Toll like receptors (TLR) in autoimmune disease and atherosclerosis

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UNIVERSITY OF ZAGREB
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Toll-Like Receptors (TLR) in Autoimmune Diseases and Atherosclerosis

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Abbreviations

ANA-antinuclear antibody

APC-antigen presenting cell

ApoE-apolipoprotein E

CLRs-C-type lectin receptors

HSP-heat shock protein

IKK- IκB kinase

IRAK-interleukin Receptor-Associated Kinase

LPS-lipopolysaccharide

LRRs-leucine rich repeats

NFκB- nuclear factor kappa-light-chain-enhancer of activated B cells

NLRs-NOD-like receptors

ODNs- oligodeoxynucleotides

PAMP-pathogen associated molecular patterns

pDC- Plasmacytoid dendritic cell

PMBC- peripheral blood mononuclear cell

PRR-pattern recognition receptor

pSS- Primary Sjögren's syndrome

RLRs-cytosolic receptors consisting of RIG-I-like receptors

SGECs-salivary gland epithelial cells

SLE-systemic lupus erythematosus

SSc-systemic sclerosis

TAK1- Transforming growth factor beta-activated kinase 1

TIRAP/MAL- TIR domain containing adaptor protain/MyD88 adaptor like protein

TIR-Toll/IL-1 receptor

TLR-toll-like receptor

TRAM-TRIF related adaptor molecule

TRIF-TIR domain containing adaptor inducing IFN-β

CONTENTS:

1. Summary

2. Introduction 1

3. Innate immunity 3

4. Pattern Recognition Receptors 4

5. Toll-like receptors 5

6. TLR 2, 4, 7, 8, 9 7

7. TLR-dependent signalling cascades 8

8. Atherosclerosis 11

9. TLRs and Atherosclerosis 12

10. Autoimmune diseases 15

11. TLRs and Autoimmune disease 16

12. Conclusion 21

13. Acknowledgements 22

14. References 23

15. Biography 33

1. Summary

Chronic inflammation and/or autoimmune responses have been associated with a number of diseases including atherosclerosis, scleroderma, Sjogren's syndrome, systemic lupus erythematosus (SLE) and others. Toll-like receptors (TLRs) are important receptors that recognize both exogenous and endogenous ligands and initiate signaling cascades, involving numerous adaptors, kinases and transcription factors, which ultimately lead to enhanced gene expression of cytokines and other factors associated with chronic inflammation or autoimmune reactions. TLRs represent a family of membrane bound receptors that are part of the larger pattern recognition receptor (PRR) family. TLRs play a role in both innate and adaptive immunity. As such TLRs are central to the processes of chronic inflammation and autoimmunity. In this review I discuss the molecular aspects of TLR signalling and how TLRs are involved in the pathogenesis of atherosclerosis and autoimmune diseases.

Key words: toll-like receptors, atherosclerosis, autoimmune disease, immunity

2. Introduction

Normal cellular physiology involves a complex network of molecular processes that involve signaling pathways and nuclear responses that maintain cellular homeostasis, respond to external stimuli and generally maintain overall equilibrium of the body state. Disease states are ultimately a reflection of dysfunctional or aberrant cellular physiology. The immune system is one of the most complex biological systems in the body. The immune system comprises two separate yet interconnected systems, 1) the innate immune response and 2) the adaptive immune response.

The innate immune response is found in nearly all forms of life including both plants and animals (Litman GW, 2005), however the adaptive immune response is specific to vertebrates (Mayer, 2006). The two types of immunity serve to protect the body from external threats such as microbial infection, viral infection and various other pathogens. The innate immune response provides an immediate but non-specific defense against infection, while the adaptive immune response leads to pathogen and antigen specific responses. If an infection is unsuccessfully eliminated by the innate immune response then the innate system can signal to the adaptive immune response as a subsequent line of defense. An important difference between the two systems is that the adaptive response has immunological memory, that is, it can mount a faster and stronger response upon subsequent infection to an antigen that has previously been encountered. Additionally, the adaptive immune response is antigen specific and is able to distinguish between self and non-self antigens. Adaptive immunity is mediated by multiple cell types including T-cells, B-cells, NK cells and gamma delta T cells. There are multiple disorders associated with dysfunctional or aberrantly functional immune systems including immunodeficient diseases, autoimmunity and cancer.

Cardiovascular diseases such as coronary heart disease and stroke are the number one cause of death worldwide (World Health Organization, 2012). Atherosclerosis is a complex inflammatory disease and is the main driver of coronary artery and cerebrovascular disease. Atherosclerosis is now known to be a disease associated with chronic inflammation driven by the innate immune system Monocytes and macrophages, cellular components of innate immunity, play a major role in atherosclerosis (Seneviratne, Sivagurunathan, & Monaco, 2011).

Autoimmune diseases include a wide range of diseases in which the adaptive immune system recognizes self-antigens and attacks normal cells and tissues. About 78% of autoimmune diseases affect women and the underlying reason for this gender specificity is unclear (DeLisa Fairweather, 2008) . Diseases with this underlying mechanism include systemic lupus erythromatosus (SLE), type I diabetes, scleroderma, and Sjogren's syndrome among others. In the case of SLE for example, patients present with a variety of symptoms as the disease can affect multiple targets including skin, joints, kidneys lungs, CNS, vascular systems and other organs. Severe renal disease and accelerated atherosclerosis are the main drivers of morbidity and mortality in SLE.

3. Innate Immunity

The innate immune system is considered the primary mechanism of defense against infections (Parham, 2009) .The innate immune system is responsible for the recognition of the existence and type of infection, as well as the regulation and choice of effector mechanisms in the adaptive immune response (Medzhitov R. , 2001). The response of innate immunity is mediated by different cells, including phagocytes, macrophages, dendritic cells (DCs) and antigen presenting cells (Mogensen T. H., 2009). In order to maintain homeostasis, the innate immune system will supervise/inspect for any exogenous pathogens or destruction through their pattern recognition receptors (PRR) (Frantz, Ertl, & Bauersachs, 2007)

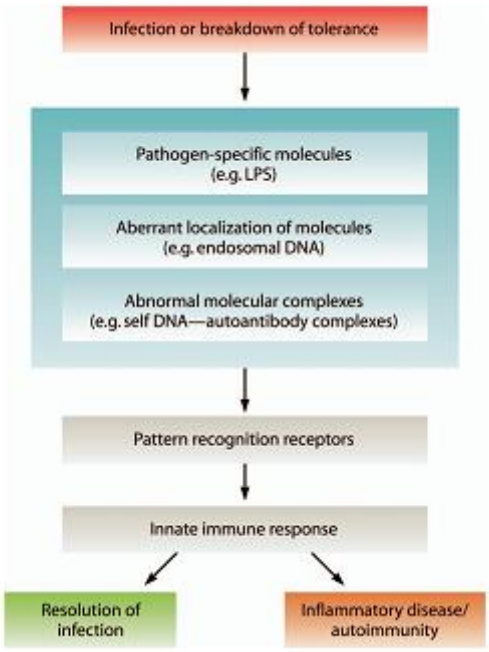


Figure 1. Detection of pathogen-specific molecules by the PRRs. Once pathogen specific molecules are detected and recognized, an immune response will be activated. The end result is either resolution of the infection or progression to inflammatory disease/autoimmunity (Mogensen T. H., 2009)

4. Pattern Recognition Receptors

Pathogen components; lipopolysaccharide (LPS), endotoxins, glycans are some of the archetypal pathogen associated molecular patterns (PAMPs) that are recognized by the pattern recognition receptors (PRRs). PRRs are located both on the cell surface and in the cytoplasm where they detect PAMPs, such as lipopolysaccharide (LPS), released from Gram-negative bacteria or viral RNA (Newton & Dixit, 2012). PRRs are expressed on a variety of cell types including among others macrophages, dendritic cells and B cells and are independent of immunological memory (Shoenfeld, 2004). Each specific type of PRR reacts with a unique PAMP, displays a specific expression pattern, activates a specific signaling pathway and results in an unequivocal anti-pathogen response (Akira, Uemtasu, & Takeuchi, 2006) Once these PAMPs are recognized, PRRs signalize to the host the presence of infection and provoke an antimicrobial response by way of activating a number of intracellular signaling pathways, involving a variety of adaptor molecules, kinases and transcription factors (Akira & Takeda, Toll-like receptor signalling, 2004). PRR-induced signal transduction pathways result in the activation of gene transcription and synthesis of immune response molecules, including cytokines, chemokines, cell adhesion molecules, and immunoreceptors (Akira, Uemtasu, & Takeuchi, 2006). Together they form an early reposnse to infection along with forming a link to the adaptive immune response. There are two broad groups of PRRs; cytosolic receptors consisting of RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs); as well as transmembrane receptors that are the C-type lectin receptors (CLRs) and Toll-like receptors (TLRs) (Takeuchi O, 2010).

5. Toll-Like Receptors

TLRs are a family of receptors and each receptor is functionally specific for a different set of microbial products. TLRs are expressed on different cell types, such as macrophages, mast cells, B cell, and dendritic cells among others. Toll receptors were first identified as important for innate immunity in the fruit fly Drosophila (Takeda, Kaisho, & Akira, 2003). TLRs are the most extensively studied of the receptors belonging to the PRR class. TLRs belong to the type 1 transmembrane protein class comprised of a transmembrane domain that determines cellular localisation, and an intracellular toll-interleukin 1 receptor (TIR) domain essential for downstream signalling (Seneviratne, Sivagurunathan, & Monaco, 2011) The TLRs exhibit a trimodular structure (Kumar, Kawai, & Akira, 2009) consisting of an extracellular N-terminal domain and a intra-cellular C-terminal region; The N-terminal is made of roughly 16-28 leucine rich repeats (LRRs) and it recognizes PAMPs (Kumar, Kawai, & Akira, 2009), while the C-terminal domain, also called Toll/IL-1 receptor (TIR) is analogous to the cytoplasmic region of IL-1R (Janeway Jr & Medzhitov, 2002., Akira, Uemtasu, & Takeuchi, 2006., Medzhitov R. , 2007., Beutler, 2009). Out of the 13 known TLRs, 10 have been identified in humans and 13 in mice (Akira, Uemtasu, & Takeuchi, 2006). Each TLR can respond to either exogenous ligands and potentially endogneous ligands to elicit a downstream signalling cascade as outlined in Table 1.

Table 1. Human and mouse TLRs with their respective endogenous and exogenous ligands. (Mingcai Li, 2009)

TLRs	Exogenous ligands	Potential endogenous ligands
TLR1	Tri-acyl lipopeptide(bacteria and mycobacteria)	
TLR2	PGN, Lipoteichoic acid(Gram-positive bacteria)	Heat shock proteins
	Lipoarabinomannan(Mycobacteria)	HMGB1
	Glycoinositolphospholipids (Trypanosomes)	Biglycan
	Glycolipids(Treponema)	EDN
	Porins(Neisseria)	
	Zymogen (fungi)	
	Atypical LPS(Gram-negative bacteria)	
	Lipoprotein/Lipopeptides	
TLR3	Double-stranded RNA(Virus)	mRMA
TLR4	LPS(Gram-negative bacteria)	HMGB1
	Taxol(Plant)	Heat shock proteins
	Fusion protein(RSV)	Fibronectin extra domain A
	Envelope proteins(MMTV)	Minimally modified-low-density lipoprotein
	Hsp60(Chlamydia pneumoniae)	Hyaluronan fragments
	Hyphae(Aspergillus)	Heparan sulfate
	Pertussis toxin	Fibrinogen
		Lung surfactant protein A
TLR5	Flagellin(Bacteria)	
TLR6	Di-acyl lipopeptides(Mycoplasma)	
TLR7	Imidazoquinoline, Loxoribine, Bropirimine(Synthetic compounds)	U1snRNP RNA
	Single-stranded RNA(Virus)	
TLR8	Imidazoquinoline(Synthetic compounds)	
	Single-stranded RNA(Virus)	
TLR9	CpG ODN (Bacteria and viruses)	Hypomethylated CpG-DNA
	Pigment hemozoin(Malaria)	
TLR10	N.D.	
TLR11	Profilin-like protein(Toxoplasma gondi)	
TLR12, TLR13	N.D.	

A division can be made based on the location of the TLRs; one group are the cell membrane TLRs (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) while the other group, which are nucleic-acid sensing; (TLR3, TLR7, TLR8 and TLR 13), are located in intracellular vesicles such as the endoplasmic reticulum lysosomes and endosomes (Cole, Mitra, & Monaco, 2010) (Kawai & Akira, 2010). The subfamilies of TLRs are related to the PAMPs they recognize; lipids are recognized by the subfamily comprised of TLR1, TLR2 and TLR6, while nucleic acids are recognized by the group of TLR7, TLR8 and TLR9. TLR gene expression is

influenced by pathogen response, a vast array of cytokines and environemntal stressors (Akira, Uemtasu, & Takeuchi, 2006).

6. TLR 2, 4, 7, 8, 9

TLR 2 and 4 are located on the cell surface and recognize a large number of ligands; TLR-2 is involved in peptidoglycan and lipoarabinomannan recognition as TLR2- deficient mice are unable to recognize these molecules (Takeuchi, et al., 1999., Takeuchi, et al., 2000). TLR-2 ligand recognition also requires related TLR family members TLR-1 and TLR-6 (Toubi & Shoenfeld, 2004). TLR4 was the first identified human TLR. TLR4 has been implicated in the recognition of viral proteins, which leads to the production of a wide range of cytokines and chemokines (Mogensen & Paludan, 2005., Shimazu, Akashi, Ogata, & a.l, 1999., Kurt-Jones, Popova, Kwinn, & al., 2000). Multiple TLR4 ligands have been identified (Table 1) and some have been proposed to function as the ligand for TLR4 in the process of atherosclerosis (Den Dekker WK, 2010). One example of a TLR4 ligand is fibronectin, a high-molecular weight extracellular matrix glycoprotein. Immuno-stimulatory activities similar to those provoked by LPS have been described for the type III repeat extra domain A of fibronectin. TLR4 signaling is activated in response to its recognition of the extra domain A of fibronectin (Okamura, Watari, Jerud, Young, & Ishizaka, 2001). Several studies have shown that TLR-2 and TLR-4 can recognize a number of self-proteins, including members of the heat shock protein (HSP) family. For example, HSP60 and HSP70 which are normally intracellular proteins, can also function as "danger" molecules when released under stress or following extensive apoptosis. These HSPs utilize the TLR-2 and TLR-4 signaling pathways to activate vascular smooth muscle cells and macrophages which leads to secretion of pro-inflammatory cytokines such as TNF-a and IL-12, and over-expression of co-stimulatory molecules on APCs (Toubi & Shoenfeld, 2004). The TLR9 subfamily including TLR7, TLR8 and TLR9 is located in the cytoplasmic compartments of the endoplasmic reticulum and endosome and they are involved

in the discrimination of nucleic acid like structures in microorganisms (Hurst & von Landenberg, 2008). TLR7 and/or TLR8 are able to recognize singlestranded (ss) guanosine and/or uridine rich RNA and ssRNA viruses. In addition, both receptors can recognize synthetic antiviral nucleoside analogs such as imidazoquinolines or loxoribine (Hurst & von Landenberg, 2008). TLR7 and TLR8 are highly homologous to TLR9 and while putative natural ligands for TLR7 and TLR8 have been suggested but not definitively shown , it is known that they participate in the discrimination of nucleic acid-like structures in microorganisms (Takeda, Kaisho, & Akira, 2003).

7. TLR-Dependent Signalling Cascades

Upon initial recognition of PAMPs by the TLR receptors a series of cascade events leads to upregulation of genes required for inflammation. Most members of the TLR family form homo-dimers with the exception of TLR2 and TLR4 which form hetero-dimers with TLR1 and TLR6 respectively (Seneviratne, Sivagurunathan, & Monaco, 2011). TLR signalling is augmented by the recruitment of five different adaptor proteins to its TIR domain. These adaptor proteins include TRIF (TIR domain containing adaptor inducing IFN-β), TIRAP/MAL (TIR domain containing adaptor protain/MyD88 adaptor like protein), TRAM (TRIF related adaptor molecule) and SARM (Steile-a and armadillo motif-containing protein) (Akira, Uemtasu, & Takeuchi, 2006). Different downstream signalling events are determined by whether the TLR interacts with MyD88 or TRIF. Previous studies have demonstrated the importance of MyD88 in the host defense against lipoproteins, CpG DNA, peptidoglycans or imidazoquinolines. In mice lacking MyD88, macrophages failed to produce any inflammatory cytokines (Takeda, Kaisho, & Akira, 2003). All TLRs, with the exception of TLR3, require MyD88 to initiate downstream signalling events. TLR2 and TLR4 signalling uses an adaptor molecule TIRAP/MAL as a bridge between the TLR and MyD88 (Fitzgerald et al.,2001; Horng et al., 2001,2002; Yamamoto et al., 2002a). Following ligand activation MyD88 recruits members of

the IL-1 receptor associated kinase (IRAK) family. The IRAKs subsequently dissociate from MyD88 and associate with TRAF6 which is an E3 ubiquitin ligase (Xia, Sun, Chen, et al., 2009) Unconjugated polyubiquitin chains activate a molecular complex consisting of TAK1 and TAK1-binding proteins which then translocate to the cytoplasm. In the cytoplasm TAK1 phosphorylates IKK-β, which is part of a complex consisting of IKKα, IKKβ and NEMO. This complex is then responsible for phosphorylation of IκBα, which is an inhibitor of the transcriptional regulator NF-kB. Once phosphorylated IkBα releases NF-κB which then translocates to the nucleus and activates genes responsible for inflammation. TAK1 additionally phosphorylates MAPK6 which results in the downstream activation of the MAPKs Erk1, Erk2, p38 and JNK. Activation of this pathway leads to activation of the transcription factor AP-1 which is involved in the control of many genes including those encoding cytokines (Seneviratne, Sivagurunathan, & Monaco, 2011).

TLR3 uses the TRIF coactivator to activate a signalling cascade the ultimately leads to the activation of the transcription factors IRF3 and NFκB (Alexopoulou L, 2001). TRIF associates with TRAF3 and TRAF6. TRAF3 activates TBK1 and IKKe which phosphorylate the cytoplasmic form of IRF3 which upon phosphorylation then translocates to the nucleus to activate gene expression (Oganesyan G, 2006., Hacker H, 2006). IRF3 activates among others genes for the production of pro-inflammatory cytokines, type 1 interferons and subsequently IFN dependent genes. NFκB activation results when a complex consisting of TRADD, FADD and RIP1 leads to TRADD dependent poly ubiquitination of RIP1. In addition, TRIF interacts with TRAF6 to activate TAK1 in a ubiquitination dependent process which leads to IKKα phosphorylation and release of NFκB (Alexopoulou L, 2001). TLR4 is a special case in that it can utilize both the MyD88 and TRIF pathways in signaling activation.Which pathway is used is determined by the receptor's cellular localisation (Tanimura N, 2008., Kagan JC, 2008). In one case, membrane bound TLR4 recruits MyD88 which binds to MAL leading ultimately to

activation of the transcriptional regulator NFkB. In the second instance, TLR4 translocates to the endosome via dynamin mediated endocytosis. In the endosome, TLR4 associates with TRAM to activate the TRIF pathway resulting in IRF3 activation and at a later stage activation of NFkB and MAPK (Tanimura N, 2008., Kagan JC, 2008., Rowe DC, 2006)

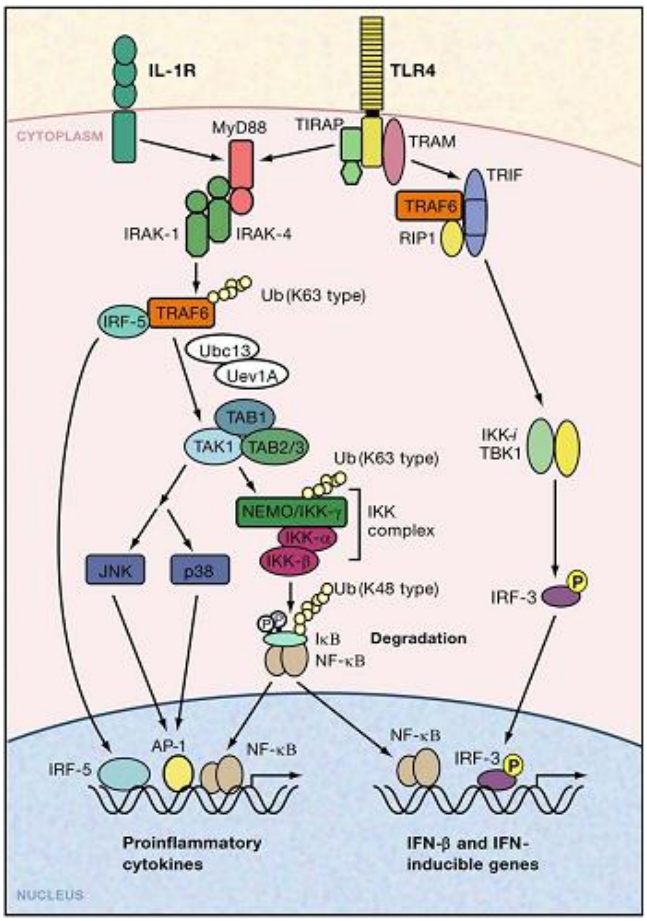


Figure 2. TLR signalling cascade showing MyD88 and TRIF dependant pathways (Akira, Uemtasu, & Takeuchi, Pathogen Recognition and Innate Immunity, 2006)

8. Atherosclerosis

Atherosclerosis is caused by many factors and is associated with risk for diseases such as coronary heart disease, cerebrovascular insults and peripheral vascular disease. The problem of atherosclerosis is not just that it is a lipid-storing disease, but it is a chronic inflammatory process as well (Curtiss & Tobias, 2009). Considering the fact that chronic inflammation is part of the etiology of atherosclerosis it has been noted that the innate immune response plays a crucial role in the progression of this disease and more specifically that certain TLRs play a role in either defending the host or advancing the disease (Seneviratne, Sivagurunathan, & Monaco, 2011., Den Dekker WK, 2010). As a result of this chronic inflammation, the presence of cells such as macrophages, monocytes, leukocyte, dendritic cells and lymphocytes is observed in the area of atherosclerotic lesions and the damaged endothelial cells demonstrate an inflamed phenotype (Biragyn A, 2002). One of the earliest lesions described in atheroslcerosis are fatty streaks. These streaks are composed of foam cells and some smooth mucsle cells. Lifestyle and genetic diseases influence the progression from fatty streaks to pathological lesions that can cause deleterious conseqeunces (Zieske A.W, 2002). The most common risk factors associated with progression of atherosclerosis are diabetes mellitus, hypertension and hypercholesterolemias, as well as lifestyle habits such as smoking, high fat diet and sedentary lifestyle. It is important to note that there is irregular distribution of atherosclerosis throughout the vascular bed which could be attributed to irregular blood flow dynamics (Wasserman & Topper, 2004), while regions of the arterial tree exposed to laminar flow are spared from endothelial activation and athersclerosis (Curtiss & Tobias, 2009). Areas that are exposed to laminar flow seem to be preserved from atherosclerosis and endothelial activation (Curtiss & Tobias, 2009). Vessel bifurcations and the lesser curvature of the aortic arch are sites that present with an inflammed phenotype even in the absence of complicating disease states (Matharu N.M, 2006., Dai G, 2004., Feintuch A, 2007). TLR expression has

been observed in endothelial cells that exhibit this proinflammatory phenotype suggesting a connection between TLR signaling and atherosclerosis (Curtiss & Tobias, 2009).

9. TLRs and Atherosclerosis

The idea that TLRs participate in the process of atherosclerosis is becoming clear, although only two, TLR2 and TLR4, have been studied in any detail. Both endogenous and exogenous ligands promote disease by activation of TLR2 signalling, which is one route for both genetic and inflammatory pathways to atherosclerotic disease (Curtiss & Tobias, 2009). Experiments with cultured human coronary artery endothelial cells exposed to conditions approximating disturbed flow conditions, similar to that found at sites of lesion development, showed up-regulated responsiveness to TLR2 agonists (Dunzendorfer, 2004) due to enhanced expression of TLR2 in response to disturbed flow (Tobias P.S, 2007). A number of studies utilizing TLR2 deleted mouse models further substantiate the role of TLR2 in atherosclerosis. *Low-density lipoprotein receptor–deficient* (*Ldlr–/–*) mice show increased susceptability for atherosclerosis and as such have been used for studies of TLR2 deficiecy TLR2 knockout in the *Ldlr–/–* background leads to complete deficiency of TLR2 and a reduction in atherosclerosis (Mullick A.E, 2005). In the specific case where BM transplantation was used, loss of TLR2 expression from BM-derived cells had no effect on disease progression. Furthermore, experiments have shown that, in the absence of a known exogenous agonist, loss of TLR2 expression on cells *not* of BM origin (such as endothelial cells) reduced atherosclerosis (Mullick A.E, 2005). Thus, it was postulated that an unknown endogenous TLR2 agonist influenced lesion progression by activating TLR2 in cells that were not of BM cell origin (Mullick A.E, 2005). Early results with double knockout LDLr−/−TLR2−/− mice showed that total deficiency of TLR2 results in decreased lesion burden after 10 or 14 weeks of consuming a high-fat diet (Yamashita, 2006) Complete loss of this receptor resulted in decreased lesion size whereas systemic exposure to a TLR2 ligand dramatically increased lesion severity (Mullick A.E, 2005). Thus, the existence

of a functional TLR2 promotes disease and suggests a clear role for TLR2 in modulating the severity of experimental atherosclerosis. As TLRs have come to be recognized as potential therapeutic targets it is of note that Arslan *et al.* reported the first humanized anti-TLR2 antibody (OPN-305) which led to reduced infarct size, preserved systolic function and prevention of myocardial damage in a pig model of ischemia/reperfusion injury (Arslan, Keogh, McGuirk, & Parker, 2010., Arslan, et al., 2012). A second report showed that the DPP-4 (CD26) inhibitor alogliptin reduced atherosclerotic lesion size in diabetic mice and inhibited TLR4-mediated pro-inflammatory cytokine expression *in vitro* (Ta, Schuyler, Li, Lopes-Virella, & Huang, 2011). The TLR4 signaling pathway is mediated by the myeloid differentiation factor 88 (MyD88) (as does TLR2) as well as the Toll/IL-1 receptor domain-related adaptor protein that induces interferon (TRIF). Loss of MyD88 leads to reductions in plaque size, lipid content, expression of proinflammatory genes, cytokines, and the chemokines IL-12 and monocyte chemoattractant protein-1 (Michelsen K.S, 2004). TLR4 has further been shown to directly interfere with cholesterol metabolism in macrophages suggesting a mechanism by which TLR4 may affect disease pathology (Castrillo A, 2003). A complete deficiency of TLR4 correlates with reductions in lesion size, lipid content, and macrophage infiltration in *ApoE*-/- (Apolipopreotein E) mice fed a high cholesterol diet for six months (Michelsen K.S, 2004). Notably, this reduction in disease severity in the absence of TLR does not seem to affect plasma cholesterol levels (Curtiss & Tobias, 2009). In at least one study *ApoE−/−* mice deficient in *TLR4* exhibited a decreased development of atherosclerotic lesions (up to 55% less), and lower levels of monocyte infiltrated atherosclerotic lesions (with a 65% decrease) in *ApoE−/−TLR4−/−* compared to *ApoE−/−* mice (Michelsen K.S, 2004., Mullick A.E, 2005). Our current understanding of these processes suggests that blocking TLR2 and perhaps TLR4 may reduce lesion formation and inflammation, while TLR2 blockade may also reduces infarct size (Falck-Hansen M, 2013). Hyperlipidaemia and infectious disease are two prominent risk

factors that point to innate immune mechanisms as potential contributors to proatherogenic inflammation. The TLRs (Toll-like receptors) being pro-inflammatory sensors of pathogens, are therefore potential connection points between inflammation, infectious disease and atherosclerosis (Tobias P.S, 2007). Michelsen et al. were the first to suggest a direct link between TLR4 and atherosclerosis formation (Michelsen K.S, 2004). In this study TLR4/apolipoprotein E (ApoE) double knockout mice developed less atherosclerosis compared to ApoE knockout controls, although the intima reduction was less prominent as compared to MyD88/ApoE double knockout mice. In both TLR4/ApoE and MyD88/ApoE double knockout mice this anti-atherogenic effect appeared to be independent of cholesterol levels (Den Dekker WK, 2010). In addition, a significant lower levels of lipids and macrophages in the plaque was observed in both TLR4/ApoE and MyD88/ApoE double knockout mice. This suggests that TLR signaling may also be involved in advanced plaques and plaque vulnerability. Michelsen et al. did not focus on progression into a vulnerable plaque, since ApoE−/− mice fail to develop, in the absence of additional maniuplation, plaques with a vulnerable phenotype (Den Dekker WK, 2010).

10. Autoimmune Diseases

Autoimmune diseases occur when the host's natural immunologic defenses start attacking healthy cells and tissue. They can affect a variety of tissues and present with different symptoms based on the particular organ or cell type in question. To date there is still no clear evidence about the exact pathogenesis of autoimmune disorders, but it is known that they are complex multifacteded disorders under the influence of genetics, environment and hormonal factors (Chen, Szodoray, & Zeher, 2015). One major characteristic of autoimmune disorders is the existence of antibodies and T cells specific for antigens expressed by a targeted tissue; these antigens being called autoantigens, which are a subset of self antigens (Parham, 2009). Many of the commonly found autoantigens are release products of dead or dying cells. Viral or bacterial infections are often associated with the onset and flare ups of systemic autoimmune disease (Banchereau J, 2004., C.J. & et al., 2005). According to one model envisions a pathogenic process whereby dendritic cells and macrophages upon activation of their PRRs upregulate production of type I IFNs and cytokines . Autoantigens presented to the immune system via APCs then activate potentially autoreactive T cells which subsequently activate and expand the population of auto-reactive B cells. These B cells undergo somatic hypermutation leading to expression and clonal expansion of pathogenic high affinity auto-antibodies. These higher affinity autoreactive B cells are then targeted by auto-antigens that increase during the course of infection (Ronnblom L, 2001) . Once self-tolerance is lost and there is a response from the adaptive immune system, an autoimmune disease can occur. In addition to microbial mechanisms of autoimmune induction, it is also possible that autoantigens not associated with infections can serve as endogenous PRR ligands and exert a proactive function in loss of tolerance (Marshak-Rothstein, 2006).

11. TLRs and Autoimmune Disease

In systemic autoimmune disorders such as SLE, scleroderma and Sjogren's syndrome a high proportion of autoantibodies associated with these diseases recognize DNA, RNA or macromolecular complexes that contain RNA or DNA (Marshak-Rothstein, 2006). An increasing body of evidence suggests that these molecules are autoantigens because they have the ability to directly activate the innate immune system and thereby promote self-directed immune responses (Marshak-Rothstein, 2006). Early clinical studies showed a correlation between aberrant expression of IFNa and SLE. There are 13 subtypes of IFNα and they represent a pleiotropic cytokine family associated with immune responses to viral infection (Theofilopoulos, 2005).

More direct evidence of the causal relationship between IFNα and SLE came from studies in which repeated administration of recombinant IFNα in patients with chronic viral infections or various cancers led to the production of antinuclear antibodies (ANA) and in some cases clinical presentation of symptoms associated with SLE or other autoimmune disorders (Ronnblom, et al.,1990., Gota & Calabrese, 2003). IFNα contributes to disease pathogenesis either directly or indirectly via effects on antigen presenting cells (APC), T cells and B cells (Utz, Gensler, & Anderson, 2000). High amounts of IFNα are produced by a small but highly efficient type of cell known as the plasmacytoid dendritic cell (pDC) (Ronnblom & Alm, 2001). The highly efficient response of pDCs to both microbial infection and SLE associated immune complexes is in large part due to the fact that pDCs constitutively express TLR7 and TLR9 (Kadowski, et al., 2001). TLR7 was originally identified as a receptor for viral single-stranded RNA (ssRNA) (Diebold, Kaisho, Hemmi, Akira, & Reis e Sousa, 2004., Heil, et al., 2004., Lund, et al., 2004), while TLR9 was initially identified as a receptor that could distinguish between bacterial/viral DNA and mammalian host DNA (Krieg, et al.,1995., Hemmi & et al., 2000). Unlike many other members of the TLR family that are membrane bound, TLR7 and TLR9 are found in the

cytoplasmic compartment, specifically the endoplasmic reticulum-endosome-lysosome array (Ahmad-Nejad & et al., 2002). DNA-containing immune complexes activate cells through TLR9, while RNA-containing immune complexes activate cells through TLR7 (Marshak-Rothstein, 2006). Activation of pDCs by these complexes stimulates IFNα production and agents such as chloroquie or bafilomycin, which disrupt acidification of endosomes and block activation of TLR7 and TLR9, lead to inhibition of IFNα production (Marshak-Rothstein, 2006). Activation of cytokine production is also inhibited by oligodeoxynucleotide (ODN) sequences that are known to block activation of TLR7 and TLR9 (Vollmer,et al., 2005., Barrat, et al.,2005., Lovgren, et al.,2006). Furthermore, immune complexes consisting of purified snRNPs and monoclonal antibodies specific for the SmD subunit of snRNP can stimulate pDCs from wild type mice but not from TLR7 deficient mice (Savarese, et al., 2006). Taken together these data suggest that nucleic acid-containing immune complexes activate TLR7- or TLR9 containing pDCs and represent a key step in the pathogenic process leading to SLE. In addition to pDCs, B cells also express TLR7 and TLR9. IFNα upregulates expression of both TLR7 and its associated adaptor MyD88 in human and mouse B cells and activates an increased response of B cells to TLR9 ligands (Marshak-Rothstein, 2006). Because TLR7 and TLR9 are cytoplasmically located a question arises as to how they recognize exogenous ligands. BCR mediated endocytosis is responsible for internalizing and presenting ligands intracellularly (Marshak-Rothstein, 2006). Several studies using in vivo mouse models of SLE have begun to provide evidence consistent with the view that TLR7 and TLR9 play a central role in the production of pathogenic autoantiboes and the development of SLE (Marshak-Rothstein, 2006).

Table 2. In vivo outcomes of aberrant TLR expression on mouse models with SLE (Marshak-Rothstein, 2006).

Mouse model*	Genetic background	Effect of deficiency or increase in Toll-like-receptor expression on autoantibody titres‡	Renal disease	Survival	Refs
Myd88$^{-/-}$ *lpr/lpr*	Mixed	• HEp-2: no ANAs • Western blotting: no Sm-specific antibodies	ND	ND	46
Tlr9$^{-/-}$ *lpr/lpr*	Mixed or MRL	• HEp-2: ↓ dsDNA-specific antibodies • *Crithidia*: ↓ dsDNA-specific antibodies • Western blotting and ELISA: ↑ Sm-specific antibodies	Exacerbated	Decreased	63,64
	MRL	• ELISA: ↑ DNA-specific antibodies	Exacerbated	Decreased	65
	C57BL/6	• HEp-2: ↓ dsDNA-specific antibodies; ↑ nucleolus-specific antibodies • ELISA: ↑ DNA-specific antibodies; ↓ nucleosome-specific antibodies	Exacerbated	Decreased	67
Tlr9$^{-/-}$ *Ali5*	C57BL/6	• HEp-2: ↓ dsDNA-specific antibodies; ↑ nucleolus-specific antibodies • ELISA: ↔ DNA-specific antibodies; ↓ nucleosome-specific antibodies	Exacerbated	ND	66
Tlr3$^{-/-}$ *lpr/lpr*	Mixed	• No effect	Unchanged	ND	63
Tlr7$^{-/-}$ *lpr/lpr*	MRL (3–6 backcrosses)	• HEp-2: ↓ speckled nuclear staining by ANAs	Reduced	ND	64
Tlr7$^{-/-}$ 564Igi	C57BL/6	• ELISA: spontaneous secretion of 564 autoantibodies	ND	ND	70
Fcgr2b$^{-/-}$*Myd88*$^{-/-}$	C57BL/6	• ELISA: ↓ DNA-, GBM- and cardiolipin-specific IgG2a and IgG2b	Reduced	Prolonged	68
Fcgr2b$^{-/-}$*Myd88*$^{-/-}$ 56R	C57BL/6	• ELISA: ↓ DNA-, GBM- and cardiolipin-specific IgG2a and IgG2b	ND	ND	68
Fcgr2b$^{-/-}$*Tlr9*$^{-/-}$ 56R	C57BL/6	• ELISA: ↓ DNA-, GBM- and cardiolipin-specific IgG2a and IgG2b	ND	ND	68
Fcgr2b$^{-/-}$ *Yaa*	C57BL/6	• HEp-2: ↑ speckled nuclear staining by ANAs; ↑ nucleolus-specific antibodies	Exacerbated	Decreased	71
sle1 Yaa	C57BL/6	• Protein array: ↑ snRNP-specific IgG	Exacerbated	Decreased	72

As a result of the likely involvement of TLR7 and TLR9 signalling in autoimmune disease, a number of studies have looked at the effect of TLR7 and TLR9 inhibitors on disease. Two main classes of TLR7 and TLR9 inhibitors exist, one are the antimalarials and second are the inhibitory oligonucleotides (ODNs) (Lenert, 2006). Antimalarials have been the standard therapeutic for autoimmune disease over the last hundred years . These compounds can inhibit the activation of both pDCs and B cells likely through inhibition of acidification and maturation of endosomes (Macfarlane & Manzel, 1998., Hacker, et al.,1998., Yi, et al., 1998). Two classes of ODNs exist, the first contain CpG rich motifs and the second class consist of a repetitive element associated with mammalian telomeres (Barrat, et a., 2005., Lau, et al., 2005., Stacey, et al., 2003). Inhibitory ODNs can antagonize both TLR7 and TLR9 directly and administering ODNs to SLE prone mice significantly reduces the severity of the disease phenotype (Patole, et al., 2005).

Systemic sclerosis (SSc) is a complex autoimmune disease involving many systems with clinical presentation resulting from tissue fibrosis and extensive vasculopathy (Saigusaa, et al., 2015). IFN regulatory factor 5 (IRF5) is a potential disease susceptibility gene for SSc. Furthermore, IRF5 is activated by TLR4 and binds to promoters of various genes leading to SSc disease pathology (Saigusaa, et al., 2015). Potential TLR4 endogenous ligands are up-regulated in SSc, skin lesions (Bhattacharyya S, 2013., Bhattacharyya, et al., 2014., Takahashi T et al., 2015) and serum levels correlating with sever organ involvement and abnormal immunological activity (Yoshizaki A, et al., 2009., Yoshizaki A, et al., 2008). HMGB1, hyaluronan and fibronectin extra domain are endogenous TLR4 ligands and these ligands are elevated in serum and dermis in SSc (Bhattacharyya S, 2013., Bhattacharyya, et al., 2014) (Yoshizaki A, et al., 2009., Yoshizaki A, et al., 2008). TLR 4 signaling enhances TGF-β signaling and blocking TLR4 represses basal and TGF-β dependant fibroblast activation (Bhattacharyya S, 2013., Bhattacharyya, et al., 2014). Moreover, in Tlr4-deficient mice bleomycin-induced dermal fibrosis is significantly decreased depite elevated levels of endogenous TLR4 ligands (Takahashi T et al., 2015). In summary, pathogenesis of dermal fibrosis appears to involve TLR4 signalling and direct activation of dermal fibroblasts (Saigusaa, et al., 2015).

Primary Sjögren's syndrome (pSS) is a chronic, inflammatory autoimmune disease in which salivary and lacrimal glands are destroyed leading to drynesss of the mouth and eyes (Jonsson, et al., 2011). TLR-family expression profiles differ greatly between pSS patients and healthy individuals (Zheng, Zhang, Yu, & Yang, 2010). TLR1, TLR2 and TLR4 mRNAs are substantially overexpressed in pSS cultured salivary gland epithelial cells (SGECs) compared to controls (Zheng, Zhang, Yu, & Yang, 2010., Spachidou MP, et al., 2007). As described earlier, peptidoglycan can activate TLR2 which in turn induces the production of IL-17 and IL-23 in peripheral blood mononuclear cells (PBMCs) of pSS patients (Kwok S-K, et al., 2012).

As for the case of SLE and scleroderma, blockade of inflammatory cytokines and chemokines as well as TLRs by neutralizing antibodies or small molecule inhibitors may be potentially useful treatments for Sjögren's syndrome (Kramer, 2014).

12. Conclusion

A growing body of evidence has shown that Toll-like receptors are impornatnt mediators of the immune response and immunity related diseases such as atherosclerosis or autoimmune disorders. Toll-like receptors, members of the larger Pattern-recognition recpetor family, recognize both exogenous and endogenous ligands to activate the immune system. Ligand binding initiates a complex cascade of molecular events leading to activation of genes important for the immune response. The amount of functional TLR seems to be related to the induction and maintenance of an autoimmune response. Chronic activation of the immune response leads to atherosclerosis or autoimmune disorders. Evidence suggests that TLR 2 and TLR 4 are critical mediators involved in atherosclerosis, while the TLR 9 subset family is important in the pathogenesis of autoimmune disorders. Preliminary evidence suggests that humanizied anti-TLR2 antibodies and ODNs maybe therapeutically effective. Future studies are now poised to expand the therapeutic potential of TLR inhibitors.

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15. Biography

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WORK EXPERIENCE

09/2013–Present

English teacher

Lingua PLUS Junior, Zagreb (Croatia)

-teaching 2-4x per week, children 5-13 years old
-organizing classes, exams and demo lessons as well as contributing to the use of quality teaching material
-teaching through verbal communication and games without using older traditional teaching methods
-monitoring and evaluating child progress while keeping parents and school director up to date

10/2014–Present

Student assistant as part of the course "History Taking and Physical Examination"

KBC Zagreb, Zagreb (Croatia)

-taking part in practicals in the course "History taking and Physical examination"on a weekly basis
-proper demonstration and assisting in taking a detailed medical history as well as demonstrating how to take a proper physical exam of a patient
-in cooperation with Assitant professor Boško SKorć, MD, PhD, KBC Zagreb

10/2007–06/2008

Volunteer

The Village of Erin Meadows, Mississauga (Canada)

-volunteering in the old age home
-organization of day activities (bingo, social programs, nature walks)
-taking part in the "Dining room" program, help feed those who are unable to do so on their own
-"Restorative walking" program, taking walks with elderly who need assistance or are suffering from dementia
-helping out with the clubs (scrap booking, card-making...) that are established at the home

EDUCATION AND TRAINING

09/2010–07/2016 University of Zagreb- Faculty of Medicine, Zagreb (Croatia)

03/2014–03/2014 Studentska Ekipa Prve Pomoci (StEPP), Zagreb (Croatia)
-active participation in the one-day course of first-aid "ABCs of Emergencies, CPR/AED"
Studentska Ekipa Prve Pomoci (StEPP), Zagreb (Croatia)

06/2015–06/2015 Student cardiology section, Zagreb (Croatia)
-active member 2014./2015.
-during the academic year 2014/2015. took part in various activities of the student cardiology section
-active participation in the one-day course "Kardio dan 2015."
-during the one day course took part in four workshops: ECG, USV, BLS and surgical suturing workshop

10/2014–Present CroMSIC (Croatian Medical Students' International Committee), Zagreb (Croatia)
-member since 2014.

PERSONAL SKILLS

Mother tongue(s) English, Croatian

Communication skills
-during my time as a student assistant I gained invaluable communication skills that I can utilize in various professional settings
-team spirit, ability of adapting to new situations and working with people from different countries/cultures are skills that I have gained working as a student demonstrator in the course "History taking and Physical Examination"

Digital competence

SELF-ASSESSMENT				
Information processing	Communic-ation	Content creation	Safety	Problem solving
Independent user	Independent user	Independent user	Independent user	Independent user

Digital competences - Self-assessment grid

-MS Office advanced (Word, Power Point, Excel, Publisher)

Other skills
-sports (tennis, swimming, hiking)
-basic German language course (starting 2016)

Driving licence B