the other PRRs including NODs and RIG-I remain to be explored. Given the complex consequences of TLR activation, further studies are required to fully elucidate the expression patterns, ligands (both endogenous and exogenous), signalling pathways and functions of PRRs in both health and at all stages of disease development. With increased knowledge, it may then be possible to design novel therapeutics targeting PRRs for the treatment of cardiovascular disease.

9. References

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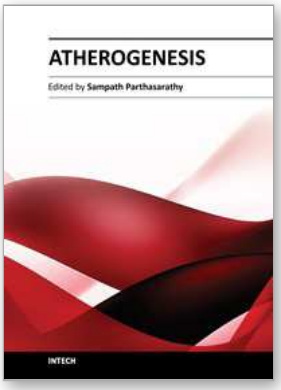

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This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

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