ABSTRACT

Toll-like receptors (TLR) are receptors with major role in activation of immune system by regulating production of chemokines and cytokines, which makes them important in different types of inflammatory reactions - bacterial, viral, parasitic, acute, chronic etc. Having in mind that atherosclerosis is a chronic inflammatory disease, it is clear then that TLRs are a group of immune system activators, producing specific immune cells. In human atherosclerotic plaque there is a markedly enhanced expression of TLR1, TLR2, and TLR4. TLRs are expressed in adrenal cells, and TLR agonists stimulate the release of steroids from human adrenal gland, as well. TLR2 deficient mice have an impaired steroid release during endotoxemia. TLR9 stimulation leads to a corticosterone and inflammatory cytokine response. The best characterized of all is TLR4. Up to date this TLR has a major role in the development of atherosclerosis. Enhanced expression of hTLR4 (human TLR4) in patients with ACS (acute coronary syndrome) was associated with elevations of IL-12 and B7-1 expression, as typical downstream effects of TLR4 activation. It is known that a certain gene polymorphism of TLR4 can slow progression of the disease. Over expression of TLR2 in mice facilitates ventricular remodeling after myocardial infarction. The CAPS study found contrary results, when in 3000 patients no connection had been found between TLR2 polymorphism (Arg 753 Gln, -16934A/T) and TLR4 polymorphism (D299G, T399I) and the process of atherosclerosis.

Key words: atherosclerosis, inflammation, toll-like receptors, gene polymorphism
**INTRODUCTION**

**TLR receptors**

Toll like receptors (TLR) are receptors with major role in activation of immune system by regulating production of chemokines and cytokines, which makes them, important in different types of inflammatory reactions- bacterial, viral, parasitic, acute, chronic etc. (1).

Having in mind that atherosclerosis is a chronic inflammatory disease, it is clear then that TLRs are a group of signal receptors that carry out an important role in progression of atherosclerosis, as well (2,3).

**Development of atherosclerosis**

The first step of atherosclerosis is endothelial denudation and consequently loss of normal endothelial functions, which are control of vascular tonus, anticoagulation characteristics of intimal layer and resistance to inflammatory mediators. Then the next step is a formation of atherosclerotic plaque by lipid accumulation in SMC (smooth muscle cells) and macrophages which is covered by fibrogenous cap. The final step is the rupture of atherosclerotic plaque with stenosis or obstruction of the arterial vessel mediated by thrombosis and loss of vascularisation with consequential ischemia of the involved region (4).

The specific etiological agent which causes the disease has not been identified yet, but what we do know is that mechanical denudation during hypertension, chemical reactions due to mechanical denudation, as well as metabolic disorders in diabetes mellitus, genetic disorders, elevated level of homocystein, infectious agents such as *Chlamydia pneumoniae*, herpes viruses, *Helicobacter pylori* (5), formation of free radicals by smoking cigarettes, and elevated levels of lipids in obesity can mediate the endothelial dysfunction and initiate the process (4). Following the loss of normal functions of endothelial layer, there is an increase in adhesiveness of the endothelium by augmented expression of adhesive molecules such as VCAM-1, resulting in higher adhesiveness of monocytes. At the same time, LDL penetrate the subendothelium through untouched endothelium after being oxidized by lipid peroxidase of endothelium and free radicals, released by denudation of endothelium as well as by macrophages and SMC (6).

![Figure 1. Function of Toll-like receptors in atherosclerosis (A. Džumhur, 2008.)](image_url)
Formation of oxidized LDL is an important moment in progression of the process because oxidized LDL is a molecule with powerful chemotactic function for monocytes, it is more cytotoxic than a non-oxidized one, and much more immunogenic. Oxidized LDL stimulates endothelium cells to produce chemokines (MCP-1) and cytokines (M-CSF), which lead to further accumulation of monocytes and their maturation into macrophages (6).

Oxidized LDL enters the macrophages and SMC through special receptors-scavengers (6). As oxidized LDL does not have regulatory mechanisms, macrophages become overfilled with oxidized LDL, resembling foam, hence they are called foam cells (6). Foam cells release cytokines such as PDGF (facilitate accumulation of LDL into SMC and then they become foam cells, too), IL-1, TNF-α and -β etc (6). Numerous T cells in subendothelium also release cytokines (6). Cytokines influence endothelium cells to release adhesive molecules, which attract much more monocytes, lymphocytes and to facilitate migration and proliferation of SMC (6).

Fatty streak is the formation of intima wall of arterial vessel which is consisted of foam cells. It is reversible state in which foam cells can disappear by acting of HDL (natural scavenger) (6).

Following the foam cells apoptosis (because there are no regulatory mechanisms), lots of free oxidized LDL molecules are released, which is toxic for endothelium cells (6). These free molecules from foam cells attract fibroblasts and can facilitate migration and proliferation of SMC in subendothelium, too (6). Cholesterol from LDL facilitates synthesis of collagen, elastine and mucopolysacharidase that format fibrous cap of the atherosclerotide plaque. The plaque thin circulation area of the vessel and production of NO in endothelium cells is lower, resulting in a spasm, which further disrupts circulation and causes hypoxia of the tissue (6).

It is not the size of atherosclerotic plaque that is important, but its consistency, because rupture prone plaque have a large lipid core, low smooth muscle cells density, high macrophage density, and thin fibrous cap (7,8).

The role of immune system in atherosclerosis

We can say that the activation of immune system happens in order to resolve the denudation of endothelium. If this mechanism is not sufficient enough, chronic immune reaction and formation of atherosclerotic plaque which develop consequentially can then be used as a marker of inflammatory reaction (9).

Immune system is considered of innate and acquired (adaptive) immunity. Innate immunity is phylogenetically older. It reacts with highly specific molecules of microorganisms and multicellular organisms like lipopolysaccharides, peptoglycans, lipoteichoic acids, mannans etc. which can not be the part of host organism, so there is no possibility for a change with host molecules (10). We can say that these structures are evolutionary highly conserved molecules. There is genetically predetermination of receptors for these structures. So, logically, innate system is also called non-specific.

On the other hand, adaptive immune system, also called specific immune system, reacts through lymphocytes T and B, which are unique, structured for every specific antigen. This specific formation of lymphocytes T and B is not encoded in germ line. Rather, an extremely diverse repertoire of receptors is generated randomly, and receptors specific for pathogens are subsequently selected.
for clonal expansion by encountering the antigens for which they happen to be specific. During this selection, crossing with host antigens is possible, and, as a consequence of it, an occurrence of an autoimmune reaction or disease also (10).

Atherosclerotic lesions are enriched with macrophages and T cells that play important roles in an early (innate) and advanced (acquired) immune responses. Progression of atherosclerosis has been associated with clonal expansion of differentiated T cells, a feature common to all adaptive immune responses (11).

The way of reacting of innate immune system is by antimicrobial peptides, phagocytes, polymorphonuclear cells, complement, dendritic cells etc. This reaction happens immediately after exposure, in a few hours, whereas the adaptive system needs some time for production of lymphocytes, up to 4 to 10 days after the first exposure. Rechallenging after priming will shorten this reaction, due to the existence of memory T cells (11).

If activation of both parts of immune system, innate and then adaptive is inefficient, a state called chronic immune reaction occurs as a final attempt to resolve the disruption of the tissue. The structures, highly specific for foreign molecules (which activate the innate immune system) are also called PAMP (pathogen-associated molecular patterns), and receptors which recognize PAMPs are called PRR (pattern recognition receptors) (10). There are a few groups of PRR: secreted – which function as opsonins; endocytic – which mediate the uptake and delivery into lysosomes in phagocytes; and signaling receptors – which regulate production of cytokines and chemokines (10).

Signaling receptors are TLR and NOD (nucleotide-binding oligomerization domain). Both have LRR (leucine reach reps). TLR recognize extracellular, and NOD intracellular structures. TLR are expressed on effector cells of the innate immune system, macrophages, dendritic cells, antigen-presenting cells, and NOD receptors in the cells where TLR are absent or expressed at low levels, like endothelium cells. So, the function of NOD receptors is to recognize microbial structures that entered the cell. The aim of both groups of signaling receptors are to induct NF-κB and by doing so, to induct transcription of pro-inflammatory genes (12).

Atherosclerosis is a chronic immune disease, and there is a strong activation of immune cells, so the major role in production of cytokines in atherosclerosis is by TLRs (11).

FUNCTION OF TLR IN ATHEROSCLEROSIS

To date, there are 13 different types of TLRs, identified both in humans and in mice. Both humans and mice have TLR1-9 (12). TLR 10 is found just in humans and TLR 11 just in mice (12). Pathogen ligands (PAMPs) and functions of TLRs 10-13 are less well understood (Figure 1) (12).

Toll like receptors (TLR) are the transmembranic receptors that can be situated on the surface, but also inside the cells, on the membrane of endoplasmatic reticulum. All of them have a big extracellular domain which is consisted of LRR and intracellular domain which is highly homologue to IL-1 receptors, and is therefore called TIR (Toll-IL-1 receptor) (12). It is assumed that TLRs function as dimers, mostly as homodimers, but some of them form heterodimers like TLR2 (which forms dimers with TLR1 or TLR6.) Most data report on TLR4, which is the first described TLR, and up to now, we know it functions as homodimer (12). CD14 and LBP facilitate presentation LPS to MD-2, and that cause full sensibility of TLR4 for recognizing LPS. There are two signaling pathways mediated via My88 or TRIF adaptor proteins (13-15). My88-dependent signaling pathway is common to all TLRs, except TLR3. Mal (it is also called TIRAP) is anchor protein between TIR and My88. These adaptor proteins activate other molecules, like some kinases, IRAK1 (IL-1R-associated kinase 1), IRAK4, TIK1 and IKK, which augment signal and lead to induction or suppression certain genes (12). My-88-independent signaling pathway is via TRIF and it is utilized by TLR4 and TLR3,
and TRAM seems to be a link between TIR and TRIF. TNFα is a My88–dependent and interferon is classified as My88–independent, and therefore TRIF dependent (16).

TLR3, TLR7 and TLR8 are intracellular receptors activated by viral RNA; TLR5 is a cell surface receptor activated by bacterial flagellin (17,18). TLR1 is also cell surface receptor activated by lipoprotein (19). TLR9 is intracellular receptor, activated by unmethylated CpG dinucleotides in bacteria (mammalian DNA in methylated). TLR2 is sensed by LTA from gram-positive bacteria, and TLR4 is sensed via LPS from gram-negative bacteria (12).

In human atherosclerotic plaque there is markedly enhanced expression of TLR1, TLR2, and TLR4 (2). There are very few facts about involvement other TLRs in atherosclerosis. What we do know is that TLR2 and TLR4 have an important role in the adrenal stress response, too. TLRs are expressed in adrenal cells, and TLR agonists stimulate the release of steroids from human adrenal gland (20). TLR2 deficient mice have an impaired steroid release during endotoxemia (3). TLR9 has important role in production of corticosteroids in adrenal gland, in other words TLR9 has major role in regulation of hypothalamic-pituitary-adrenal axis during bacterial infection and enhanced expression of TNF-α, IL-1β, -6, -10, -12, both in vivo and in vitro (21).

An important role of infectious, but also noninfectious agents - endogenous ligands like HSP, products of oxidative stress (reactins, oxygen radicals) in development of atherosclerosis has already been explained. (22). There is a possible connection between exogenous signals and progression of atherosclerosis- there are hypothesis about mild endotoxemia (level of endotoxine beyond 50pg/mL) influencing the development of the disease. Gram-negative bacteria, which, colonize gastrointestinal, genitourinary and respiratory tract can lead to subclinical infection (bacteremia, activation of immune system, but no signs of illness) like periodontitis, sinusitis, bronchitis, diverticulitis etc. (23). Brunick study showed that subclinical infection is a strong risk factor for the development of atherosclerosis, mostly in cigarette smokers, both current and ex-smokers (23). The risk caused by high endotoxin (ETX) levels was mainly confined to levels beyond 50 pg/mL and applied to subjects with chronic infection and cigarette smoking. The findings fit well into the concept that it is not smoking itself (exposure to nicotine and other smoke ingredients) but chronic bronchial infections and/or bacterial colonization that represent the actual atherogenic culprit (23).

Beside that, endotoxemia can cause lowering of HDL (which is a natural scavenger of endotoxins (24). It can also induce formation of foam cells by lipid accumulation and proteins involved in atherosclerotic plaque development such as adipocyte fatty acid-binding protein (aP2) in macrophages (25); minimally oxidized LDL will trigger a TLR4-dependent pro-inflammatory response in macrophages (26).

On the other hand, there are number of endogenous anti-inflammatory mediators such as IL10, TGF-beta, CD200 (which send „stop signal“ to macrophages suppressing production of pro-inflammatory mediators) (27) and lipid mediators (lipoxin and cyclopentenone prostaglandins) which carry out an important role in negative regulation of inflammatory process. Gene disruption and pharmacological inhibition can abrupt this process.

TLRs are capable of inducing expression of these anti-inflammatory mediators, but recent studies have shown that induction of TRAF3, and in the absence of it, expression of the TRAF6-dependent, pro-inflammatory cytokines IL6 and IL12 are dramatically up-regulated (28) and that this is critical for induction of IL10 expression. Any aberrations in these negative regulatory pathways can lead to chronic inflammation (4).

Endogenous (host-derived) ligands for TLRs have also been identified. Heat-shock protein 70 (HSP70), described as a TLR4 agonist (29) and chromatin component (HMG-B1), which appears to activate macrophages through the receptor for advanced glycation products (RAGE) (30), are released in the context of tissue injury and necro-
sis. This observation suggests that even in the absence of infection, TLRs and other PRRs may play a role in inflammation and immune homeostasis through recognition of endogenous ligands, which are presented as a result of tissue damage. Confirming this theory, it is observed that TLR4-deficient mice are resistant to development of diet-induced atherosclerosis, a chronic inflammatory disease of the vasculature (31).

After ischaemia there is an increased expression of TLR2 in both medial layer and adventitia (32). TLR9 stimulation leads to a corticosterone and inflammatory cytokine response (21).

The best characterized of all is TLR4. Up to day this TLR has major role in development of atherosclerosis. Several exogenous and endogenous ligands for TLR4 such as lipopolysaccharide (LPS), fibrinogen, minimally modified LDL and heat shock proteins (HSP) 60 have been identified. Immunohistochemical staining of murine and human atherosclerotic tissue revealed prominent expression of TLR4, especially, at the lipid-rich, macrophage-infiltrated shoulder region of plaques (33). Enhanced expression of hTLR4 (human TLR4) in patients with ACS (acute coronary syndrome) was associated with elevations of IL-12 and B7-1 expression, as typical downstream effects of TLR4 activation.

In concert with antigen presentation it has been shown to activate T cells and drive their differentiation into T-helper 1 cells (34) for which role is increasing plaque instability (35).

Furthermore, upregulation of hTLR4 expression in patients with ACS was accompanied by enhanced MyD88 transcript levels in circulating monocytes. My88 is a subject of inflammatory stimuli in monocytes. If they have only My88-deficiency but not CD14-deficiency, mice show a marked reduction in early stages of atherosclerosis (36).

It is known that a certain gene polymorphism of TLR4 can slow progression of the disease. A mutation of TLR4 (Asp299Gly and Thr399Ile) results in weaker reaction to LPS, so lower activation of immune system. That mutation is less present in patients with ACS, and more in old, healthy people (37). It is also associated with reduced risk of acute coronary events, independent of standard coronary risk factor. Plasma fibrinogen and soluble VCAM-1 levels were also lower in Gly299 heterozygotes. On the contrary, other studies did not find a connection between that polymorphism and atherosclerosis (38,39). TLR4 deficient mice had smaller myocardium infarction size after one-hour ligation of coronary artery and less lipid peroxides and complement deposition compared to controls (40).

In a recent study conducted on Chinese population another TLR4 polymorphism C119A (11,7%) was found, which was more present in people who had ischaemic stroke. In that population, there was no Asp299Gly mutation (41). Double negative (both for apolipoprotein E and TLR4) mice had lower risk for developing atherosclerosis (28%) compared to mice which were deficient just for apoE, (31).

Based on this evidence, we can postulate that this mutation is present in very low percent, and therefore the conclusions are still debatable.

The second important TLR in atherosclerosis is TLR2, a transmembranic receptor with LRR extracellulary and TIR intracellulary. Over-expression of TLR2 in mice facilitates ventricular remodeling after myocardial infarction (32). Certain gene polymorphism of TLR2 play a significant/substantial role in coronary disease-for example, a gene polymorphism for Arg753Gln SNP, which results in non-functional receptor, is elevated in patients with restenosis after PTCA and stent implantation. At the same time, a common TLR4 SNP was similarly distributed among patients, and we can say that functional TLR2 is protective preventing restenosis (42). The CAPS study found contrary results, when in 3000 patients no connection had been found between TLR2 polymorphism (Arg 753 Gln,-16934-A/T) and TLR4 polymorphism (D299G, T399I) and the process of atherosclerosis (43).

Finally, we can conclude that TLRs are important in developing atherosclerosis. Two most important ones among these receptors are TLR2
and TLR4. To date, they remain best characterized and their role in this disease is best described and explained. What remains uncertain is the role of other TLRs?

It is known that certain TLRs are important in viral infectious (for example TLR3, TLR7, TLR8), and we know that viruses play an important role in the development of atherosclerosis (viruses were isolated from the plaques and elevated levels of antibodies were connected with progression of the disease). On the other hand, activation of stress mechanisms during any kind of stress (infectious or non-infectious cause) is also mediated by TLRs (TLR2, TLR4 and TLR9). Hence, we can assume (judging by these mechanisms) that certain TLRs are involved in progression of the atherosclerosis as well (particularly TLR9) (Figure 2). The gene polymorphism should help us understand these mechanisms better, but there is still a big problem with analyzing and interpreting this data due to low percentages of representation of certain gene polymorphisms, and big differences in sort and percent of these polymorphisms between nations. Namely, these differences tell us how groups of people (nations) developed protection which helped them survive, and how this protection is connected with their way of life, their diet (animal or plant food, food with low or lot fats), climate conditions etc.

In conclusion, we may say that this field has opened an entirely new angle on understanding atherosclerosis, and it will be interesting to see where it is going to take us.

REFERENCES


