UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
DEPARTMENT OF BIOLOGY

Jasna LOJK

THE INFLUENCE OF CHOSEN MICROENVIRONMENTAL FACTORS ON TOLL-LIKE RECEPTOR 5 SIGNALLING IN HUMAN CELL LINE HEK293

GRADUATION THESIS
University studies

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VPLIV IZBRANIH MIKROOKOLJSKIH DEJAVNIKOV NA SIGNALIZIRANJE TOLLU-PODOBNEGA RECEPTORJA 5 ČLOVEŠKE CELIČNE LINIJE HEK293

DIPLOMSKO DELO
Univerzitetni študij

Ljubljana, 2011

The graduation thesis is a completion of the university studies of biology. The work was carried out in the laboratory of Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

Diplomsko delo je zaključek univerzitetnega študija biologije. Opravljeno je bilo v laboratoriju Oddelka za infekcijske bolezni in imunologijo Fakultete za veterinarsko medicino, Univerza v Utrechtu, Utrecht, Nizozemska.

The Council of the Department of Biology appointed Assistant Professor Marjanca Starčič Erjavec, PhD, as a supervisor, Professor Jos van Putten, PhD, as co-advisor and Professor Jasna Štrus, PhD, as reviewer.

Študijska komisija dodiplomskega študija biologije je za mentorja diplomskega dela imenovala doc. dr. Marjanco Starčič Erjavec, za somentorja prof. dr. Josa van Puttna in za recenzentko prof. dr. Jasno Štrus.

Supervisor (mentor): Assist. Prof. Dr. Marjanca Starčič Erjavec

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Reviewer (recenzentka): Prof. Dr. Jasna Štrus

Podpisana se strinjam z objavo svoje naloge v polnem tekstu na spletni strani Digitalne knjižnice Biotehniške fakultete. Izjavljam, da je naloga, ki sem jo oddala v elektronski obliki, indentična tiskani verziji.

Jasna Lojk

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AB	The microenvironment of the cell has an important role in establishing a proper cellular phenotype and can also have a significant effect on cell function. Toll-like receptors (TLR), important receptors of the innate immune system, are also subject to environmental influences and this can result in a modified cellular response to TLR ligands. In this study, several microenvironmental factors like extracellular matrix components, hormones, cytokines, growth factors and metabolites were assessed for their potential regulatory effect on TLR5-dependent NF-κB activation in the absence (auto-activation) and presence of TLR5 ligand (FliC). For this purpose we used our newly established two step transfection method combined with the NF-κB luciferase reporter system. Results showed that butyrate, propionate, epidermal growth factor, a matrix metalloproteinase inhibitor and disruption of lipid rafts altered TLR5 function. The results also indicated distinct differences between auto-activation and ligand stimulation of TLR5. qRT-PCR assays showed that ligand stimulation but not auto-activation induces cytokine gene transcription. Most of the other microenvironmental factors tested had no influence on TLR5 signalling at the level of NF-κB activation, indicating that the function of this receptor is relatively stable. This confirms and underlines the importance of TLR5 signalling and its tight regulation for proper host defence and immune responsiveness.

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AI Mikrookolje ima pomembno vlogo pri vzpostavljanju pravega celičnega fenotipa, pomembno pa lahko vpliva tudi na funkcijo celic. Tudi Tollu-podobni receptorji (TLR), receptorji pridobljenega imunskega sistema, so podvrženi vplivu okolja, ki lahko spremeni njihovo delovanje in s tem odgovor celice na vezavo ustreznih ligandov TLR. V okviru te raziskave smo preverili potencialni vpliv številnih molekul, ki se običajno nahajajo v mikrookolju celic, na regulacijo in modulacijo aktivacije transkripcijskega faktorja NF-κB, odvisne od TLR5, v odsotnosti (avtoaktivacija) in prisotnosti (stimulacija) liganda TLR5 flagelina (FliC). Testirane so bile nekatere komponente zunajceličnega matriksa, hormoni, citokini, rastni dejavniki in metaboliti. Vsi poskusi so bili izvršeni z uporabo nove transfekcijske metode v dveh korakih in NF-κB luciferaznega reporterskega sistema. Rezultati so pokazali, da butirat, proprionat, epidermalni rastni faktor, inhibitor matriksnih metaloproteinaz in komponente, ki razbijejo lipidne rafte, vplivajo na funkcijo TLR5. Rezultati so nakazali tudi razliko med procesoma avtoaktivacije in aktivacije receptorja v prisotnosti liganda. qRT-PCR je pokazal, da samo stimulacija z ligandom, ne pa tudi avtoaktivacija, sproži transkripcijo gena za IL-8. Na relativno stabilnost delovanja receptorja nakazuje dejstvo, da večina ostalih mikrookoljskih dejavnikov ni imela vpliva na signaliziranje TLR5 na nivoju aktivacije NF-κB. To potrdi in poudari pomen signaliziranja TLR5 in ustrezne regulacije za prave imunske odgovore in obrambo gostitelja.

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ABREVATIONS AND SIMBOLS

AP-1	Activator protein 1
c/EBPβ	CCAAT/enhancer-binding protein β
CMP-NANA	Cytidine-5'-monophospho-N-acetylneuraminic acid sodium salt
DAMP	Damage associated molecular pattern
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FCS	Fetal calf serum
FliC	Flagellin
GM-CSF	Granulocyte macrophage – colony stimulating factor
GPCR	G-protein coupled receptor
HDACi	Histone deacetylase inhibitor
HEK293	Human embryonic kidney cell line
hTLR5	Human Toll-like receptor 5
IL	Interleukin
IPAF	IL-1-converting enzyme protease-activation factor
LacZ	β-galactosidase assay
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MMP9	Metrix metalloproteinase 9
MPI	Matrix metalloproteinase inhibitor
MyD88	Myeloid differentiation primary-response protein 88
MβC	Methyl-β-cyclodextrin
Neu4	Neuroaminidase 4
NF-κB	Nuclear factor-κB
OSM	One-step transient transfection method
PAMP	Pathogen associated molecular pattern
PLL	Poly-L-lysin

PRR	Pathogen recognition receptor
qRT-PCR	Quantitative real-time polimerase chain reaction
RLU	Relative luminiscence units
RNA	Ribonucleic acid
SCFA	Short chain fatty acids
STAT	Signal transducer and activator of transcription
TIR	Toll/IL-1 receptor-like domain
TLR	Toll-like receptor
TN	Tenascin
TNFα	Tumor necrosis factor α
TQ	Thymoquinone
TSM	Two-step transient transfection method

1 INTRODUCTION

Many recent studies increased awareness that the cellular microenvironment has an important role in establishing proper cell functions. The extracellular matrix is not only meant for structural support but can, through specific receptors, induce signalling cascades, which can influence cell behaviour. Signalling molecules of different, functionally non-related cascades have been shown to interact and influence one another, thus altering cell responses to certain stimuli in the environment. The cell response is not only dependent on single molecules, but also on combinations of factors in a certain moment, once again confirming the complexity of the whole system.

Propagating cells *in vitro* means isolating them from their usual microenvironment which may alter the cellular phenotype. Lack of crucial signals from the environment may also cause a different gene expression pattern which creates an artificial cell type, otherwise not found *in vivo*. *In vitro* experiments can of course give very important discoveries, that can also be confirmed *in vivo*, but the whole picture can be distorted because of general different cell behaviour.

Lack of a natural microenvironment can also effect the expression and function of proteins of the immune system such as the pathogen recognition receptors (PRR). These receptors are important for recognition of conserved pathogen associated molecular patterns (PAMPs). One class of PRR is the Toll-like receptor (TLR) family which is composed of 10 different receptors in humans, each recognizing different conserved parts of microbes, like lipopolysaccharides (LPS), DNA, lipopeptides and others. Tight regulation of the function of those receptors is crucial as their activation and signalling induces an immune response which can be also harmful for the host, if not tightly controlled. It has been reported that the expression and function of these receptors is regulated by external and internal factors, but many regulatory factors are still unknown. Growing of cells in a controlled *in vitro* laboratory environment enables to study the effects of single or multiple environmental factors on a TLR functions.

One of the most important TLRs is TLR5. This receptor recognises flagellin, the main component of bacterial flagella. Human TLR5 is epresent on many cell types but is especially important in the intestine, where it is a major sensor of invading pathogens. Exact mechanisms involved in TLR5 signalling and its modulation are still unclear, thus making it a good subject to study.

The objective of this study was to assess the dynamics of TLR5 function in presence of different molecules usually found in the microenvironment of the cells. The effect of several extracellular matrix components, cytokines, hormones, growth factors and metabolites was tested on the auto-activation of the receptors in absence of the ligand and on the activation upon stimulation of TLR5 by its natural ligand. Knowledge of the status and regulation of the immune response is crucial to better understand the basics of inflammation and immunity and the host defence against microorganisms.

2 STATE OF ART

2.1 INNATE IMMUNITY

The immune system is one of the oldest and most complex organ systems. Its physiologic function is the distinction of self and non-self and in this way protection against infectious microbes. Molecules that are recognised by the immune system may elicit reactions of innate immune system and at a later stage a response of the adaptive immune system. Nevertheless, the two systems are tightly connected and many immune responses of one system are built on the foundation of the other.

Innate immunity is the first line of defence against infections. The system exists in a functional state before encounter with microbes and is rapidly activated by them before the development of an adaptive immune response. In many instances, the innate immune response alone can eliminate the microbes. Activation of innate immunity can also stimulate the adaptive immune response and can influence the nature of the adaptive responses to make them optimally effective against different types of microbes. Innate immunity thus plays an essential role in human host defence (Abbas and Lichtman, 2003, Janeway et al., 2001, Roitt and Delves, 2001).

2.1.1 Mechanisms of innate immunity

The innate immune system consists of epithelial barriers, circulating cells and proteins that recognise microbes or substances produced in infections. Anatomical and physiologic barriers provide the crucial first line of defence against pathogens. These barriers include intact skin covered with antimicrobial secretions like lysozyme and toxic lipids, mucus membranes and vigorous mucociliary clearance mechanisms, low stomach pH and body temperature. An important part of host defence is also carried out by bacterial microbiota, which can secrete different antimicrobial molecules, thus inhibiting growth of potential pathogens.

If pathogens manage to overcome the barriers, they are encountered by the second line of defence: cells and soluble proteins. The principal effector cells of innate immunity are neutrophils, mononuclear phagocytes, dendritic cells (DCs), mast cells, and natural killer (NK) cells. Phagocytes, including neutrophils, monocytes, macrophages and DCs, are cells whose primary function is to identify, ingest and destroy microbes. These cells are

strategically located at all the sites where microbes may gain entry into the host, like subepithelial connective tissue, in the interstitial of parenchymal organs and in the lymphatic sinuses of lymph nodes (Abbas and Lichtman, 2003, Janeway et al., 2001, Roitt and Delves, 2001).

If the microbes enter the blood, they are often attacked by proteins of the complement system and other plasma proteins like LPS binding protein, C-reactive protein and other antimicrobial peptides that bind microbial structures, thus helping their recognition by immune cells (Turvey and Broide, 2010).

2.1.1.1 PRR and PAMPs

Invading microorganisms are first recognised by the innate immune system through germline-encoded pattern recognition receptors (PRRs). These receptors recognise distinct microbial components, known as pathogen-associated molecular patterns (PAMPs) and directly activate immune cells through intracellular signalling cascades that rapidly induce the expression of a variety of overlapping and unique genes involved in inflammatory and immune responses (Akira et al., 2006). The recognition works on two principles. On one hand, PAMPs are evolutionary conserved structures, shared between different microbial species and not present on host cells, e.g. double-stranded RNA (found in replicating viruses) or lipopolysaccharides (LPS) in Gram-negative bacteria. On the other hand, cells can sense aberrant locations of molecules that can be identical to host molecules, notably nucleotide structures. Microbial infections can be associated with the introduction of nucleotides into endosomes and the cytoplasm, which are abnormal localisations for these structures. By having nucleotide sensing PRRs in the endosomes and the cytoplasm, the innate immune system thus uses the aberrant location of specific molecules as a means of microbial recognition (Rasmussen et al., 2009).

There are several classes of PRRs. One type of PRRs, such as complement receptor 3 (CR3) and C-type lectins, work as opsonins and facilitate receptor-mediated phagocytosis by binding to PAMPs. Other PRRs, such as TLRs, can trigger inflammation after recognizing a variety of different microbial motifs at either the cell surface or lysosome/endosome membranes (Takeda et al., 2003). Pathogens that have invaded the cytosol are recognised by NLRs (NOD like receptor family, NOD nucleotide-binding and oligomerization domain) (Rasmussen et al., 2009). NOD1 and NOD2 are the best-characterized NLRs recognizing γ-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) found in bacterial peptidoglycan, respectively (Akira et al.,

2006). For detection of viral dsRNA in the cytosol, where viral replication takes place, retinoid acid inducible gene-I (RIG)-like receptors (RLRs) are responsible. DNA in cytoplasm is detected on similar principles by DNA-dependant activator of IFN regulatory factors (IRFs) (DAI) (Rasmussen et al., 2006).

A second approach used by the innate immune system next to recognising PAMPs, is to detect immunologic danger in the form of damage-associated molecular patterns (DAMPs). These molecules are released during cell lysis and tissue damage that occurs in the context of both infectious and sterile inflammation.

2.1.2 Toll-like receptors

Toll-like receptors (TLRs) are a family of type I transmembrane signalling receptors, which are one of the most important receptors for discrimination among groups of pathogens. TLRs exist as dimeric proteins, either being heterodimers or homodimers. The ectodomain of TLRs are composed of leucine-rich repeat motifs, whereas the cytosolic component, called Toll/IL-1 receptor–like (TIR) domain, is involved in signalling.

In humans, TLRs are present on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and on non-immune cells such as fibroblasts and epithelial cells. The expression of TLRs is not static but is rather modulated rapidly in response to pathogens, a variety of cytokines and environmental stress (Akira et al., 2006). Furthermore, TLRs can be divided into two subgroups depending on their cellular localisation. One group is composed of TLR1, TLR2, TLR4, TLR5 and TLR6, which are present on cell surfaces and recognise mainly microbial membrane components such as lipids, lipoproteins and proteins. The other group is composed of TLR3, TLR7, TLR8 and TLR9, present mainly in intracellular vesicles such as the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes, where they recognise microbial nucleic acids (Kawai and Akira, 2010).

Individual TLRs recognise a distinct but limited repertoire of conserved microbial products (Table 1).

Table 1: TLR receptors, their ligands and effect of activation (adapted from Takeda et al., 2003)

Tabela 1: TLR receptorji, njihovi ligandi in posledice aktivacije *(prilagojeno po Tekada et al., 2003)*

TLR	Functional receptor	Ligand of TLR	Immune response
TLR1/2	Heterodimers of TLR2-TLR1	Tri-acyl lipopeptides	Secretion of inflammatory cytokine(s)
TLR2	Heterodimers of TLR2-TLR1 and TLR2-TLR6	Lipoprotein/lipopeptides Peptidoglycan, lipoteichoic acid, porins, lipoarabinomannan, glicolipid, zymosan, host HSP70, glycoinositolphospholipids, hemagglutinin protein	Secretion of inflammatory cytokine(s)
TLR3	Homodimer[a]	Double-stranded RNA (mainly from viruses)	Induces type I interferon (IFN-α/IFN-β)
TLR4	LPS-LPS binding protein complex associated with CD14 and MD-2	LPS, host HPS60, HPS70, fibronectin, oligosaccharides of hyaluronic acid, polysaccharides fragments of heparin sulfate and fibrinogen	Induces secretion of inflammatory cytokines and chemokines
TLR5	Homodimer	flagellin	Inflammatory cytokines
TLR6/2	Heterodimer TLR2-TLR6	Di-acyl lipopeptides	Inflammatory cytokines
TLR7	Not known	Synthetic compounds like loxoribine (guanosine analog), imidazoquinoline, and bropirimine, synthetic poly(U) RNA, certain small interfering RNAs, ssRNA	Secretion of inflammatory cytokine, type I IFN and DC maturation
TLR8	Homodimer[b]	Resiquimod, R-848, ssRNA	Not known
TLR9	homodimer (localised in endosomal compartment)	Unmethylated CpG DNA, crystal hemozoin	Activates Th1 type immune response, induces proliferation of B-cell, activates macrophage and DCs
TLR10	Heterodimer TLR2-TLR10[c]	triacylated lipopeptides[c]	Not known

[a] Wang et al., 2010

[b] Zhu et al., 2009

[c] Guan et al., 2010

The first TLR member to be discovered was TLR4, a receptor for bacterial lipopolysaccharide (LPS), the outer membrane component of Gram-negative bacteria. TLR2 recognises peptidoglycan, in addition to the lipoproteins and lipopeptides of Gram-positive bacteria and mycoplasma lipopeptides. TLR2 also appears to collaborate with TLR1 and TLR6 (forms heterodimers) to discriminate between the molecular structures of diacyl and triacyl lipopeptides, respectively. Furthermore, TLR2 also binds other non-TLR receptors, like dectin-1 for recognition of zymosan, a cell-wall preparation of yeast (Kawai and Akira, 2005). Additionally, TLR2 binds to the C-type lectin that binds

fungus β-glucan and induces its internalisation (Kawai and Akira, 2010). TLR5 recognises flagellin, the major protein component of bacterial flagella.

The synthetic imidozoquinoline-like molecules imiquimod and resiquimod, guanosine analogs such as loxoribine and single stranded RNA have potent antiviral activities and activate TLR7. TLR8 is closely related to TLR7 and a transfection study has demonstrated that human TLR8 confers responsiveness to resiquimod. The structural similarities between these compounds and ribonucleic acids suggest the possibility that both TLR7 and TLR8 are responsible for virus detection. TLR9 mediates the recognition of the unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs found in bacteria and DNA viruses. TLR9 also recognises other molecules, like hemozion, a hydrophobic heme polymer derived from digested host hemoglobin. TLR3 is involved in recognition of double-stranded (ds) RNA generated during virus replication and has a protective role against viral infection (Turvey and Broide, 2010). TLR10 has been only recently characterised and studies indicate common sensing properties with TLR1, thus recognising triacylated lipopolypeptides (Guan et al., 2010). Collectively, the complete TLR family allows the host to detect infection by most types of microbial pathogens. The activated receptor interacts with a range of downstream signalling molecules to activate an inflammatory cascade (Turvey and Broide, 2010).

Apart from ligands of microbial origin, some TLRs also recognise host molecules like heat-shock proteins and many fragments of extracellular matrix proteins. The presence of these molecules is usually a sign of damaged tissue.

2.1.3 TLR5

TLR5 is a homodimeric receptor, responsible for recognition and response to flagellin. Flagellin is the major protein constituent of bacterial flagella, the motility apparatus used by many microbial pathogens, and is a potent activator of innate immune response. TLR5 specifically recognises the constant domain D1 (the central α helix chain) of flagellin, which is relatively conserved among different species. TLR5 is present on epithelial cells, monocytes, and immature DCs. Since TLR5 is positioned basolaterally on intestinal epithelia, flagellin is recognised by the host only when bacteria have invaded the epithelial barrier (Akira et al., 2006).

In spite of its cytoprotective attributes, the ability of TLR5 signalling to drive inflammation is likely potentially dangerous to its host. Non controlled activation can result in

overproduction and secretion of pro-inflammatory cytokines, triggering an exaggerated immune response, which may result in host tissue damage. TLR5 activation thus also triggers several mechanisms for reduction of pro-inflammatory gene expression and additional TLR5 activation (Vijay-Kumar et al. 2007).

2.1.4 TLR5 signalling cascade

After flagellin binding, TLR5 dimerises and undergoes conformational changes required for the recruitment of TIR-domain-containing adaptor molecule myeloid differentiation primary-response protein 88 (MyD88) to the intracellular TIR domain of the receptor, which in turn recruits IL-1R-associated kinase (IRAK) 4, thereby allowing the association of IRAK1. IRAK4 then induces the phosphorylation of IRAK1, thus enabling TNFR-associated factor 6 (TRAF6) binding. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with transforming-growth-factor-β-activated kinase (TAK1), TAK1-binding protein (TAB) 1 and TAB2 at the plasma membrane, which induces phosphorylation of TAB2 and TAK1. IRAK1 is degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1 and TAB2) translocates to the cytosol, where it associates with the ubiquitin ligase ubiquitin-conjugating enzyme 13 (UBC13) and ubiquitin-conjugating enzyme E2 variant 1 (UEV1A). This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. TAK1, in turn, phosphorylates both mitogen-activated protein (MAP) kinases and the inhibitor of nuclear factor – κB (IκB)-kinase complex (IKK complex), which consists of IKK-α, IKK-β and IKK-γ, also known as IKK1, IKK2 and nuclear factor -κB (NF-κB) essential modulator (NEMO), respectively. The IKK complex then phosphorylates IκB, an inhibitor protein bound to NF-κB, which leads to its ubiquitinylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes (Akira and Tekada, 2004). TLR5 activated genes include those with direct antibacterial function (e.g. defensins), immune cell chemoattractants, cytokines and a number of more general stress-induced genes such as heat-shock proteins (Vijay-Kumar et al. 2007).

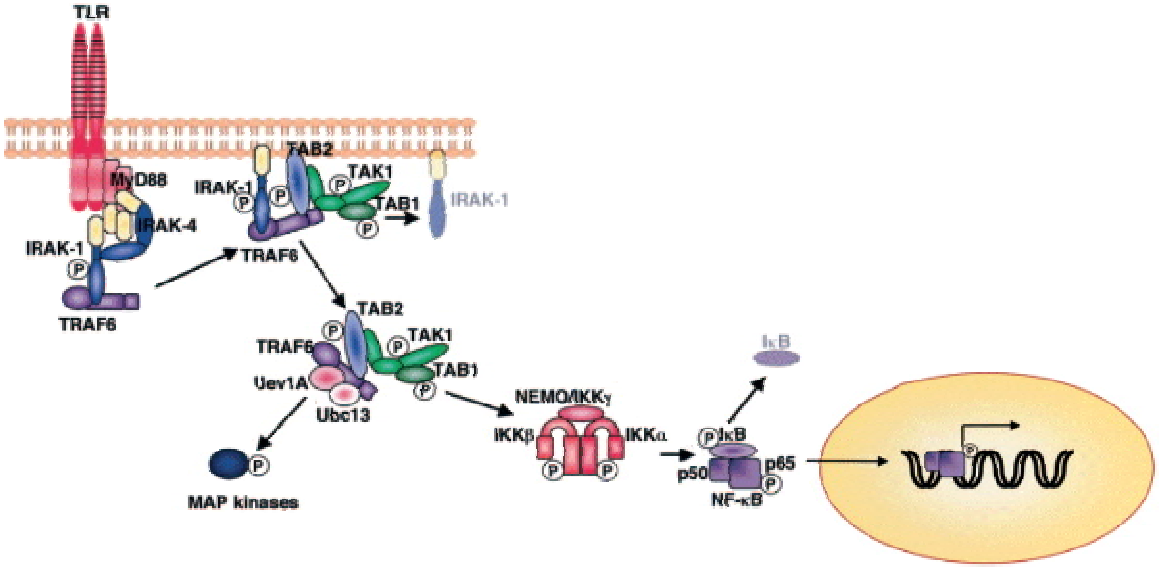

Figure 1: TLR5 signalling pathway. See the text for description (adopted from Akira, 2003)

Slika 1: Signalizacijska kaskada receptorja TLR5 (prilagojeno po Akira, 2003)

2.2 THE CELLULAR MICROENVIRONMENT

The cellular microenvironment consists of all factors in the immediate vicinity of the cell. Only those that are detectable can influence cell behaviour, thus only molecules, which bind to a cell receptor and trigger activation, or diffuse into the cell and alter *e.g.* metabolism, play a role. The receptor repertoire differs from cell type to cell type, being dependant also on the state of differentiation, stage of the cell cycle and other molecules present in the microenvironment. Certain signals are constantly present and are required for normal cell function, like signals inhibiting apoptosis or signals from extracellular matrix components, reporting the state of cell attachment, while others are present only temporarily and alter cell usual gene expression and metabolism.

Physical and chemical conditions, like temperature, pH, oxygen conditions and the amount of water available, can also affect cell behaviour by altering the solubility and activity of molecules, especially proteins. A change in these conditions can also induce a change in gene transcription in an attempt to nullify the effects of suboptimal conditions on cell metabolism.

2.2.1 The extracellular matrix

The extracellular matrix (ECM) is a network of macromolecules, composed of a variety of polysaccharides and proteins secreted by the cell. The major components of the ECM include cell adhesive or anti-adhesive molecules, such as fibronectin, vitronectin, laminin and tenascin and, on the other hand, the structural components, such as collagens and elastin, and proteoglycans, a complex array of proteins with glycosaminoglycans side-chains. ECM molecules interact with each other and with their specific receptors on the cell surface (Lin and Bissell, 1993), mostly integrins. Integrins are heterodimeric transmembrane receptors that also enable a two-way communication between the intracellular and extracellular environment. Activation and clustering of integrins upon binding to ECM proteins initiate focal adhesion formation and the activation of cytoskeletal signalling cascades involved in cell growth, proliferation, migration, differentiation and gene expression. The combinations of α and β integrin subunits that forms the functional heterodimer largely determine the binding of integrins. At least 17 α and 8 β subunits have been determined so far, and they associate non-covalently to form more than 20 heterodimers with various signalling and substrate binding properties (Lukashev and Werb, 1998, Barczyk et al., 2009).

The ECM is also the origin of tension forces, which can physically alter the conformation of extracellular receptor complexes, stretching or compressing domains within these adhesion complexes, and therefore eliciting alterations in the structure and function of the ECM receptor complex to actively influence signalling. Forces can also modify the activity and function of other membrane complexes such as growth factor receptors, cytokine receptors, ion channels and cell-cell junctional complexes (Lopez et al., 2008).

The extracellular matrix can also act as a molecular filter, preventing the passage of macromolecules, or as a reservoir for growth factors and cytokines. Binding to the ECM could significantly affect the bioavailability of these cytokines: growth factors and other intercellular mediators are probably secreted in a quick burst after a given stimulus, which would cause a sharp local increase in concentration of the mediator, followed by its rapid disappearance. Binding to the ECM retains some of the mediator locally and enables a gradual release (Schnaper and Kleinman, 1992).

2.2.2 Hormones and growth factors

Hormones and growth factors are molecular messengers, usually released from specialised cells and tissues and mainly utilized for slower, long-term transmission of signals. Endocrine hormones are carried by the blood to target structures in all parts of the body, while paracrine hormones only act on cells in the immediate vicinity of the cells from which they were released. Cells, that have high affinity receptors for specific hormones, can respond to these hormones and are called target cells. Because of the extremely high affinity between the hormone and its cognate receptor, hormones can be active in very low concentrations. The secretion of the hormones is under positive and negative feedback control, thus maintaining optimal concentration levels.

Glycoprotein and peptide hormones, like insulin and growth hormones, bind to cell surface receptors, leading to release of secondary messengers that forward the signal within the cell. Their membrane receptors are usually coupled to G proteins. The triggered signalling cascade can be modified (inactivated or amplified) by other signals or pathways. Steroid and thyroid hormones are lipid-soluble substances and can freely penetrate the cell membrane, activating an intracellular receptor. This binding leads to the dissociation of the inhibitory proteins from the receptor and the hormone-receptor protein complex then migrates to the cell nucleus, where it induces or inhibits the transcription of certain genes.

Hormones work closely with the nervous system to regulate digestion, metabolism, growth, maturation, physical and mental development, reproduction, adaptation and homeostasis. Hormones exhibit those functions by altering enzyme activity and their transcription, by controlling transport processes, proliferation, apoptosis and secretion of other regulatory molecules (Despopoulos and Silbernagl, 2003).

2.2.3 Cytokines and chemokines

Cytokines and chemokines are soluble mediators of the immune system but also of other cell types (*e.g.* intestinal epithelial cells). They are low molecular weight proteins that mediate cell growth, inflammation, immunity, differentiation, migration and repair. Because they regulate the amplitude and duration of the immune inflammatory response, cytokines and chemokines must be produced in a transient and tightly regulated manner. Unlike endocrine hormones, the majority of cytokines normally act locally in a paracrine or even autocrine fashion. They are mostly produced by cells of the immune system, but also by non-lymphoid cells, triggered by bacterial products. Cytokine receptors have a really high affinity for their ligands, so femtomolar concentrations are enough to induce changes in the pattern of RNA and protein synthesis.

Cytokines work in complex networks; one cytokine may trigger or dampen the production of another, interactions may occur through trans-modulation of the receptor for another cytokine and through synergism or antagonism of two cytokines acting on the same cell. The final response of the cell is thus dependant on the set of cytokines, present in a certain moment.

Chemokines are cytokines enhancing the recruitment of immune cells to the site of inflammation. These can be produced by a variety of cell types and are divided into four families based on their structure: CXC, CX3C, CC and C chemokines. Chemokines bind to G-protein-coupled transmembrane receptors. Despite the fact that a single chemokine can sometimes bind to more than one receptor, and a single receptor can bind several chemokines, many chemokines exhibit a strong tissue and receptor specificity. They play important roles in inflammation, lymphoid organ development, cell trafficking, cell compartmentalization within lymphoid tissues, Th1/Th2 cell development, angiogenesis and wound healing.

2.2.4 Metabolites and cell secretions

The cellular interstitial space is the area that surrounds cells of a given tissue and is filled with interstitial fluids. The main component of this fluid is water, along with solutes like amino acids, sugars, fatty acids, coenzymes, transport proteins, proteins of immune system (complement, antibodies), proteins for coagulation, vitamins, hormones, neurotransmiters, salts (sodium, potassium, calcium and chloride), and waste products, like creatinine, uric acid and ammonium salts. The composition is regulated by body homeostasis in order to keep the right electrolyte and nutrient balance. However, although the basic composition of these fluids remains the same, its composition is tissue specific. The fluid is constantly being renewed by new plasma from blood vessels thus keeping the concentration of waste products and other cell secretions low, but nevertheless, those molecules can still influence the cells.

Epithelial cells, especially those in mucosa, are also in contact with the external environment including bacteria. Commensal bacteria have evolved mechanisms to avoid detection by the host immune system and induce the secretion or secrete metabolites and other molecules that directly or indirectly influence the immune system, and also substance, that prevent growth of other bacterial species.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Cell lines

Human embryonic kidney cell line 293 (HEK293) cells were used in all experiments in this research. This cell line has a functional TLR5 and can respond to flagellin. The cell line was kindly provided by Dr. B. van der Burg (Hubrecht Laboratory, Utrecht, The Netherlands).

3.1.2 Expresion plasmids

For this research, pFlag-hTLR5, p4xNF-kB-luciferase, pTK-LacZ and empty pFlag expression plasmids were used. pFlag-hTLR5 and the empty pFlag plasmids were kindly provided by Dr. M.R. de Zoete (Utrecht University, Utrecht, The Netherlands) (Keestra et al., 2007); normalisation vector pTK-LacZ and the p4xNF-kB-luciferase reporter plasmid (Rodriguez et al., 1999) were provided by Dr. Bart van der Burg (Hubrecht Laboratory, Utrecht, The Netherlands).

3.1.3 Reagents

3.1.3.1 Microenvironmental factors

A list ob microenvironmental factors, tested for an effect on TLR5 dependant NF-κB activation is given below.

Table 2: Microenvironmental factors tested in this research

Tabela 2: Seznam testiranih sestavin mikrookolja

ECM components	
Poly-L-lysine solution (PLL)	Sigma-Aldrich (St. Louis, MO, USA)
Mucin from bovine submaxillary glands	Sigma-Aldrich (St. Louis, MO, USA)
Collagen VI	Sigma-Aldrich (St. Louis, MO, USA)
Collagen IV	Sigma-Aldrich (St. Louis, MO, USA)
Human tenascin	InvivoGen (San Diego, CA, USA)

'*continued overleaf*'

'continuation'

Cytokines	
Recombinant human GM-CSF	PeproTech (Rocky Hill, NJ, USA)
Human IL-1β	PeproTech (Rocky Hill, NJ, USA)
Recombinant Human TNFα	BD Biosciences (Breda, the Netherlands)
Recombinant Human IL-10	BD Biosciences (Breda, the Netherlands)
Human IL-12	BioSource Europe (S.A., Nivelles, Belgium)
Human IL-6	BioSource Europe (S.A., Nivelles, Belgium)
Human IL-4	Miltenyi Biotec GmbH (Germany)
Hormones and metabolites	
Human insulin	Sigma-Aldrich (St. Louis, MO, USA)
Human epidermal growth factor	Sigma-Aldrich (St. Louis, MO, USA)
Sodium butyrate	Sigma-Aldrich (St. Louis, MO, USA)
Sodium proprionate	Sigma-Aldrich (St. Louis, MO, USA)
Sodium acetate trihydrate	Sigma-Aldrich (St. Louis, MO, USA)
D(+)-glucose	Merck (Germany)
Role of the properties of the cell membrane in TLR5 activation	
Methyl-β-cyclodextrin	Sigma-Aldrich (St. Louis, MO, USA)
Thymoquinone	Sigma-Aldrich (St. Louis, MO, USA)
Filipin III	Sigma-Aldrich (St. Louis, MO, USA)
Cytidine-5'-monophospho-N-acetylneuraminic acid sodium salt (CMP-NANA)	Sigma-Aldrich (St. Louis, MO, USA)
Matrix metalloproteinase inhibitor (Galardin)	Enzo Life Science (NZ, USA)
Ligands	
Flagellin from *Salmonella enteritidis*	Dr. M.R. de Zoete (Utrecht University, Utrecht, The Netherlands)

3.1.3.2 Chemicals and kits

A list of other chemicals, used in cell culturing and experiments is given below.

Table 3: Other chemicals used for this research

Tabela 3: Ostale uporabljene kemikalije

Cell culturing	
DMEM	PAA Laboratories (Germany)
Fetal Calf Serum (FCS)	PAA Laboratories (Germany)
Trypsin- EDTA	PAA Laboratories (Germany)
Experiments	
FuGene 6 Transfection reagent	Roche Diagnostic Systems
Dulbecco's PBS (pH 7,4) with Ca^{2+} and Mg^{2+}	PAA Laboratories
Dulbecco's PBS (pH 7,4) without Ca^{2+} and Mg^{2+}	PAA Laboratories
Reporter lysis buffer	Promega, Leiden, the Netherlands
Luciferase assay system	Promega
β-galactosidase assay	Promega
qRT-PCR	
RNA-Bee	Bio-Connect, Huissen, The Netherlands
DNase	Fermentas
One-Step qRT-PCR MasterMix kit for probe assays	Eurogentec
RNA-free water	Quiagen
Plasmid isolation	
QIAGEN HiSpeed Plasmid Midi Kit	Qiagen
LB medium	KD Medical (Maryland, USA)
Ampicillin	Sigma-Aldrich (St. Louis, MO, USA)
Other	
DMSO	Sigma-Aldrich (St. Louis, MO, USA)
BSA	Sigma-Aldrich (St. Louis, MO, USA)
Double distilled water	

3.1.3.3 PCR primers and probes

Primers and probes (Table 4) were designed using Primer Express software (Applied Biosystems) (de Zoete et al., 2010b). Probes, labeled with the reported dye carboxyfluorescein (FAM) and the quencher tetramethyl-6-carboxyrhodamine (TAMRA), were purchased from Eurogentec, while primers for human IL-8 and human β-actin were purchased from ISOGEN Biosciense BV and Invitrogen, respectively.

Table 4: qRT-PCR primers and probes used

Tabela 4: Začetni nukleotidi in sonde za qRT-PCR

Gene	Primer (orientation) or probe	Sequence (5'→3')
β-actin	Forward	ACCGAGCGCGGCTACAG
	Reverse	CTTAATGTCACGCACGATTTCC
	Probe	(FAM)-TTCACCACCACGGCCGAGC-(TAMRA)
IL-8	Forward	CTGGCCGTGGCTCTCTTG
	Reverse	CCTTGGCAAAACTGCACCTT
	Probe	(FAM)-CAGCCTTCCTGATTTCTGCAGCTCTGTGT-(TAMRA)
IL-1β	Forward	CGAATCTCCGACCACCACTAC
	Reverse	TCCATGGCCACAACAACTGA
	Probe	(FAM)-AGGGCTTCAGGCAGGCCGC-(TAMRA)

3.1.4 Laboratory equipment

A list of machines and other equipment used in this research is given below.

Table 5: Machines and other equipment used

Tabela 5: Uporabljene aparature in ostali pripomočki

Machines		
	Luminometer	TD-20/20, Turner Designs
	NanoDrop ND-1000 spectrophotometer	Thermo Scientific
	LightCycler 480 System	Roche, Woerden, the Netherlands
Other equipment		
	25 cm^2 tissue culture flasks	Corning
	48-well plate	Corning
	6-well plate	Corning
	12-well plate	Corning
	Eppendorf tubes	Eppendorf (Hamburg, Germany)

3.2 METHODS

3.2.1 Cell lines

HEK293 cells were maintained in DMEM supplemented with 5% heat-inactivated FCS (HI-FCS; heat-inactivated by incubation at 65 °C for 40 min), at 37 °C in a 10% CO_2-rich atmosphere and were routinely propagated in 25 cm^2 tissue culture flasks. Cells were propagated every third day when confluency of 85–100% was reached. Cells were detached by using 0.05% trypsin- EDTA and propagated in 1:3 and 1:4 dilutions.

3.2.2 Plasmid isolation

All plasmids were isolated from *E. coli* by using the QIAGEN HiSpeed Plasmid Midi Kit. A single colony from a freshly streaked selective plate was inoculated in a starter culture of 50 ml LB Broth containing a final concentration of 100 μg/ml ampicilli and left overnight at 37 °C with vigorous shaking (300 rpm). Bacterial cells were harvested by centrifugation at 3500 rpm for 15 min at 4 °C. The rest of the isolation was carried out according to the manufacturer's protocol.

3.2.3 One-step transient transfection

HEK293 cells were grown in a 48-well plate in DMEM + 5% FCS 24 h prior to transfection. Cells at 50–60 % confluence were transfected using FuGene 6 Transfection reagent according to the manufacturer's instructions. A transfection mixture of 3:1 lipid-to-DNA was prepared, using in total 500 ng of DNA per well. The total plasmid DNA mixture consisted of 167 ng of the reporter plasmid p4xNF-κB-luciferase, 167 ng of normalisation vector pTK-LacZ, and 167 ng of either pFlag-hTLR5 or empty control vector pFlag. The DNA-Fugene mixture was incubated for 30 min at room temperature. Per well, 20 μl of the mixture was added dropwise and mixed briefly by shaking the plate. After 48 h of incubation, the medium was replaced with fresh serum-free DMEM followed by stimulation of the cells.

3.2.4 Two-step transient transfection

HEK293 cells were first grown in a 6-well plate and transfected with 120 μl of the plasmid DNA transfection mixture as described above in the one step transient transfection. Twenty four hours post-transfection, cells were trypsinised, resuspended and seeded in a 48-well

plate which, when applicable, was coated with ECM components prior to seeding. After 24 h of incubation, medium was changed to serum-free DMEM and the cells were stimulated.

3.2.5 Luciferase and LacZ assays

TLR signaling was assessed using the NF-κB-luciferase reporter system. HEK293 cells were, when applicable, pre-incubated with different microenvironmental factors for 30 min and stimulated with flagellin. After 5 h incubation, cells were rinsed once with 0.5 ml of PBS with Ca^{2+} and Mg^{2+} and immediately lysed in 0.1 ml reporter lysis buffer. Firefly luciferase activity was measured with the luciferase assay system using a luminometer. For normalisation of the efficiency of transfection and cell numbers in individual wells, luciferase values were adjusted to β-galactosidase values as determined with the β-galactosidase assay, performed according to manufacturer's instructions. The corrected data are displayed as Relative luminescence units (RLU) or as a percentage of the RLU measured for the control non-stimulated auto-activating cells.

3.2.6 Coatings

In this study, cells were grown on different extracellular matrix components and other materials in 48-well plates by using the two step transient transfection method as described above. The following components were used: Poly-L-lysin (PLL), tenascin, mucin, collagen IV and VI.

Poly-L-lysin coating was prepared by incubating 0.5 ml 0.01% PLL solution in the wells for 5 min at room temperature. The solution was removed and plates were dried at room temperature overnight.

Tenascin was prepared by diluting the protein to 10 μg/ml in PBS with Ca^{2+} and Mg^{2+}. Per well, 0.1 ml Tenascin solution was added and incubated for 1 hour at 37 °C. After removal of the solution, wells were washed three times with PBS with Ca^{2+} and Mg^{2+}, followed by blocking with 1 mg/ml of BSA in PBS with Ca^{2+} and Mg^{2+} for 1 hour at 37 °C. After blocking wells were washed once with PBS with Ca^{2+} and Mg^{2+}.

The mucin solution was prepared in 2×PBS with Ca^{2+} and Mg^{2+} at a concentration of 1 mg/ml. 0.1 ml of the solution was added to wells and incubated 20 h at room temperature. Subsequently, wells were rinsed three times with PBS with Ca^{2+} and Mg^{2+} to remove unbound mucin and allowed to dry.

Collagen was diluted in 0.02 M acetic acid and added to the wells followed by 30 min incubation at 37 °C. Wells were washed with PBS. Plates were used immediately.

3.2.7 RNA isolation and quantitative RT-PCR

HEK293 cells were grown in DMEM + 5% FCS in a 12-well plate until 90-95% confluence was reached. Prior to stimulation with flagellin (15 ng/ml), medium was changed to serum free DMEM. After 3 h incubation with flagellin, cells were rinsed once with PBS with Ca^{2+} and Mg^{2+} and lysed with RNA-Bee. RNA was isolated according to manufacturer's instructions. The nucleotide yield was measured on the NanoDrop ND-1000 spectrophotometer. RNA was stored at –80 °C until use. Prior to quantitative reverse transcription-PCR (qRT-PCR), the RNA was treated with 1 µg of DNase per 1 µg of RNA for 30 min at 37 °C. The DNase was inactivated by adding EDTA (2.5 mM, final concentration) and heating at 65 °C for 10 min. RNA transcript levels were assessed by the LightCycler 480 System using a One Step qRT-PCR MasterMix kit for probe assays. Per reaction, 50 ng of DNase-treated RNA was used.
Real-time cycler conditions were 30 min at 48 °C, followed by 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The mRNA levels for the target gene (IL-8) corrected to those for the housekeeping gene (β–actin) were calculated by subtracting their corresponding Ct (threshold cycle) before and after treatment using the following formulae:

1) before treatment

$$\Delta Ct\ control = Ct\ target\ gene\ control - Ct\ \beta\text{-}actin\ control \quad \ldots (1)$$

2) after treatment

$$\Delta Ct\ treated = Ct\ target\ gene\ treated - Ct\ \beta\text{-}actin\ treated \quad \ldots (2)$$

The fold change in mRNA was determined by:

1) for positive $\Delta Ct(control) - \Delta Ct(treated)$ values:

$$Fold\ change = 2^{\wedge} (\Delta Ct(control) - \Delta Ct(treated)) \quad \ldots (3)$$

2) for negative $\Delta Ct(control) - \Delta Ct(treated)$ values:

$$Fold\ change = -\ [2^{\wedge} - (\Delta Ct(control) - \Delta Ct(treated))] \quad \ldots (4)$$

The experiment was performed twice.

4 RESULTS

4.1 SETUP AND VALIDATION OF THE EXPERIMENTAL PROTOCOL

4.1.1 The effect of FCS on NF-κB activity

All cell lines used were grown in different media containing FCS, which provides essential growth factors and other mediators indispensable for proper cell growth. To determine the actual effect of FCS on NF-κB activation following TLR5 signalling, HEK293 cells were transiently transfected with a pFlag-hTLR5 expression plasmid and stimulated with the TLR5 ligand flagellin (FliC) in medium either containing 5% heat inactivated (HI) or 5% non-HI serum. Serum free medium and cells transfected with an empty pFlag expression vector were used as a control.

Upon stimulation with flagellin all the HEK293 cells responded with increased NF-κB activity as expected. The response to flagellin was also seen in control cells, indicating the presence of a functional endogenous TLR5 in the HEK293 cells.

Both stimulated and non-stimulated cells in presence of 5% HI or non-HI FCS showed altered and more variable NF-κB activity among experiments compared to cells incubated with serum free DMEM: HI serum non-specifically induces NF-κB activation, while the stimulation of hTLR5 transfected cells resulted in lower activation in presence of non-HI FCS (Figure 2). Overall, we here show that the presence of serum either HI FCS or non-HI FCS is capable of altering the NF-κB activity. To exclude any effects of FCS which may alter cell responses all stimulations in the next experiments were performed in serum-free stimulation conditions.

The results also showed higher background levels of NF-κB activity in unstimulated cells transfected with pFlag-hTLR5, compared to cells transfected with an empty control vector. The HEK293 cells already possess TLR5, thus transfection with extra hTLR5 results in TLR5-dependent NF-κB activation most probably due to auto-activation of TLR5 receptors. Auto-activation, as flagellin independent TLR5 signalling pathway activity, will also be used as a read out method.

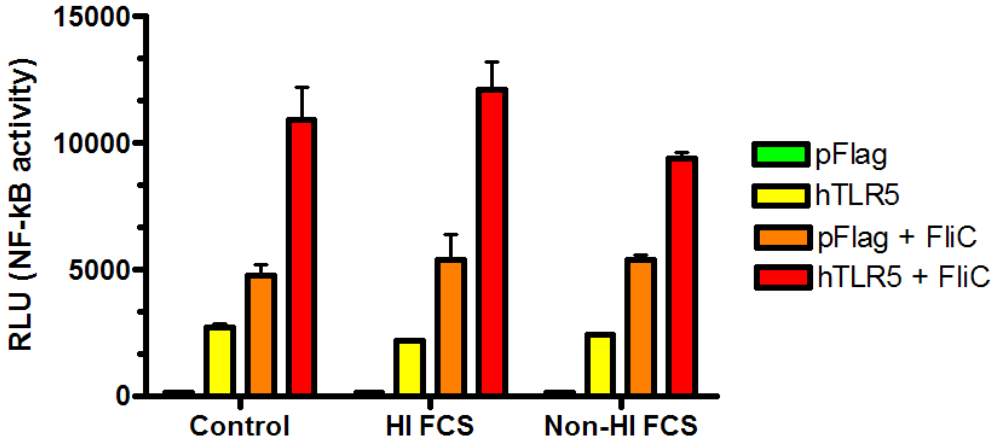

Figure 2: The NF-κB activity can be modified by FCS. HEK293 cells, transfected with an empty control pFlag or with a pFlag-hTLR5 expression plasmid were incubated in different media. Cells were stimulated with 20 ng/ml of flagellin (FliC) for 5 h or left unstimulated as control. The data depict the Relative luminescence units (RLU), correlating to NF-κB activity. Data are presented as mean ± SEM of RLU of both parallels of the same conditions. The experiment was performed twice giving similar results.
Legend: Control – cells incubated in DMEM only as a negative control; HI FCS – cells incubated in DMEM containing 5% heat inactivated FCS; Non-HI FCS – cells incubated in DMEM containing 5% FCS; pFlag – cells transfected with an empty pFlag vector (control); hTLR5 – cells transfected with pFlag-hTLR5; pFlag + FliC – cells, transfected with an empty pFlag vector, stimulated with falgellin; hTLR5 + FliC – cells, transfected with pFlag-hTLR5, stimulated with flagellin.
***Slika 2: FCS lahko vpliva na aktivnost NF-κB**. Celice HEK293, z vnešenim praznim kontrolnim plazmidom ali ekspresijskim plazmidom z zapisom za hTLR5, so bile inkubirane v različnih gojiščih. Aktivacija je podana v Relativnih enotah luminescence (RLU), kar kolerira z aktivnostjo NF-κB. Predstavljeni podatki so povprečje ± SD RLU za dve vzporedni meritvi v istem poskusu. Poskus je bil izveden dvakrat, rezultati obeh poskusov so podobni.*
Legenda: Control – celice inkubirane samo v DMEM, negativna kontrola; HI FCS – celice inkubirane v DMEM s 5% toplotno inaktiviranega FCS; Non-HI FCS – celice inkubirane v DMEM s 5% FCS; pFlag – celice transficirane s praznim plazmidom pFlag; hTLR5 – celice transficirane s plazmidom pFlag-hTLR5; pFlag + FliC - celice transficirane s praznim plazmidom pFlag in stimulirane s flagelinom; hTLR5 + FliC – celice transficirane s plazmidom pFlag-hTRL5 in stimulirane s flagelinom.

4.1.2 Auto-activating cells do not secrete NF-κB modulating mediators

TLR5 activation and subsequent dimerisation eventually triggers activation of NF-κB, an important transcription factor for induction of many regulatory cytokines. Since TLR5 auto-activation is also able to activate NF-κB it may also induce cytokine secretion.

Some cytokines, like TNFα and IL-1β which are secreted upon TLR activation, also activate NF-κB. Measuring NF-κB activity following TLR5 stimulation may not be solely the result of TLR5 activation but may be a cumulative event of auto-activation, cytokine induced activation and TLR5 activated NF-κB activity.

To assess whether auto-activating cells induce cytokines or other mediators capable of inducing NF-κB activation, cells transfected with an empty plasmid (HEK293-pFlag) were incubated in presence of supernatant of cells transfected with TLR5 (HEK293-TLR5, auto-activated supernatant) (C_2 to T). HEK293-pFlag incubated in HEK293-pFlag supernatant were used as a control (C_1 to C_2). Additionally, HEK293-TLR5 cells were incubated in supernatant of HEK293-pFlag (T to C_1). The experiment was performed in DMEM + 5% FCS in which cells, used for the experiment already grew for two days to allow accumulation of cell secretions. After 30 min pre-incubation with the supernatants, cells were stimulated with flagellin. Luciferase assays showed that NF-κB activity was only slightly higher in cells incubated with HEK293-TLR5 supernatant compared to the control

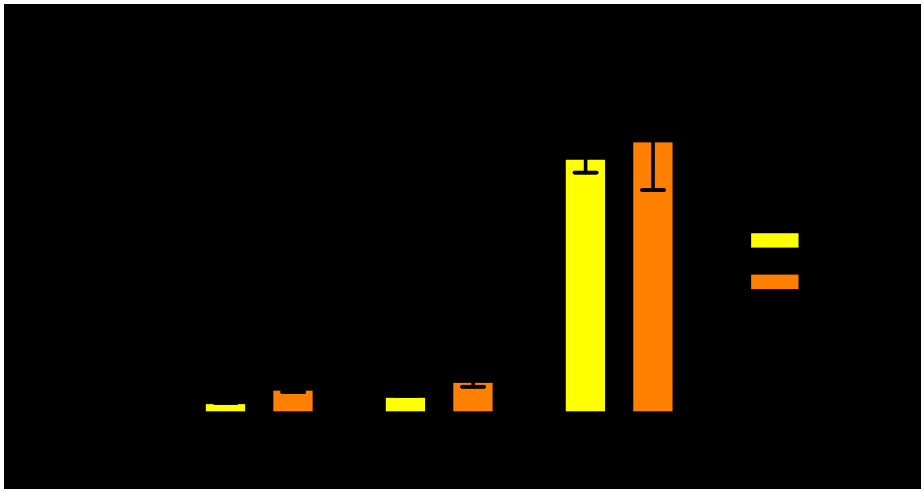

Figure 3: Auto-activating cells do not secrete mediators which signal via NF-κB. HEK293 cells were seeded in 48-well plate and transfected with control pFlag or pFlag-hTLR5 expression vectors. After 48 h, medium was collected and added to the cells in a different order as indicated. Cells were stimulated with 2 ng/ml FliC, non-stimulated cells were used as a control. After 5 h incubation, cells were analysed for NF-κB activity. Data represent Relative luminescence units (RLU), correlating to NF-κB activity. The data are presented as mean ± SEM of RLU of both parallels of the same conditions. The experiment was performed twice giving similar results.
Legend: C_1 to C_2 – control C_1 cells were incubated in supernatant of control C_2 cells, so in a supernatant of other cells transfected with the same empty vector; C_2 to T – C_2 cells were incubated in supernatant of auto-activating hTLR5 transfected cells; T to C_1 - hTLR5 transfected cells were incubated with C_1 supernatant; - FliC – not stimulated cells; + FliC – stimulated cells.
Slika 3: Avtoaktivirajoče celice ne izločajo mediatorjev, ki signailzirajo preko NF-κB. *Celice HEK293 smo nacepili na mikrotitrsko ploščo z 48 luknjicami in transficirali s praznim kontrolnim plazmidom ali ekspresijskim plazmidom z zapisom za hTRL5. Po 48 urah inkubacije smo odstranili gojišče in ga takoj vrnili v luknjice v v drugačnem zaporedju, kot je nakazano. Celice so bile stimulirane z 2 ng/ml flagelina in inkubirane za 5 ur. Za kontrolo smo uporabili nestimulirane celice. Aktivacija je podana v Relativnih enotah luminescence (RLU), kar korelira z aktivnostjo NF-κB. Predstavljeni podatki so povprečje ± SD RLU za dve vzporedni meritvi v istem poskusu. Poskus je bil izveden dvakrat, rezultati obeh poskusov so podobni.*
Legenda: C_1 to C_2 – kontrolne celice C_1 smo inkubirali v supernatantu kontrolnih celic C_2, torej v supernatantu celic, transficiranih z enakim praznim plazmidom; C_2 to T – kontrolne celice C_2 smo inkubirali v supernatantu avtoaktivirajočih celic; T to C_1 – avtoaktivirajoče celice smo inkubirali v supernatantu kontrolnih celic C_1; - FliC – nestimulirane celice; + FliC – stimulirane celice.

cells, indicating that auto-activating cells apparently secrete some mediators, but in low quantities (Figure 3). It may also be that secreted mediators directly bind to their receptors, inducing their autocrine and paracrine effects, and are thus not present in the supernatant or are in lower concentration.

To determine cytokine gene transcription levels, a qRT-PCR of IL-8 and IL-1β was performed on non-transfected cells, HEK293-pFlag and HEK293-TLR5 cells. The results showed that there was no IL-8 and IL-1β mRNA present in all non-stimulated cells, while stimulation with flagellin induced mRNA transcription of IL-8 (Figure 4), but not IL-1β (data not shown). IL-8 mRNA levels in non-transfected cells and HEK293-pFlag were the same, indicating that transfection itself does not alter cell response to flagellin, while the response of HEK293-TLR5 cells to flagellin was stronger, as expected due to more TLR5 receptors present on the surface. These results are in line with the NF-κB responses in Figure 3: auto-activating cells do not secrete mediators such as IL-8 or IL-1β which may modulate via NF-κB. Moreover, the results also suggest that auto-activation probably works via a different process compared to activation upon TLR-ligand stimulation.

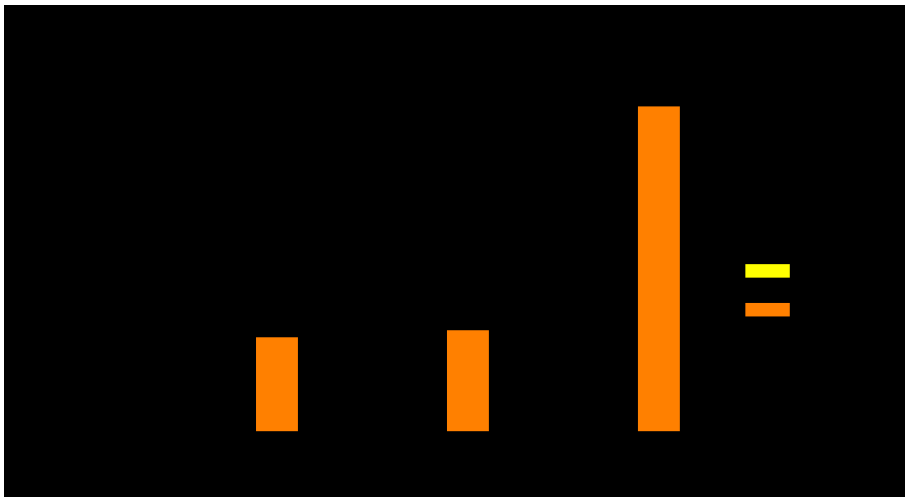

Figure 4: IL-8 mRNA induction in non-transfected HEK293 and HEK293 transfected with control vector pFlag orpFlag-hTLR5. Cells were left unstimulated or were stimulated with 15 ng/ml flagellin (FliC) for 3 h. IL-8 mRNA transcripts were analysed by qRT-PCR and are presented as fold increase in mRNA levels in stimulated versus non-stimulated (control) cells. The correction was done separately for non-transfected and transfected cells. The experiment was performed twice giving similar results.
Legend: Non transfected – non transfected cells; pFlag – cells transfected with an empty control vector; hTLR5 – cells transfected with pFlag-hTLR5 plasmid; - FliC – not stimulated cells; + FliC – stimulated cells.
Slika 4: Indukcija transkripcije mRNA za IL-8 v netransficiranih HEK293 in HEK293 transficiranih s praznim kontrolnim vektorjem ali vektorjem z zapisom za hTLR5. *Celice smo stimulirali s 15 ng/ml flagelina (FliC) ali jih pustili nestimulirane kot kontrolo ter jih inkubirali 3 h. Transkripte mRNA za IL-8 smo kvantificirali s qRT-PCR in jih predstavili kot veckratno povečanje v količini mRNA med stimuliranimi in nestimuliranimi (kontrolnimi) celicami. Popravek je bil narejen ločeno za netransficirane in transficirane celice. Poskus je bil izveden dvakrat, rezultati obeh poskusov so podobni.*
Legenda: Non transfected – netransficirane celice; pFlag – celice, transficirane s praznim kontrolnim plazmidom; hTLR5 – celice transficirane s plazmidom z zapisom za hTLR5; - FliC – nestimulirane celice; + FliC – stimulirane celice.

4.1.3 Method evaluation and validation

In order to determine the effect of certain microenvironmental factors, like extracellular matrix components (ECM), cells needed to be cultured in coated wells. When using the standard one step transient transfection method (OSM), cells were seeded into coated wells prior to transfection. However, the presence of coating could affect transfection efficiency by binding to DNA-FuGene 6 complexes or by altering susceptibility of the cells for transfection.

To circumvent this problem, a two step transient transfection method (TSM) was established, enabling the cells to be transfected before seeding onto coated wells. In this method, cells were first seeded in 6-well plates and transfected with the standard transfection mixture. After 24 h, cells were trypsinised, resuspended and seeded in coated wells of 48-well plate (Table 6).

Here, a comparative experiment was performed using the OSM and TSM. Cells were either seeded in a 6-well plate or directly in poly-L-lysine (PLL) pre-coated 48-well plate. Both plates were transfected simultaneously after 24 h. Twenty-four hours post-transfection, cells in 6-well plate were trypsinised, resuspended and seeded in the same pre-coated 48-well plate. After an additional 24 h, cells were lysed and LacZ and Luciferase assays were performed. Non-coated wells were used as a control in both transfection methods.

Table 6: Comparison of one step (OSM) and two step (TSM) transient transfection methods.
Tabela 6: Primerjava med metodo transfekcije v enem koraku (OSM) in metodo transfekcije v dveh korakih (TSM)

Day	OSM	TSM
1	Seeding cells (48-well plate)	Seeding cells (6-well plate)
2	Transfection, incubation	Transfection, incubation
3	Incubation	Trypsinisation, re-plating cells (48-well plate)
4	Stimulation, lysis	Stimulation, lysis

The results showed that the transfection efficiency (LacZ values) were similar and in the same range among the two methods in cells grown on coated or non-coated wells (data not shown), indicating that the same transfection efficiency can be achieved using the OSM or TSM. Luciferase measurements showed that pFlag-hTLR5 transfected cells still auto-activate after trypsinisation in the TSM, although background and auto-activation NF-κB activity were slightly lower, perhaps due to a certain degree of damaged receptors by trypsinisation, which were not replaced in the following 24 h. In general, from both methods the NF-κB activity in cells, grown on PLL, was lower compared to the same

treatments in non-coated wells (Figure 5). In OSM, part of the decrease in activity might be due to lower transfection efficiency, but the difference between TSM in PLL-coated and non-coated wells is most likely due to the effect of PLL coating.

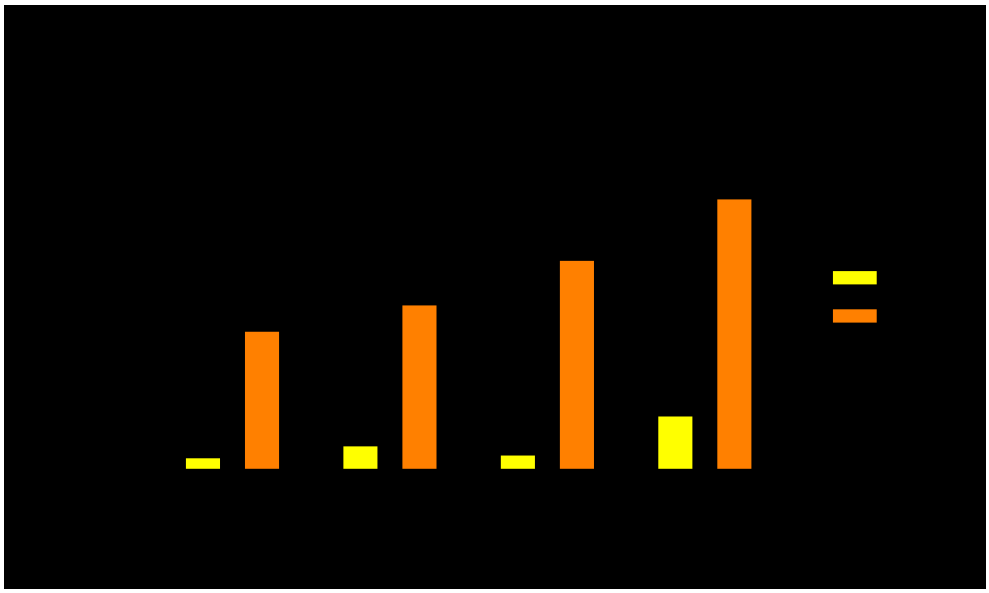

Figure 5: Comparison of the two step transient transfection method to the one step transfection method. The efficiency of the novel two step transient transfection method (TSM) was compared to standard one step transfection method (OSM) by performing both methods at the same time. For OSM, cells were seeded directly into PLL coated 48-well plate, while for TSM, cells were first seeded in a 6-well plate, transfected, and 24h post-transfection resuspended by trypsinisation and seeded in the same PLL coated 48-well plate. Non-coated wells were used as a control. The experiment was done using hTLR5 auto-activating cells and pFlag transfected cells as a control. Data represents Relative luminescence units (RLU), correlating to NF-κB activity. Data are presented as mean ± SEM of RLU for duplicates in the same experiment. The experiment was performed twice giving similar results.
Legend: PLL-coated – cells grown in wells, coated with PLL; Non-coated – cells grown in non-coated wells; pFlag – cells transfected with an empty pFlag vector (control); hTLR5 – cells transfected with pFlag-hTLR5.
Slika 5: Primerjava transfekcijske metode v enem koraku (OSM) in metode v dveh korakih (TSM). *Učinkovitost na novo vzpostavljene metode TSM smo primerjali s standardno metodo OSM s sočasnim izvajanjem obeh metod. Celice za OSM smo nacepili direktno na mikrotitrsko ploščo z 48 luknjicami, prevlečeno s PLL, celice za TSM pa smo najprej nacepili na mikrotitrsko ploščo s 6 luknjami in vse celice sočasno transficirali. Celice za TSM smo 24 ur po transfekciji resuspendirali in nacepili na mikrotitrsko ploščo z 48 luknjicami prevlečeno s PLL. Celice v neprevlečenih luknjicah smo uporabili kot kontrole. Celice smo transficirali z vektorjem z zapisom za hTLR5 in praznim kontrolnim vektorjem. Aktivacija je podana v Relativnih enotah luminescence (RLU), kar korelira z aktivnostjo NF-κB. Predstavljeni podatki so povprečje ± SD RLU za dve vzporedni meritvi v istem poskusu. Poskus je bil izveden dvakrat, rezultati obeh poskusov so bili podobni.*
Legenda: PLL-coated – celice v luknjicah, prevlečenih s PLL; Non-coated – celice v neprevlečenih luknjicah; pFlag – celice transficirane s praznim plazmidom pFlag (kontrola); hTLR5 – celice transficirane s plazmidom pFlag-hTLR5.

Although NF-κB activity is lower in the TSM, auto-activation is still present, so the method proved suitable for the purpose of this research. In TSM transfection efficiency is the same as in OSM and resuspension of the cells after trypsinisation abolishes the

variation in transfection efficiency among wells. LacZ values in TSM correct only for differences in cell number among wells. For this reason, the TSM was used for all experiments in this research.

4.2 MICROENVIRONMENTAL MODULATION OF TLR5 FUNCTION

4.2.1 Extracellular matrix components

ECM is an important part of the cellular microenvironment providing support for cells and enabling many cell interactions, which can affect cell behaviour and function. Here, several ECM components were tested in order to assess their influence on TLR5-dependent NF-κB activity. The following ECM components and concentrations were used: mucin (1 mg/ml), human Tenascin (5 µg/ml), Collagen VI (9 µg/ml) and Collagen IV (9 µg/ml). PLL (0.01%), which is usually used for coatings of plates and microscope slides, was taken along as an additional adhesion molecule.

Twenty-four hours after seeding in the coated 48-well plate, the cells were first examined by microscope: cells grown on mucin were round and loosely attached, but still alive, indicating that HEK293 cells can not attach to mucin. Culturing cells on mucin resulted in loss of cells during subsequent cellular procedures (data not shown).

Cells cultured in other ECM coated wells grew better (up to 100% confluence) compared to the non-coated control (85-90% confluence) (data not shown), indicating that HEK293 could adhere to the tested ECM components and that this has an effect on cell proliferation.

Despite the fact that cells bound to the tested ECM components, luciferase assay results indicated that the ECM did not have an effect on auto-activation initiated NF-κB activity or background NF-κB activation (Figure 6). The effect of the selected components on flagellin induced NF-κB activation was determined with a flagellin concentration curve on pFlag transfected cells, thus stimulating only the endogenous TLR5. No evident differences in NF-κB activity were observed (Figure 7). Collagen type IV was also tested, showing same cell response (data not shown).

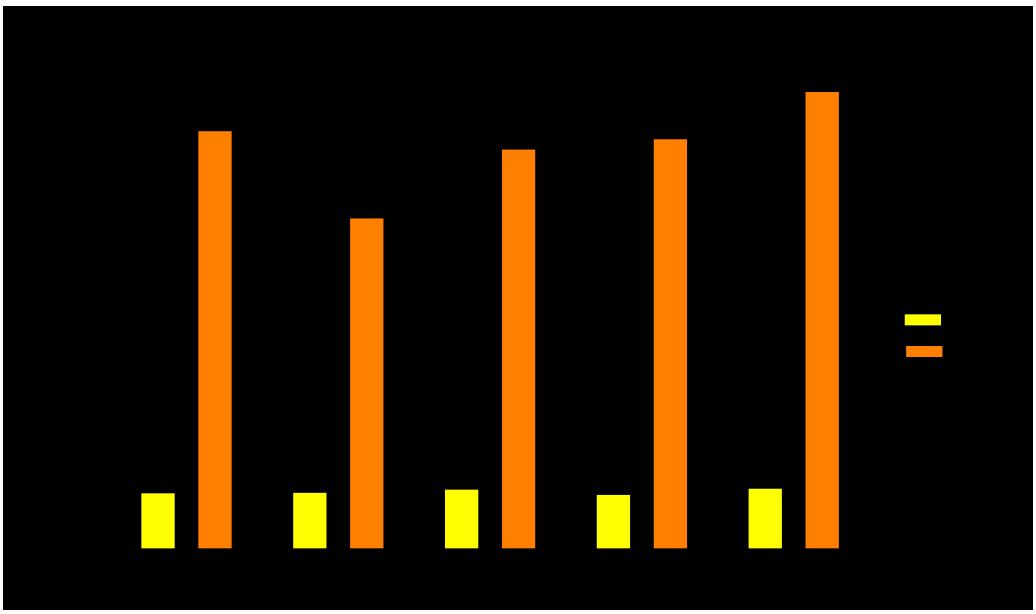

Figure 6: ECM components have little to no effect on hTLR5-dependent auto-activation. HEK293 were grown on different coatings for 24 h and analysed for NF-κB activity. Cells grown in non-coated wells were used as a control. Data are presented as mean ± SEM of cell responses compared to non-stimulated auto-activating cells from two experiments performed in duplicate.
Legend: Control – cells grown in non-coated wells, negative control; PLL – cels grown on PLL (0.01%); hTenascin – cells grown on human Tenascin (5 µg/ml); Collagen IV – cells grown on collagen IV (9 µg/ml); Collagen VI – cells grown on collagen VI (9 µg/ml); pFlag – cells transfected with an empty pFlag vector (control); hTLR5 – cells transfected with pFlag-hTLR5.
Slika 6: Komponente zunajceličnega matriksa imajo malo ali nič vpliva na avtoaktivacijo, pogojeno s hTLR5*. Celice HEK293 smo 24 ur gojili v luknjicah, prevlečenih z različnimi snovmi, in analizirali aktivnost NF-κB. Kot kontrole smo uporabili celice v neprevlečenih luknjicah. Aktivacija je podana v deležu aktivacije celic glede na avtoaktivacijo kontrolnih celic. Podano je povprečje razmerij ± SD štirih meritev (dva poskusa, v vsakem dve vzporedni meritvi).*
Legenda: Control – celice v neprevlečenih luknjicah, negativna kontrola; PLL – celice v luknjicah, prevlečenih s PLL (0,01%); hTenascin – celice v luknjicah, prevlečenih s človeškim tenascinom (5 µg/ml); Collagen IV – celice v luknjicah, prevlečenih s kolagenom IV (9 µg/ml); Collagen VI – celice v luknjicah, prevlečenih s kolagenom VI (9 µg/ml); pFlag – celice transficirane s praznim plazmidom pFlag (kontrola); hTLR5 – celice transficirane s plazmidom pFlag-hTLR5.

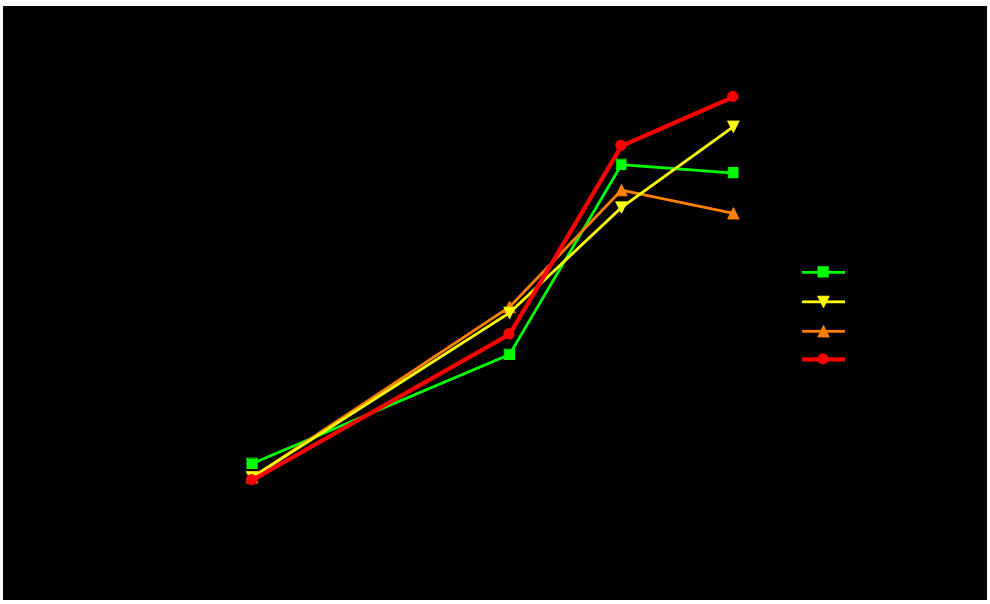

Figure 7: ECM components have no effect on flagellin-dependent NF-κB activation. HEK293 were grown on different coatings for 24 h and stimulated with increasing concentration of flagellin (FliC; 2 ng/ml, 20 ng/ml and 200 ng/ml). After 5 h incubation, cells were analysed for NF-κB luciferase activity. Cells grown in non-coated wells and not stimulated were used as a control. Data represent Relative luminescence units (RLU), correlating to NF-κB activity. Data presented are results from one of two experiments giving similar results.
Legend: Control – cells grown in non-coated wells, negative control; PLL – cels grown on PLL (0.01%); hTenascin – cells grown on human Tenascin (5 µg/ml); Collagen VI – cells grown on collagen VI (9 µg/ml).
Slika 7: Komponente zunajceličnega matriksa nimajo vpliva na aktivacijo NF-κB ob stimulaciji. Celice *HEK293 smo 24 ur gojili v luknjicah, prevlečenih z različnimi snovmi, in jih po 24 urah stimulirali z naraščajočimi koncentracijami flagelina (FliC; 2 ng/ml, 20 ng/ml in 200 ng/ml). Po petih urah inkubacije smo izmerili aktivnost NF-κB. Za kontrole smo uporabili celice v neprevlečenih luknjicah. Aktivacija je podana v Relativnih enotah luminescence (RLU), kar korelira z aktivnostjo NF-κB. Predstavljeni podatki so RLU enega od dveh izvedenih poskusov. Rezultati obeh poskusov so podobni.*
Legenda: Control – celice v neprevlečenih luknjicah, negativna kontrola; PLL – celice v luknjicah, prevlečenih s PLL (0,01%); hTenascin – celice v luknjicah, prevlečenih s človeškim tenascinom (5 µg/ml); Collagen VI – celice v luknjicah, prevlečenih s kolagenom VI (9 µg/ml).

4.2.2 Cytokines

In the cellular microenvironment many different cell secreted products such as cytokines, can be found. Cytokines are important soluble mediators of innate and adaptive immune responses. These molecules are mostly secreted by immune cells, but are also produced by non immune cells when activated by microbes. Usually they target immune cells, but they can also activate non-immune cells and prepare them for possible encounter with pathogens. In this section, several pro- and anti-inflammatory cytokines have been tested for their possible effect on TLR5-dependent auto-activation and TLR ligand induced NF-κB activation. The following cytokines were tested: IL-4 (0.5 μg/ml), IL-6 (2.7 ng/ml), IL-10 (2 ng/ ml), IL-12 (1.1 ng/ml), GM-CSF (0.2 μg/ml), TNFα (20 ng/ml) and IL-1β (0.1 ng/ml). Human TLR5 transfected cells and control pFlag transfected cells were pre-incubated with the cytokines for 30 min before flagellin stimulation.

The luciferase measurements showed that most of the tested interleukins (IL-4, IL-6, IL-10 and IL-12) and GM-CSF have no effect on the NF-κB activation either induced by auto-activation or flagellin-dependent activation (Figure 8 and 9).

TNFα and IL-1β are pro-inflammatory cytokines that signal also via NF-κB, thus activating it independently of TLR5. TNFα induced a really strong response, completely masking the TLR5- and flagellin-dependent NF-κB activation. IL-1β increased NF-κB activity in all four conditions, but the increase was much stronger in non-stimulated cells (100% increase in non-stimulated compared to 30% increase in stimulated cells compared to the same conditions in control cells) (Figure 9). Since the TLR5 and IL-1β signalling pathways largely use the same molecular pathways and the transcription factor NF-κB, the simultaneously signalling events may probably affect each other. It is difficult to dissect the effects of TNFα and IL-1β on TLR5 activation and signalling using our method.

Overall, the results indicate that, of the cytokines tested, TNFα and IL-1β influenced NF-κB activation, suggesting a direct interaction with the TRL5 signalling pathway either via auto-activation or ligand induced activation.

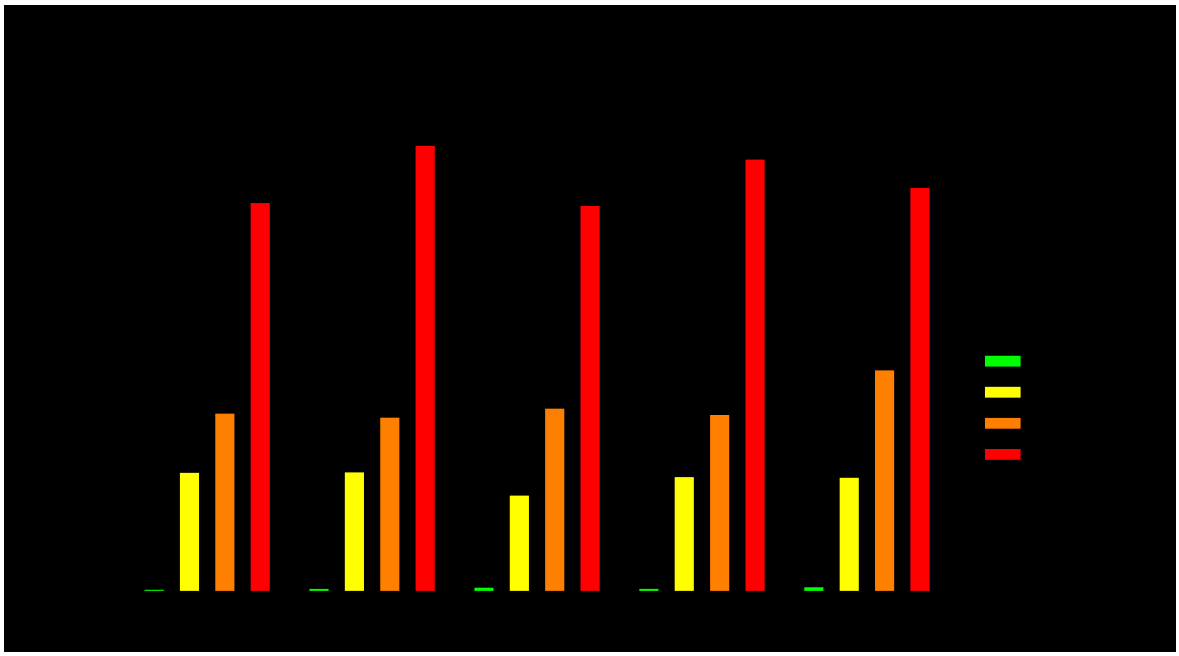

Figure 8: Cytokines IL-4, IL-6, IL-10 and GM-CSF do not affect TLR5-dependent NF-κB activation. pFlag-hTLR5 or control pFlag transfected cells were incubated in different cytokines for 30 min prior to stimulation with 20 ng/ml of flagellin (FliC). Data are presented as mean ± SEM of cell responses compared to non-stimulated auto-activating cells from duplicates in one experiment. Data presented are results from one of the two experiments giving similar results.
Legend: Control – cells incubated without a cytokine, negative control; IL-4 – cells incubated with interleukin-4 (0.5 µg/ml); IL-6 – cells incubated with interleukin-6 (2.7 ng/ml); IL-10 – cells incubated with interleukin-10 (2 ng/ ml); GM-CSF – cells incubated with granulocyte macrophage-colony stimulating factor (0.2 µg/ml) ; pFlag – cells transfected with an empty pFlag vector (control); hTLR5 – cells transfected with pFlag-hTLR5; pFlag + FliC – cells, transfected with an empty pFlag vector, stimulated with falgellin; hTLR5 + FliC – cells, transfected with pFlag-hTLR5, stimulated with flagellin.
Slika 8: Citokini IL-4, IL-6, IL-10 in GM-CSF nimajo vpliva na aktivacijo NF-κB, odvisno od TLR5*. Celice, transficirane s plazmidom z zapisom za hTLR5 ali kontrolnim plazmidom smo predhodno inkubirali z IL-4, IL-6, IL-10 ali GM-CSF za 30 min pred stimulacijo z 20 ng/ml flagelina (FliC). 5 ur po stimulaciji smo izmerili aktivnost NF-κB. Nestimulirane celice in celice, ki niso bile inkubirane s citokini, smo uporabili kot kontrole. Aktivacija je podana v deležu aktivacije celic glede na aktivacijo avtoaktivirajočih nestimuliranih kontrolnih celic. Podano je povprečje razmerij ± SD za dve vzporedni meritvi v enem poskusu. Poskus je bil izveden dvakrat, rezultati obeh poskusov so podobni.*
Legenda: Control – celice inkubirane brez citokinov, negativna kontrola; IL-4 – celice inkubirane z interlevkinom-4 (0,5 µg/ml); IL-6 – celice inkubirane z interlevkinom-6 (2,7 ng/ml); IL-10 – celice inkubirane z intelreukinom-10 (2 ng/ ml); GM-CSF – celice inkubirane z granulocitne in makrofagne kolonije stimulirajočim faktorjem (0,2 µg/ml); pFlag – celice transficirane s praznim plazmidom pFlag; hTLR5 – celice transficirane s plazmidom pFlag-hTLR5; pFlag + FliC - celice transficirane s praznim plazmidom pFlag in stimulirane s flagelinom; hTLR5 + FliC – celice transficirane s plazmidom pFlag-hTRL5 in stimulirane s flagelinom.

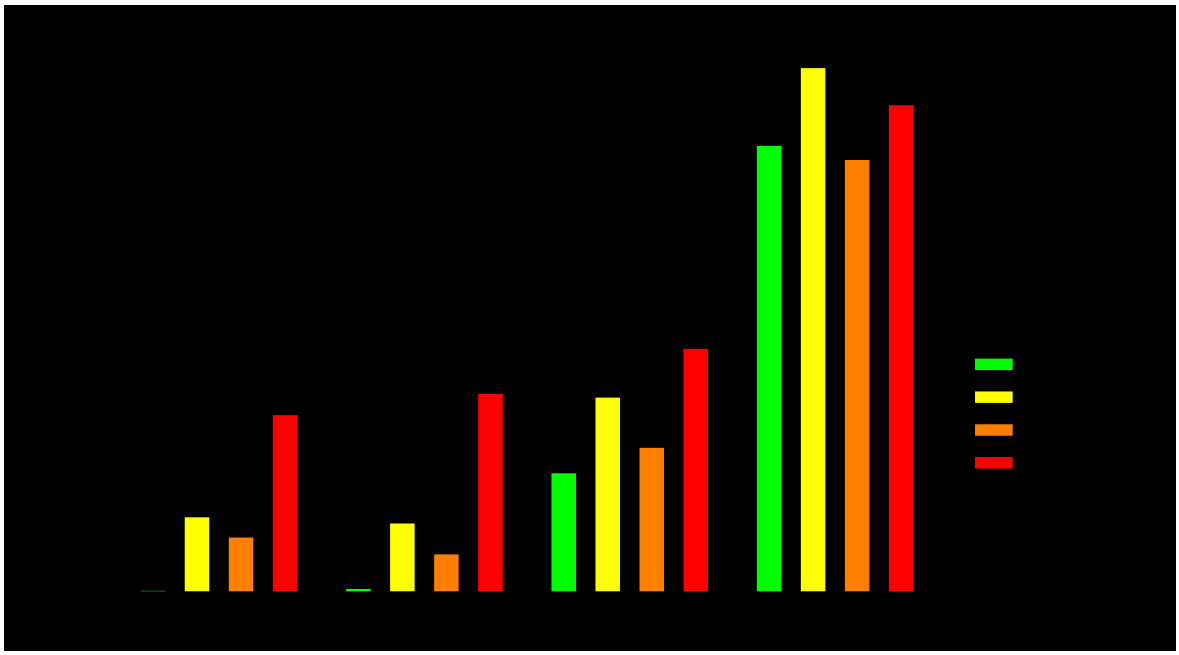

Figure 9: The effects of IL-12, TNFα and IL-1β on TLR5-dependent NF-κB activation. pFlag-hTLR5 or control pFlag transfected cells were incubated with different cytokines for 30 min prior to stimulation with 20 ng/ml of flagellin (FliC). Data are presented as mean ± SEM of cell responses compared to non-stimulated auto-activating cells from duplicates in one experiment. Data presented are results from one of the two experiments giving similar results.
Legend: Control – cells incubated without a cytokine, negative control; IL-12 – cells incubated with interleukin-12 (1.1 ng/ml); IL-1β – cells incubated with interleukin-1β (0.1 ng/ml); TNFκ – cells incubated with tumor necrosis factor α (20 ng/ ml); pFlag – cells transfected with an empty pFlag vector (control); hTLR5 – cells transfected with pFlag-hTLR5; pFlag + FliC – cells, transfected with an empty pFlag vector, stimulated with falgellin; hTLR5 + FliC – cells, transfected with pFlag-hTLR5, stimulated with flagellin.
Slika 9: Vpliv citokinov IL-12, TNFα in IL-1β na aktivacijo NF-κB, odvisno od TLR5*. hTLR5transficirane in kontrolne celice smo predhodno inkubirali z različnimi citokini za 30 min pred stimulacijo z 20 ng/ml flagelina (FliC). 5 ur po stimulaciji smo izmerili aktivnost NF-κB. Za kontrole smo uporabili nestimulirane celice in celice, ki niso bile inkubirane s citokini. Aktivacija je podana v deležu aktivacije celic glede na avtoaktivacijo nestimuluranih kontrolnih celic. Podano je povprečje razmerij ± SD za dve vzporedni meritvi v istem poskusu. Poskus je bil izveden dvakrat, rezultati obeh poskusov so podobni.*
Legenda: Control – celice inkubirane brez citokinov, negativna kontrola; IL-12 – celice inkubirane z interlevkinom-12 (1,1 μg/ml); IL-1β – celice inkubirane z interlevkinom-1β (0,1 ng/ml); TNFα – celice inkubirane s tumor nekroznim faktorjem α (20 ng/ ml); pFlag – celice transficirane s praznim plazmidom pFlag; hTLR5 – celice transficirane s plazmidom pFlag-hTLR5; pFlag + FliC - celice transficirane s praznim plazmidom pFlag in stimulirane s flagelinom; hTLR5 + FliC – celice transficirane s plazmidom pFlag-hTRL5 in stimulirane s flagelinom.

4.2.3 Metabolites and hormones

Besides being exposed to cytokines, cells are also surrounded by other cell secretions, like hormones and cellular metabolites, either host derived or of microbial nature. These metabolites can function as nutrient source for cells. One group of metabolites abundantly derived from commensal bacteria are the short chain fatty acids (SCFAs) butyrate, propionate and acetate. They have an impact on the metabolism of the host: acetate and propionate influence cholesterol production, propionate is also gluconeogenic (Wolever et al., 1991) whereas butyrate is a carbon source for colonocytes (Roediger et al., 1982). Another available energy source for cells is glucose.

Here, SCFAs and glucose were tested at a concentration of 10 mM. Transfected HEK293 cells were incubated with the metabolites for 30 min prior to flagellin stimulation. The data showed that acetate and glucose do not have an effect on measured NF-κB activation. The presence of butyrate and propionate increased flagellin-dependant NF-κB activation in pFlag control cells but had no effect on non-stimulated cells (Figure 10). Interestingly, butyrate and propionate did not enhance NF-κB activation in auto-activating hTLR5 transfected cells.

Next to the metabolites, two hormones, which are constantly present in the microenvironment, were tested. Insulin is a metabolic hormone, regulating glucose concentration and uptake, while epidermal growth factor (EGF) controls growth, proliferation and differentiation of epithelial cells.

Cells were pre-incubated with different range of hormones (concentration curves were performed in range of 0–140 μg/ml for insulin and 0–5 μg/ml for EGF). After 30 min preincubation, cells were stimulated with 2 ng/ml of flagellin for 5h. Figure 11 shows that increasing insulin or corresponding HCl concentration had no effect on the background of pFlag transfected cells or on flagellin induced NF-κB activation.

EGF, on the other hand, increased NF-κB activity in a dose dependent manner with the strongest effect on auto-activating pFlag-hTLR5 transfected cells stimulated with flagellin, while there was no effect on the background (pFlag) activity (Figure 12), indicating that EGF does not induce NF-κB activity on its own, but rather modulates the activity of already activated NF-κB. This is also evident from the fact, that the higher the basal NF-κB activity in control cells (0 μg/ml EGF) was, the higher the increase in activity in presence of EGF could be observed.

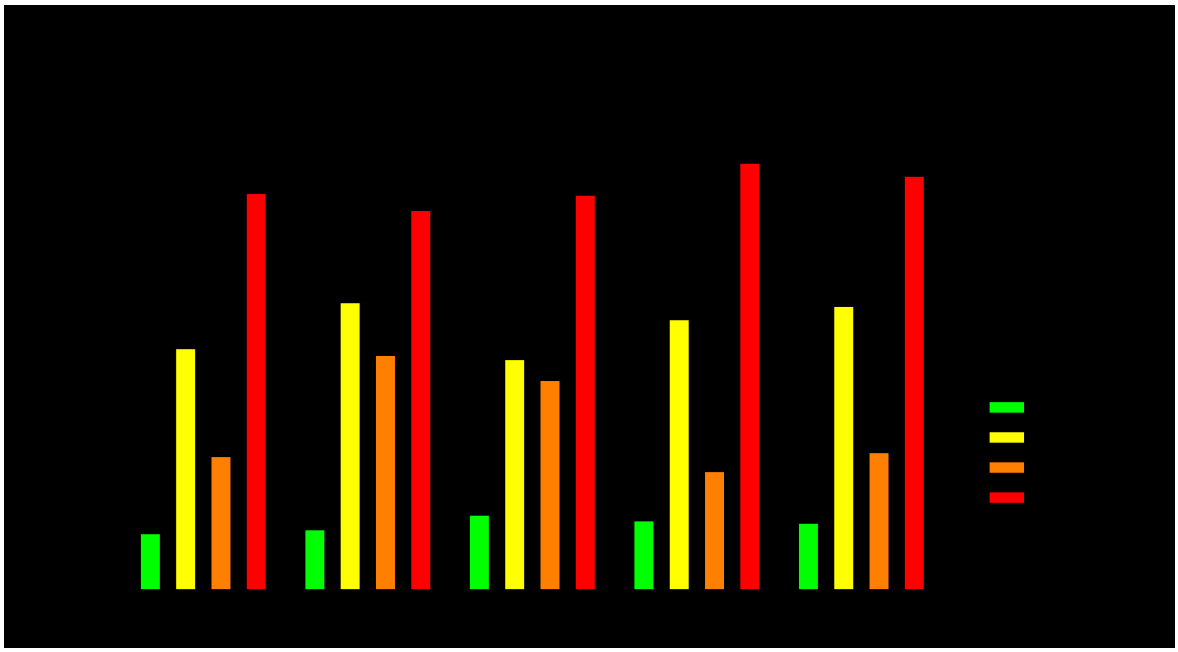

Figure 10: The effect of metabolites on NF-κB activation. pFlag andpFlag-hTLR5 transfected cells were preincubated with different metabolites for 30 min prior to stimulation with 2 ng/ml flagellin (FliC) (5h). Data are presented as mean ± SEM of cell responses compared to non-stimulated auto-activating cells from duplicates in one experiment. Data presented are results from one of the two experiments giving similar results.
Legenda: Control – cells incubated without metabolites, negative control; Butyrate – cells incubated with 10 mM sodium butyrate; Propionate – cells incubated with 10 mM sodium propionate; Acetate – cells incubated with 10 mM sodium acetate; Glucose – cells incubated with 10 mM glucose; pFlag – cells transfected with an empty pFlag vector (control); hTLR5 – cells transfected with pFlag-hTLR5; pFlag + FliC – cells, transfected with an empty pFlag vector, stimulated with falgellin; hTLR5 + FliC – cells, transfected with pFlag-hTLR5, stimulated with flagellin.
Slika 10: Vpliv metabolitov na aktivnost NF-κB. *Celice smo transficirali s kontrolnim ali plazmidom s hTLR5 in jih predhodno inkubirali za 30 min z različnimi metaboliti, preden smo jih stimulirali z 2 ng/ml flagelina (FliC). Po 5 urni inkubaciji smo izmerili aktivnost NF-κB. Kot kontrole smo uporabili nestimulirane celice in celice, ki niso bile inkubirane z metaboliti. Aktivacija je podana v deležu aktivacije celic glede na avtoaktivacijo nestimuliranih kontrolnih celic. Podano je povprečje razmerij ± SD dve vzporedni meritvi enega od dveh izvedenih poskusov. Rezultati obeh poskusov so podobni.*
Legenda: Control – celice inkubirane brez dodanih metabolitov, negativna kontrola; Butyrate – celice inkubirane z 10 mM natrijevega butirata; Propionate – celice inkubirane z 10 mM natrijevega propionata; Acetate – celice inkubirane z 10 mM natrijevega acetata; Glucose – celice inkubirane z 10 mM glukoze; pFlag – celice transficirane s praznim plazmidom pFlag; hTLR5 – celice transficirane s plazmidom pFlag-hTLR5; pFlag + FliC - celice transficirane s praznim plazmidom pFlag in stimulirane s flagelinom; hTLR5 + FliC – celice transficirane s plazmidom pFlag-hTRL5 in stimulirane s flagelinom.

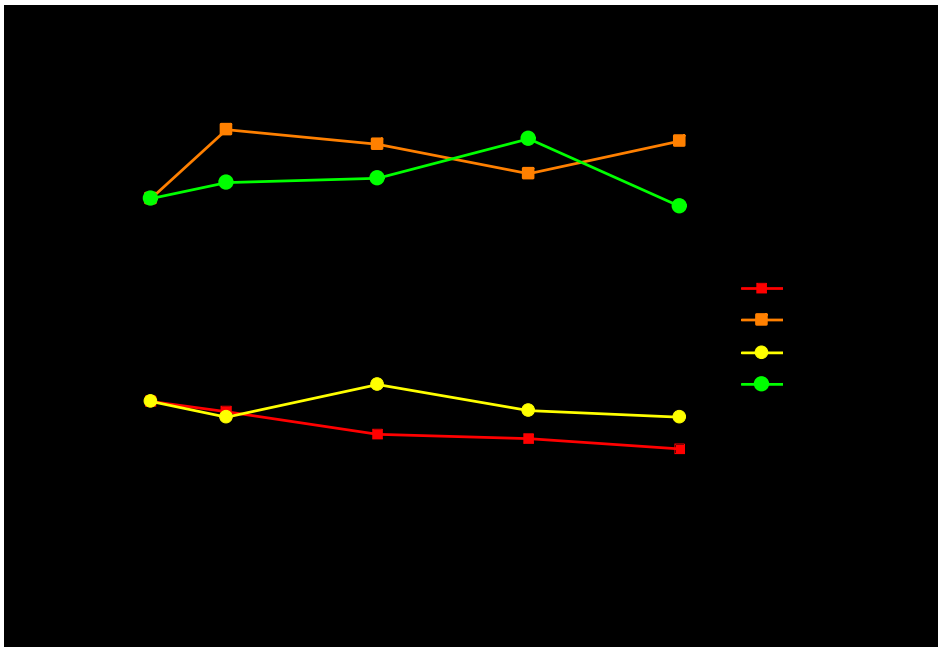

Figure 11: Insulin does not affect TLR5-dependent NF-κB activation. pFlag transfected HEK293 cells were preincubated with increasing insulin (dissolved in 0.01 M HCl) and HCl (control) concentration for 30 min before stimulation with 2 ng/ml flagellin (FliC). Data represents Relative luminescence units (RLU), correlating to NF-κB activity. The experiment was performed twice giving similar results.
Legend: Insulin – cells incubated with hormone insulin; Insulin + FliC – cells incubated with hormone insulin and stimulated with flagellin; HCl – cells incubated with HCl; HCl + FliC – cells incubated with HCl and stimulated with flagellin.
Slika 11: Inzulin ne vpliva na aktivacijo NF-κB, odvisno od TLR5*. Celice HEK293, transficirane s kontrolnim vektorjem, smo predhodno inkubirali za 30 min z naraščajočo koncentracijo inzulina (raztopljen v 0,01 M HCl) in ustreznimi koncentracijami HCl (negativna kontrola) pred stimulacijo z 2 ng/ml flagelina (FliC). Aktivacija je podana v Relativnih enotah luminescence (RLU), kar korelira z aktivnostjo NF-κB. Poskus je bil izveden dvakrat, rezultati obeh poskusov so podobni.*
Legenda: Insulin – celice inkubirane s hormonom inzulinom; Insulin + FliC – celice inkubirane s hormonom inzulinom in stimulirane s flagelinom; HCl – celice inkubirane s HCl; HCl + FliC – celice inkubirane s HCl in stimulirane s flagelinom.

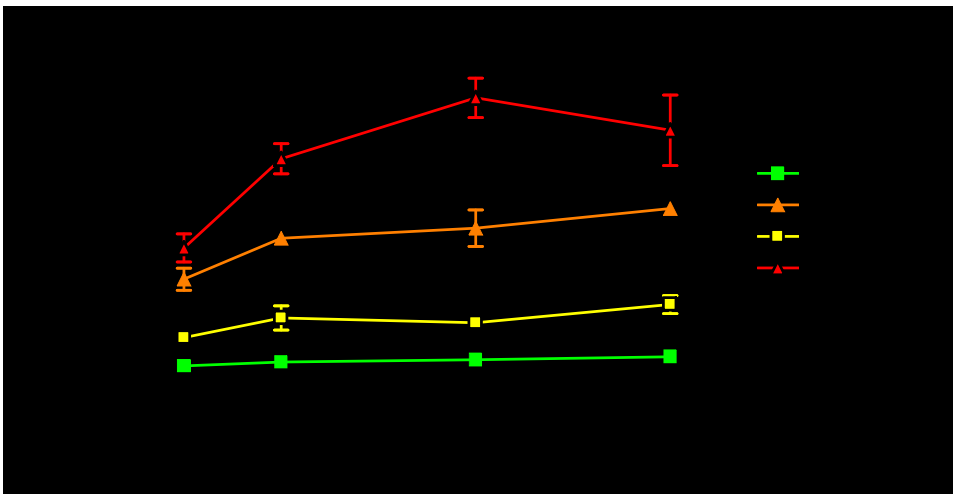

Figure 12: EGF increases TLR5-dependent NF-κB activation in a dose dependent manner. pFlag andpFlag-hTLR5 transfected HEK293 cells were pre-incubated with increasing EGF concentration 30 min before stimulation with 2 ng/ml flagellin (FliC). Data represents Relative luminescence units (RLU), correlating to NF-κB activity. Data are presented as mean ± SEM of RLU for duplicates in the same experiment. The experiment was performed twice giving similar results.
Legend: pFlag – cells transfected with an empty pFlag vector (control); hTLR5 – cells transfected with pFlag-hTLR5; pFlag + FliC – cells, transfected with an empty pFlag vector, stimulated with falgellin; hTLR5 + FliC – cells, transfected with pFlag-hTLR5, stimulated with flagellin.
***Slika 12: EGF (epidermalni rastni faktor) ojača aktivacijo NF-κB**. Celice HEK293, transficirane s plazmidom z zapisom za hTLR5 ali praznim kontrolnim plazmidom, smo predhodno inkubirali za 30 min z naraščajočo koncentracijo EGF pred stimulacijo z 2 ng/ml flagelina (FliC). Aktivacija je podana v Relativnih enotah luminescence (RLU), kar korelira z aktivnostjo NF-κB. Predstavljeni podatki so povprečje ± SD RLU za dve vzporedni meritvi v istem poskusu. Poskus je bil izveden dvakrat, rezultati obeh poskusov so podobni.*
Legenda: pFlag – celice transficirane s praznim plazmidom pFlag; hTLR5 – celice transficirane s plazmidom pFlag-hTLR5; pFlag + FliC - celice transficirane s praznim plazmidom pFlag in stimulirane s flagelinom; hTLR5 + FliC – celice transficirane s plazmidom pFlag-hTRL5 in stimulirane s flagelinom.

4.3 ROLE OF THE PROPERTIES OF THE CELL MEMBRANE IN TLR5 ACTIVATION

The cell membrane is a dynamic structure, separating the inside of the cell from its environment, but at the same time, enabling communication of the cell with its environment via many membrane receptors, adopting cell behaviour to the microenvironmental conditions. A proper membrane structure, its fluidity and membrane proteins are thus essential for proper sensing of the environment and signal transduction.

TLR5 is a type I transmembrane receptor found in lipid rafts in the plasma membrane. Previously it has been reported that other molecules, like co-receptors or proteolytic enzymes may be necessary for TLR activation. To determine the importance of membrane fluidity and other possibly involved factors on TLR5 activation, several inhibitors and activators have been tested.

The first component tested was filipin III. Filipin III (5 μg/ml) is a chemical compound that binds to cholesterol and forms globular deposits that disrupt the planar organization of the membrane. It also induces changes in membrane fluidity and permeability. When applied to HEK293 cells, it caused a slight reduction in NF-κB levels in both control andpFlag-hTLR5 transfected cell, regardless of stimulation (Figure 13).

Galardin, a metalloproteinase inhibitor (MPI, used at a concentration of 2.5 μg/ml), is a potent inhibitor of collagenases and was tested to determine, if TLR5 activation is associated with any matrix metalloproteinases, like it has been proposed for some other TLRs. The MPI caused a decrease in NF-κB activity inpFlag-hTLR5 transfected auto-activating cells both stimulated and non-stimulated, but had only a small effect on pFlag transfected control cells. Results indicate that metalloproteinases might have a role in auto-activation (Figure 13).

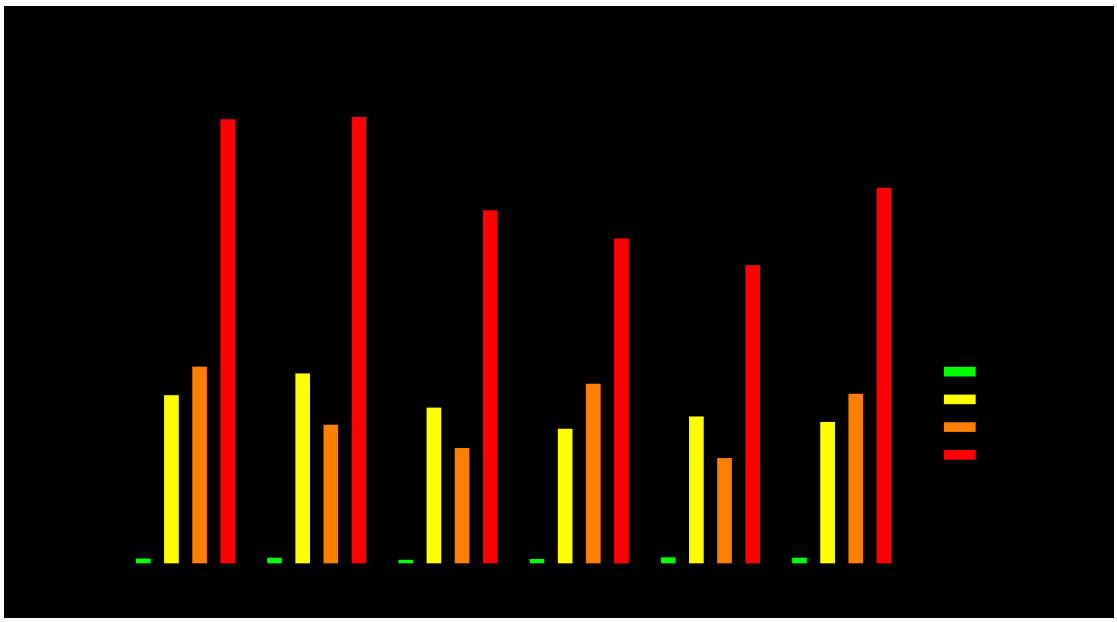

Figure 13: The cellular membrane plays a role in TLR5 activation. HEK293 cells were preincubated with DMSO, Fillipin III, MPI and MβC for 30 min or with CMP-NANA for 19 h prior to stimulation with 20 ng/ml flagellin (FliC). Data represent Relative luminescence units (RLU), correlating to NF-κB activity. Data are presented as mean ± SEM of RLU for duplicates in the same experiment. The experiment was performed once.
Legend: Control – cells incubated without any chemical components, negative control; DMSO – cells incubated with a final concentration of 5µl/ml dimethyl sulfoxide, a negative control for Fillipin III; Fillipin III - cells incubated with Fillipin III (5 µg/ml); MPI – cells incubated with metalloproteinase inhibitor Galardin (5 µg/ml); MβC – cells incubated with Methyl-β-cyclodextrin (5 µg/ml); CMP-NANA – cells incubated with Cytidine-5′-monophospho-N-acetylneuraminic acid (200 µg/ml) ; pFlag – cells transfected with an empty pFlag vector (control); hTLR5 – cells transfected with pFlag-hTLR5; pFlag + FliC – cells, transfected with an empty pFlag vector, stimulated with falgellin; hTLR5 + FliC – cells, transfected with pFlag-hTLR5, stimulated with flagellin.
Slika 13: Celična membrane sodeluje pri aktivaciji TLR5*. Celice HEK293 smo predhodno inkubirali za 30 min z DMSO, Fillipinom III, MPI in MβC oz. za 19 ur z CMP-NANA pred stimulacijo z 20 ng/ml flagelina (FliC). Aktivacija je podana v Relativnih enotah luminescence (RLU), kar korelira z aktivnostjo NF-κB. Predstavljeni podatki so povprečje ± SD RLU za dve vzporedni meritvi v istem poskusu. Poskus je bil izveden samo enkrat.*
Legenda: Control – celice inkubirane brez dodanih kemikalij, negativna kontrola; DMSO – celice inkubirane s končno koncentracijo 5 µl/ml dimetil sulfoksida, negativna kontrola za Filipin III; Filipin III – celice inkubirane z Filipinom III (5 µg/ml); MPI – celice inkubirane z inhibitorjem metaloproteinaz Galardinom (5 µg/ml); MβC – celice inkubirane z metil-β-ciklodekstrinom (5 µg/ml); CMP-NANA – celice inkubirane s citidin-5-monofosfo-N-acetilneuroaminično kislino (200 µg/ml); pFlag – celice transficirane s praznim plazmidom pFlag; hTLR5 – celice transficirane s plazmidom pFlag-hTLR5; pFlag + FliC - celice transficirane s praznim plazmidom pFlag in stimulirane s flagelinom; hTLR5 + FliC – celice transficirane s plazmidom pFlag-hTRL5 in stimulirane s flagelinom.

Methyl-β-cyclodextrin (MβC, used at a concentration of 5 μg/ml) is a seven sugar ring molecule that forms soluble inclusion complexes with cholesterol, thereby enhancing its solubility in aqueous solutions. It removes cholesterol from membranes and disrupts lipid rafts. MβC caused a reduction in NF-κB in flagellin stimulated cells, but did not affect the rate of auto-activation. This indicates that auto-activation is not dependent on membrane fluidity or lipid raft structure but the flagellin induced response is (Figure 13).

Cytidine-5′-monophospho-N-acetylneuraminic acid (CMP-NANA, used at a concentration of 200 μg/ml) is a precursor for sialic acid usually found on proteins and also on TLRs, where it may sterically inhibit spontaneous dimerization. In order to determine whether auto-activation is caused by a lack of glycosylation, cells were incubated with CMP-NANA for 19 h before stimulation. Before stimulation, medium was changed to serum free DMEM. Incubation with CMP-NANA caused a small decrease in auto-activation and flagellin induced NF-κB activity indicating that glycosylation might play a role in receptor activation (Figure 13). However, more experiments are needed to confirm these results.

The importance of receptor glycosylation was assessed also by removing sialic residues from receptors. Thymoquinone (TQ) activates Neu4 sialidase (Neu4), which removes sialic acids from proteins. TQ has been shown to induce TLR4-dependent NF-κB activation by inducing Neu4 and matrix metalloproteinase 9 (MMP9) activity (Finlay et al., 2010b). To determine the effect of Neu4 activity on TLR5 auto-activation and activation, a TQ concentration curve has been performed, using concentrations of 0–2 μg/ml (dissolved in DMSO). TQ induced a strong decrease in NF-κB activity ofpFlag-hTLR5 transfected cells in a dose dependent manner, but there was no effect on the basal NF-κB activity in control cells (Figure 14). Phenotypically, cells incubated in TQ became round and loosely attached in higher concentrations of TQ (1 μg/ml and higher). Eosin staining showed that TQ induces cell death in a dose and time dependent manner (data not shown). This may be the reason for the decrease in NF-κB activity.

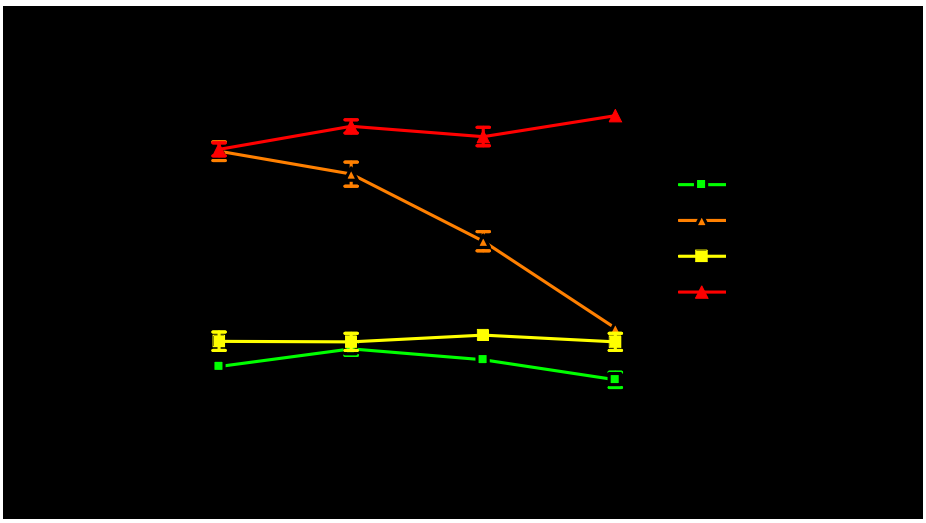

Figure 14: TQ decreases NF-κB activity in a dose dependant manner. Control pFlag andpFlag-hTLR5 transfected cells were incubated in increasing TQ concentration for 5 h. Data represents Relative luminescence units (RLU), correlating to NF-κB activity. Data are presented as mean ± SEM of RLU for duplicates in the same experiment. The experiment was performed twice giving similar results.
Legend: pFlag + TQ – cells transfected with an empty pFlag vector incubated in thymoquinone; hTLR5 + TQ – cells, transfected with pFlag-hTLR5, incubated in thymoquinone; pFlag + DMSO – cells, transfected with an empty pFlag vector, incubated with DMSO (control); hTLR5 + DMSO – cells, transfected with pFlag-hTLR5, incubated with DMSO.
Slika 14: TQ zmanjša aktivnost NF-κB, odvisno od koncentracije TQ. *HEK293, transficirane s kontrolnim in hTLR5 plazmidom, smo inkubirali v naraščajoči koncentraciji TQ za 5 ur. Aktivacija je podana v Relativnih enotah luminescence (RLU), kar korelira z aktivnostjo NF-κB. Predstavljeni podatki so povprečje ± SD RLU za dve vzporedni meritvi v istem poskusu. Poskus je bil izveden dvakrat s podobnimi rezultati.*
Legenda: pFlag + TQ – celice transficirane s praznim plazmidom pFlag, inkubirane s timokinonom; hTLR5 + TQ – celice transficirane s plazmidom pFlag-hTLR5, inkubirane s timokinonom; pFlag + DMSO - celice transficirane s praznim plazmidom pFlag, inkubirane z DMSO; hTLR5 + DMSO – celice transficirane s plazmidom pFlag-hTRL5, inkubirane z DMSO.

5 DISCUSSION

5.1 AUTO-ACTIVATION IN hTLR5 TRANSFECTED HEK293

The immune system is one of the oldest and most complex organ systems, responsible for recognition and defence against invading pathogen microbes. Recognition occurs via pathogen recognition receptors, which usually trigger inflammation and an immune response. The important function of those receptors requires tight regulation, which is in many instances derived from signals in microenvironment of the cell. Growing cells *in vitro* in the laboratory means isolating them from their usual microenvironment, but at the same time this offers a controlled environment for research to investigate the influence of isolated environmental factors on cell function.

The goal of this study was to determine the influence of different molecules usually found in the microenvironment of the cells, on TLR5 signalling. The effect of some extracellular matrix components, cytokines, hormones, growth factors and metabolites was tested on NF-κB activation following activation upon stimulation of TLR5 with flagellin. Next to this ligand induced activation, the NF-κB activation following auto-activation of TLR5 (in the absence of ligand) was assessed.

Transfection of HEK293 cells, which also possess functional endogenous TLR5, with hTLR5 resulted in measurable higher NF-κB activity, most probably due to auto-activation of TLR5. Additional introduced copies of the TLR5 gene probably resulted in higher concentration of the receptor at the cell surface, resulting in spontaneous dimerisation and triggering of the signalling pathway, leading to activation of NF-κB (Figure 2). This phenomenon has been observed in HEK293 cells previously, but the underlying mechanisms are still unknown. TLR5-dependent NF-κB auto-activation indicates that the TLR signalling pathway is constantly active, thus offering a flagellin independent way to determine possible effect of microenvironmental factors on TLR5 signalling. Because the differences between auto-activation and activation upon stimulation are not known, it was decided to use both assays in this study.

Auto-activation in TLR5 transfected cells (HEK293-TLR5) induces NF-κB, a key transcription factor in immunity. NF-κB together with other transcription factors, *e.g.* AP-1, induces transcription of many pro-inflammatory genes including important cytokines like tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), interleukin 6 (IL-6) and interleukin 8 (IL-8) (Tak and Firestein, 2001). Since TNFα and IL-1β also signal via NF-κB and may induce additional NF-κB activity, auto-activating cells were tested for

cytokine production. qRT-PCR (Figure 4) and transient transfection assay results (Figure 3) showed that auto-activation alone does not induce transcription of IL-8 mRNA while flagellin stimulation induced IL-8 cytokine transcripts (Figure 3). These results suggested that auto-activation and activation upon ligand binding are at least partially different processes. To initiate transcription, many activated transcription factors are necessary. It has been shown, that transcription of the IL-8 gene requires at least the transcription factors NF-κB, AP-1 and c/EBPβ (D'Aversa et al., 2008, Khan et al., 2004). Auto-activation probably does not induce all the signalling pathways, which are activated in presence of flagellin, resulting in the unavailability of an essential transcription factor. It may also be the case that auto-activation induces NF-κB activation through a different signalling pathway or that cytokine transcription requires activation of additional signalling pathways. It has been shown that flagellin can also bind to other receptors, like interleukin (IL)-1-converting enzyme protease-activation factor (IPAF), inducing additional signalling pathways, resulting in a different cell response compared to just TLR5 activation (Carvalho et al,. 2011). This may also be the case with auto-activation: dimerisation of TLR5 receptors might induce the regular TLR signalling pathway, but does not trigger other flagellin dependant signalling pathways, resulting in lack of active transcription factors necessary for cytokine transcription. Further investigation of the differences between the two responses may elucidate the mechanism behind auto-activation. By using these two different read-out methods we hoped to gain insight into the role of potential modulatory factors in the microenvironment on receptor function.

5.2 MICROENVIRONMENTAL FACTORS

5.2.1 Extracellular matrix

All adherent cells, especially epithelial cells, are in constant contact with the extracellular matrix (ECM), offering a continuous signal that could regulate immune response. Previous reports indicated a possible role for the polysaccharide heparan sulfate in TLR4 signalling (Brunn et al., 2005) or a role of certain integrins in affecting TLR signalling (Means and Luster, 2010, Marre et al., 2010, Han et al., 2010, Isberg and Leong, 1990). The ECM factors tested in the present study show no effect on TLR5-dependent NF-κB activation (Figure 6 and 7), but did show some interesting results. Surprisingly, HEK293 cells attached and spread on tenascin coated wells as well as in non-coated or collagen coated wells. Tenascin even induced cell growth and proliferation (100% confluency on tenascin compared to 85% in non-coated wells, results not shown), indicating that HEK293 possess tenascin receptors. Tenascin (TN) is a polymeric high molecular mass ECM, mainly present during morphogenesis in embryonic life (Chiquet-Ehrismann et al., 1986). In adults

it reappears in malignant tumors (Mackie et al., 1987a, Mackie et al., 1987b) and during inflammation and tissue repair (Mackie et al., 1988). It alters the adhesion properties of human monocytes, B and T cells and inhibits attachment of fibroblasts to fibronectin, laminin and Arg-Gly-Asp peptides with integrins (Chiquet-Ehrismann et al., 1988). Tenascin is thus generally classified as an anti-adhesive protein.

Tenascin can bind to at least 5 members of the integrin family ($\alpha_9\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_6$, $\alpha_v\beta_3$, $\alpha_2\beta_1$) through fibronectin type II repeats (Schnapp et al., 1995, Sriramarao et al., 1993, Yokosaki et al., 1996). Thus, results suggest that HEK293 possess at least one of the tenascin binding integrins. Li et al. (2001) showed that HEK293 express α_v, α_5, α_1 and β_1 subunits, while Jin and co-workers (2007) showed that HEK293 do not express β_3, but until now, no one confirmed the presence of any of the five TN binding integrins on the surface. Observations that tenascin is frequently present *in vivo* under conditions associated with high levels of cell turnover suggest a role for tenascin in stimulating cell proliferation (Yokasaki et al., 1996), which coincides with cell response in our experiments. The presence of TN binding integrins might be due to the fact, that HEK293 are embryonic cells.

Similar conclusions could be drawn for collagens, the main and most abundant components of extracellular matrix and an important source of attachment sites. Cells bind to Collagen IV and VI mainly by integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Kuhn and Ebel, 1994, Pfaff at al., 1993). Both collagens tested stimulated cell attachment, spreading and proliferation in HEK293 cells, indicating the presence of at least one of the two integrins, most probably $\alpha_1\beta_1$, since the presence of both subunits has been reported (Li et al., 2001). This integrin is linked via the Shc adaptor protein to the mitogen-activated protein kinase pathway and is responsible for regulation of cell survival and cell cycle progression (Wary et al., 1996), explaining better cell growth in collagen coated wells compared to the non-coated wells. Since collagens are an essential part of the ECM, HEK293 cells most probably secrete them on their own, thus reducing the effect of the coatings. Better growth of the cells could be also attributed to the fact that binding of already present collagens via $\alpha_1\beta_1$ also reduced collagen secretion (Heino, 2000), thus providing space for synthesis of other molecules.

ECM components do not comprise mucins, the major macromolecular constituent of the mucus secretions that coat the oral cavity and the respiratory, gastrointestinal and urogenital tracts of animals. Mucins are important environmental components that are responsible for the viscoelastic properties of the secretions, providing protection for the exposed epithelial surface against microbial and physical insults. Their functions are very diverse, from acting as ‘decoy’ receptors for the prevention of the binding of pathogens to

epithelial cells (Carlstedt et al., 1985, Rose, 1992, Carraway and Fregien, 1995, Roussel et al., 1988), to binding to cells or extracellular matrix by means of their carbohydrate structure (Straus and Dekker, 1992). Since mucins usually cover the apical cell surface and reduce adhesion of microorganisms to cell surface, it explains why cells could not adhere well to mucin coated wells. Interactions between cells and mucin may have formed, but at least cells were unable to adhere firmly and spread hence it was for us not possible to assess their role in NF-κB modulation. In future experiments addition of mucin after adherence of the cells may indicate their potential regulatory role on TLR5 function.

Poly-L-lysin (PLL), an artificial non-ECM molecule, surprisingly reduced NF-κB activity induced by auto-activation. PLL is a homopolypeptide of L-lysine residues, which is positively charged and widely used as a coating to enhance cell attachment and adhesion. Cell adhesion to PLL is nonspecific and based on the negatively charged cell membrane. Since cells do not have receptors for PLL, the observed reduction in NF-κB activity (Figure 6) might be due to better adherence, which may limit lateral movement of the membrane proteins on the attached side of the cell, thus possibly limiting the amount of spontaneous dimerisation and auto-activation. Adhesion to highly positive coating might also displace other positive molecules, bound on the surface, which may cause auto-activation, thus reducing spontaneous dimerisation. Next, there was no effect on NF-κB activation upon flagellin stimulation (Figure 7).

5.2.2 Cytokines

Cytokines are soluble mediators of immunity secreted mostly by immune cells to affect other immune cells by activating them, attracting cells to sites of infection or regulating cellular differentiation. In this way the overall immune response can be directed by cytokines. Non-immune cells can also respond to cytokines, mostly by up or down regulation of genes involved in immunity, thus activating the cell for possible encounter with pathogen microbes (Mueller et al, 2006). Regarding the results for the tested cytokines, HEK293 cells possess the receptors for IL-4 (Hebenstreis et al., 2005), IL-6 (von Laue et al., 2000), TNFα and IL-1β, but there is no reference of HEK293 cells having IL-10R, IL-12R or GM-CSFR. Despite the presence of the receptors, IL-4 and IL-6 had no effect on NF-κB signalling, similarly seen for IL-10, IL-12 and GM-CSF (Figure 8 and 9). The method used might be not appropriate for testing the effect of cytokines, since certain mechanisms of regulation, like change in the transcription of certain cytokine induced genes, require longer periods of exposure than the used 5 hours here. Our method did show

however that these cytokines do not interact directly with NF-κB signalling or the TLR5 signalling pathway either via auto-activation or flagellin binding.

As mentioned above, TNFα and IL-1β signal via NF-κB and thus may mask the specific TLR5-induced NF-κB activation (Figure 9). This made it difficult to assess specifically the influence of those two cytokines on TLR5 activation.

5.2.3 Hormones and metabolites

Hormones and growth factors are molecular messengers, usually released from specialized cells or tissues and carried by blood to target cells, possessing high affinity receptors. Two hormones, insulin and epithelial growth factor (EGF) have been tested for an effect on NF-κB activation.

Insulin is a metabolic hormone, central in regulation of carbohydrate and fat metabolism in the body. Many reports indicated a connection between insulin and TRL receptor signalling. It has been shown that TLR signalling induces insulin resistance, chronic TLR signalling even resulted in type 1 diabetes (Zeyda and Stulnig, 2009), suggesting c-Jun N-terminal kinase as a key component, connecting both pathways (Davis et al., 2009). In our experiment, the insulin signalling pathway was activated first by pre-incubating HEK293 cells 30 min with insulin, followed by flagellin stimulation. Insulin had no effect on TLR5-induced NF-κB activation (Figure 11), suggesting that the connection between the two pathways only works in one way.

The growth hormone EGF controls cell growth, proliferation, differentiation and survival. The activated signalling cascade results in a variety of biochemical changes within the cell: a raise in intracellular calcium levels, increased glycolysis and protein synthesis and increased gene expression. The effects of EGF are signalled through several pathways. Binding of EGF results in receptor dimerisation and auto-phosphorylation. Active EGF receptor (EGFR) in turn activates Ras and the MAPK pathway, ultimately causing phosphorylation of transcription factors such as c-Fos to induce AP-1 and ELK-1. Activation of STAT-1 and STAT-3 transcription factors by Jak kinases in response to EGF contributes to proliferative signalling. NF-κB transcription factor can be activated by Phospholipase-C-Gamma, which activates Protein kinase C (Carpenter and Cohen, 1990). Since EGF had no effect on background NF-κB activity, it probably does not induce NF-κB activity on its own in HEK293 cells, but rather facilitates or prolongs NF-κB activity, triggered by an additional signal, like TLR signalling (Figure 12). Facilitation is

probably triggered by the active components of other EGF activated signalling pathways or by their effects. So far we could show that the level of increased NF-κB activity by EFG was proportional to the height of NF-κB activation.

So far, several metabolites, either host-derived or bacteria-derived molecules have been shown to influence the TLR response: certain yeast and bacterial secretions can down regulate the immune response of intestinal epithelial cells (Romanin et al., 2010). Similarly effects have been reported for pyruvate, a final metabolite in glycolysis (Wang et al., 2009). Vitamin D3 reduces TLR expression (Dickie et al., 2010) and exposure to high glucose has been reported to induce TLR2 and TLR4 expression (Dasu et al., 2008).

The set of bacteria derived metabolites tested here were acetate, propionate and butyrate. These are short-chain fatty acids (SCFA), major products of the microbial fermentation of plant fiber polysaccharides in the human colon (Cummings, 1983, Wolin and Miller, 1983). It is known from literature that butyrate and acetate are histone deacetylase inhibitors (HDACi) (Kien et al., 2008). They block histone deacetylases, a class of enzymes that remove acetyl groups from ε-N-acetyl lysines on histones and other non histone proteins, including the transcription factor NF-κB. The presence of acetyl groups on those proteins increases or represses their activity; in case of the histones, HDACi increase the availability of chromatin for transcription. As demonstrated here, butyrate and propionate can increase the flagellin response in pFlag transfected cells, but not in auto-activating cells (Figure 10).

Dasu and co-workers (2008) showed that high glucose induces TLR2 and TLR4 expression. We could not show any influence of high glucose levels on TLR5 signalling activity. These differences may be explained by the different media used (our experiment was performed in DMEM instead of RPMI), read-out method, or the analysis of different TLR receptors. In our hands, glucose (10 mM) did not influence TLR5 induced NF-κB activity (Figure 10).

5.3 ROLE OF THE MEMBRANE IN TLR5 ACTIVATION

Dimerisation and activation of TLR receptors occurs in lipid rafts in cell membranes, thus making cell membrane a key component in immune response. Previous reports have shown, that dimerisation of TLR receptors is not the only requirement for activation. TLR9 and TLR7 need to be cleaved in the endolysosomes, so they can signal upon ligand binding (Ewald et al., 2008). TLR2, TLR3 and TLR4 require neuroaminidase Neu1 activity, which

cleaves sialyl residues from the receptors, enabling proper dimerisation and signalling (Finlay et al., 2010a, Finlay et al., 2010b). Finlay and co-workers (2010a) also showed that TQ induces Neu4 activity via G-protein coupled receptors, Gαi proteins and matrix metalloproteinase 9, which can also cleave sialyl residues, causing NF-κB activation. Our experiments with the MMP inhibitor revealed similar findings for TLR5, indicating that one of the matrix metalloproteinases, inhibited by Galardine, might also have a role in TLR5 activation (Figure 13). The effect was observed only for the auto-activation process. Incubation with TQ, on the other hand, indicated a strong down regulation of NF-κB activity (Figure 14). This may be due to apoptosis/cell death after 5 h of incubation to the drug. Both apoptosis and Neu4 activation might be triggered by TQ through GPCR, activating p53 (Gali-Muhtasib et al., 2004).

Experiments involving changing membrane properties using Filipin III and Methyl-β-cyclodextrin indicated that the membrane played a role in FliC-induced activation of TLR5, but did not affect auto-activation (Figure 13). Both components disrupted lipid rafts and reduced the response to stimulation, but that had no effect on auto-activation. The effect of TLR5 is consistent with previous studies with different TLRs (Islam et al., 2011). Islam and co-workers (2011) also showed that TLR2 and TLR4 can also activate and signal independently of lipid rafts when stimulated with titanium particles with adherent PAMPs.

5.4 CONCLUSION

In conclusion, in this study we first of all developed a new method for transient transfection, reducing the variation among wells due to different transfection efficiency. The method was used to test many microenvironmental factors for an effect on TLR5 activation and auto-activation. The majority of the tested factors did not influence TLR5 signalling indicating that it is relatively stable. This confirms and underlines the importance of TLR5 in the tight regulation of the immune response.

Among the tested factors, SCFA butyrate and propionate increased NF-κB activity in stimulated control cells, but had no effect on the rate of auto-activation. Epidermal growth factor increased TLR5-triggered NF-κB activity. Disruption of the lipid rafts reduced activation following flagellin stimulation, but had no effect on auto-activation, while inhibition of matrix metalloproteinase activity only reduced auto-activation. The differences of the response of auto-activating and flagellin-stimulated cells to certain factors indicated that the two events are not identical. This hypothesis is supported by the

qRT-PCR results showing that auto-activation does not induce IL-8 mRNA transcription while flagellin stimulation does.

Overall, this study provides insight into the steadiness of the TLR5 response to microenvironment. If TLR5 would be influenced by many environmental factors, the immune response would depend on a stochastic array of conditions in a certain moment and not only on the type of infection. This is consistent with the assumed important regulatory role of TLR5 in innate immunity. Knowledge of the status and regulation of the immune response is crucial to better understand the basics of inflammation and host defence against microbes.

6 SUMMARY

6.1 SUMMARY

The immune system is one of the oldest and most complex organ systems. Its physiologic function is the distinction of self and non-self and in this way protection against infectious microbes. Innate immunity is the first line of defence against infections. This system exists in a functional state before encounter with microbes and is rapidly activated by them before the development of an adaptive immune response. The innate immune system consists of epithelial barriers, circulating cells and proteins that recognise microbes or substances produced during infections. Among those proteins are also germline-encoded patter recognition receptors (PRR), mostly found on immune, but also non-immune cells and tissues. These receptors recognise distinct microbial components, known as pathogen-associated molecular patterns (PAMPs) and directly activate immune cells through intracellular signalling cascades that rapidly induce the expression of a variety of overlapping and unique genes involved in inflammatory and immune responses.

Among PRRs are Toll-like receptors (TLRs), a family of type I transmembrane signalling receptors, which are one of the most important receptors for discrimination among groups of pathogens. TLRs exist as dimeric proteins, either being heterodimers or homodimers. The ectodomain of TLRs are composed of leucine-rich repeat motifs, whereas the cytosolic component, called Toll/IL-1 receptor–like (TIR) domain, is involved in signalling. In humans, TLRs are present on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and on non-immune cells such as fibroblasts and epithelial cells. The expression of TLRs is not static but is rather modulated rapidly in response to pathogens, a variety of cytokines and environmental stress.

TLR5 is a homodimeric receptor, responsible for recognition and response to flagellin. Flagellin is the major protein constituent of bacterial flagella, the motility apparatus, and is a potent activator of innate immune response. TLR5 is present on epithelial cells, monocytes, and immature DCs. After flagellin binding, TLR5 dimerises and undergoes conformational changes required for activation of the signalling pathway, leading to the activation of the transcription factor NF-κB. NF-κB translocates to the nucleus and induces the expression of its target genes, thus regulating the immune response of the activated cell.

Many recent studies showed that the microenvironment can influence the response of the cells to TLR5 activation. Factors of the microenvironment can alter cell response by

binding to certain receptors, thus triggering additional signalling cascades, or by interfering with other active pathways and their components. Certain signals are constantly present and are required for normal cell function, like signals inhibiting apoptosis or signals from extracellular matrix components, reporting the state of cell attachment, while others are present only temporarily and alter cell usual gene expression and metabolism.

The objective of this study was to assess the dynamics of TLR5 function in presence of different molecules usually found in the microenvironment of the cells. The effect of several extracellular matrix components, cytokines, hormones, growth factors and metabolites was tested on the auto-activation of the receptors in absence of the ligand and on the activation upon stimulation of TLR5 by its natural ligand.

Experiments were performed using human cell line HEK293, transiently transfected with p4xNF-kB-luciferase reporter plasmid, normalisation vector pTK-LacZ and pFlag-hTLR5 or empty pFlag expression plasmid as a control. Since the presence of certain microenvironmental factors (mostly extracellular matrix components) could affect transfection efficiency, a new transient transfection method was established, enabling the cells to be transfected before seeding onto coated wells. In this method, cells were first seeded in 6-well plates and transfected with the standard transfection mixture. After 24 h, cells were trypsinised, resuspended and seeded in coated wells of 48-well plate. The new method had the same efficiency as the standard one and was used for all transfections in this research. HEK293 cells were, when applicable, pre-incubated in serum free medium with different microenvironmental factors for 30 min and stimulated with flagellin. After 5 h incubation, luciferase and LacZ assays were performed. For normalisation of the efficiency of transfection and cell numbers in individual wells, luciferase values were adjusted to β-galactosidase values as determined with the β-galactosidase assay. Activation and auto-activation, as flagellin independent TLR5 signaling pathway activity, were used as a read out method.

HEK293 cells already possess their endogenous TLR5 and transfection with an extra hTLR5 results in TLR5-dependent NF-κB activation, most probably due to auto-activation of TLR. Additional introduced copies of the TLR5 gene probably resulted in higher concentration of the receptor at the cell surface, resulting in spontaneous dimerisation and triggering of the signalling pathway, leading to activation of NF-κB. Some cytokines, like TNFα and IL-1β, which are secreted upon TLR activation, can in turn activate NF-κB, thus indeterminably altering the measured NF-κB activity. To assess whether auto-activating cells induce cytokines or other mediators capable of inducing NF-κB activation, qRT-PCR for IL-8 mRNA and a transient transfection assay were performed, showing that auto-

activation alone does not induce transcription of IL-8 mRNA or any NF-κB activating cytokines while flagellin stimulation induces IL-8 cytokine transcripts. This suggests that besides TLR activation an additional, flagellin dependent mechanism is required for cytokine production, which most likely provides other transcription factors necessary for transcription. These results suggest that auto-activation and activation upon ligand binding are at least partially different processes.

Next, using the activation and auto-activation read-out methods, several microenvironmental factors were tested for an effect on NF-κB activity: extracellular matrix components (ECM), cytokines, hormones and growth factors, metabolites and certain inhibitors of membrane functions.

The extracellular matrix (ECM) is an important part of the cellular microenvironment providing support for cells and enabling many cell interactions which can affect cell behaviour and function. Previous reports already showed that certain ECM components, like heparin sulphate and several integrins can affect TLR signalling. Nevertheless, factors tested in this research (poly-L-lysin (PLL), mucin, human tenascin, collagen VI and collagen IV) showed no influence on NF-κB activity (auto-activation or activation upon flagellin stimulation), although cells, cultured on PLL, tenascin and both collagens grow better compared to the non-coated control, indicating that HEK293 could adhere to the tested ECM components and that this has an effect on cell proliferation. These results suggest that integrins or other receptors for these ECM components are present on HEK293 cell, that have not jet been reported for this cell line.

Cytokines are soluble mediators of immunity secreted mostly by immune cells to affect other immune cells by activating them, attracting cells to sites of infection or regulating cellular differentiation. In this way the overall immune response can be directed by cytokines. Cytokines tested in this research were IL-4, IL-6, IL-10, IL-12, GM-CSF, TNFα and IL-1β. A 5 h incubation in IL-4, IL-6, IL-10, IL-12 and GM-CSF showed no effect on NF-κB signalling, on the other hand, TNFα and IL-1β signal via NF-κB and thus mask the specific TLR5-induced NF-κB activation, which made it difficult to assess specifically the influence of those two cytokines on TLR5 activation. The method used might be not appropriate for testing the effect of cytokines, since certain mechanisms of regulation, like change in the transcription of certain cytokine induced genes, require longer periods of exposure than the used 5 h here. Our method did show however that these cytokines do not interact directly with NF-κB signalling or the TLR5 signalling pathway either via auto-activation or flagellin binding.

Besides being exposed to cytokines, cells are also surrounded by other cell secretions, like hormones and cellular metabolites, either host derived or of microbial nature. The set of bacteria derived metabolites tested here were acetate, propionate and butyrate. These are short-chain fatty acids (SCFA), major products of the microbial fermentation. Butyrate and acetate are histone deacetylase inhibitors (HDACi), blocking histone deacetylases thus keeping histones and other non-histone proteins like NF-κB acetylated. The presence of acetyl groups on those proteins increases or represses their activity; in case of the histones, HDACi increases the availability of chromatin for transcription. As demonstrated here, butyrate and propionate can increase the flagellin response in pFlag transfected cells, but not in auto-activating cells.

Next to the metabolites, insulin and epidermal growth factor (EGF) were tested. Cells were pre-incubated with different range of hormones and stimulated with flagellin. Increasing insulin concentration had no effect on the background of pFlag transfected cells or on flagellin induced NF-κB activation. EGF, on the other hand, increased NF-κB activity in a dose dependent manner with the strongest effect on auto-activating hTLR5 transfected cells stimulated with flagellin, while there was no effect on the background (pFlag) activity, indicating that EGF does not induce NF-κB activity on its own, but rather modulates the activity of already activated NF-κB.

Dimerisation and activation of TLR receptors occurs in lipid rafts in cell membranes, thus making cell membrane a key component in immune response. Previous reports have shown, that dimerisation of TLR receptors is not the only requirement for activation; certain TLRs need to be cleaved in the endosomes, or require neuroaminidase Neu1 activity, which cleaves sialyl residues from the receptors, enabling proper dimerisation and signalling. Incubation with MPI, matrix metalloproteinase inhibitor, a protein inhibitor of collagenases which cleave sialyl residues, decreased NF-κB activity in hTLR5 transfected auto-activating cells both stimulated and non-stimulated, but had only a small effect on pFlag transfected control cells. This indicates that certain metaloproteinases, inhibited by used inhibitor, might have a role in TLR5 activation. Incubation with TQ, on the other hand, indicated a strong down regulation of NF-κB activity. This may be due to apoptosis/cell death after 5 h of incubation with TQ.

Experiments involving changing membrane properties using Filipin III and Methyl-β-cyclodextrin indicated that the membrane plays a role in FliC-induced activation of TLR5, but does not affect auto-activation. Both components disrupted lipid rafts and reduced the response to stimulation, but that had no effect on auto-activation.

In conclusion, this study provided insight into the steadiness of the TLR5 response regarding microenvironment. The majority of the tested factors did not influence TLR5 signalling indicating that it is relatively stable. This confirms and underlines the importance of TLR5 in the tight regulation of the immune response.

6.2 POVZETEK

Imunski sistem je eden od najstarejših in najbolj kompleksnih organskih sistemov. Njegova osnovna naloga je ločevanje med lastnim in tujim, da lahko organizem obrani pred tujki. Najpreprostejši način obrambe predstavljajo mehanizmi pridobljenega imunskega sistema, ki so funkcionalni še pred stikom z mikrobi in tako zaustavijo številne okužbe še pred njihovim izbruhom. Aktivacija prirojenega sistema lahko sproži tudi pridobljeni imunski sistem, ki se odzove počasneje, a bolj specifično in s tem učinkoviteje. Sistema sta tesno povezana in odvisna eden od drugega.

Mehanizmi prirojenega imunskega sistema vključujejo epitelije, imunske celice in proteine, ki lahko prepoznajo mikrobe ali snovi, sproščene ob infekciji. Anatomske (koža, sluznice) in fiziološke bariere (telesna temperatura, nizek pH v želodcu) so prva linija obrambe, ki mikrobom preprečuje vstop na fizičen način. Drug način obrambe so imunske celice in proteini v telesnih tekočinah in tkivih, ki so podvržena vdoru mikrobov. Glavne efektorske celice imunskega sistema so nevtrofilni levkociti, mononuklearni fagociti, dendritične celice, mast celice in celice ubijalke (NK) celice. Te celice mikrobe prepoznajo, fagocitirajo in onesposobijo. Med imunske proteine štejemo komponente komplementnega sistema in številne druge proteine, ki se vežejo na značilne mikrobne strukture in tako olajšajo njihovo prepoznavanje.

Prepoznavanje mikrobov poteka s pomočjo v genih zapisanih receptorjev (pattern recognition receptors, PRR), ki se vežejo na evolucijsko ohranjene dele za mikrobe esencialnih molekul, imenovane s patogeni povezani molekularni vzorci (pathogen-associated molecular patterns, PAMPs). Vezava receptorja na ustrezen ligand sproži signalno kaskado, ki spremeni ekspresijo številnih ligandno specifičnih in drugih splošnih z imunskim odgovorom in vnetji povezanih genov. Prepoznava poteka na osnovi dveh principov; bodisi se na receptorje vežejo molekule, ki niso prisotne pri gostitelju (npr. dsRNA ali flagelin), ali pa se so prisotne tudi pri gostitelju, a se v primeru okužbe pojavljajo na neobičajnih lokacijah v celici (npr. DNA v endosomu ali citoplazmi).

Pomembnejši tip receptorjev za razlikovanje med tipi patogenov so Tollu podobni receptorji (Toll-like receptors, TLR). So transmembranski dimerni proteini, ki se večinoma pojavljajo na imunskih celicah, nahajajo pa so tudi na nekaterih neimunskih celicah, kot so npr. fibroblasti in epitelijske celice. Izpostavljanje na površini in delovanje teh receptorjev ni statično, temveč je podvrženo vplivu prisotnih molekul v mikrookolju in stresu.

Razlikovanje med patogeni omogoča aktivacijo najustreznejšega obrambnega mehanizma glede na patogenost mikroba. Pri človeku poteka razlikovanje na osnovi specifičnosti vezave desetih tipov receptorjev. TLR4 tako prepozna bakterijski lipopolisaharid (LPS), komponento zunanje membrane po Gramu negativnih bakterij, TLR2 v kombinaciji s TLR1 in TLR6 prepozna različne tipe lipopeptidov po Gramu pozitivnih bakterij. TLR5 se veže na flagelin, osnovni gradnik bakterijskega bička, TLR3, TLR7, TLR8 in TLR9 vežejo različne oblike nukleinskih kislin in tako večinoma prepoznajo viruse. Celotna družina TLR omogoča prepoznavo in obrambo pred večino patogenov, prepoznavajo pa tudi nekatere lastne molekule, kot npr. proteine vročinskega šoka (Heat-Shock proteins, HSP) in fragmente zunajceličnega matriksa, saj je prisotnost teh molekul navadno znak poškodovanega tkiva.

TLR5 je homodimerni receptor, ki prepozna flagelin, osnovni gradnik bakterijskega flagela (bička). TLR5 imajo epitelijske celice, monociti in nezrele dendritične celice. Vezava flagelina sproži dimerizacijo in konformacijsko spremembo, ki omogoči vezavo adaptorske molekule MyD88 (myloid differentioation primary-response protein 88), nanjo se vežejo in aktivirajo sledeče komponente v aktivacijski kaskadi, ki na koncu z razgradnjo inhibicijske molekule IκB sprostijo in aktivirajo transkripcijski faktor NF-κB (nuclear factor κB). Aktiven NF-κB potuje v jedro, kjer sproži prepis tarčnih genov. TLR5 je močan aktivator imunskega sistema, zato je natančna regulacija izražanja njegovega gena in delovanja zelo pomembna.

Mikrookolje celice lahko vpliva na njeno splošno delovanje, kot tudi na specifične procese. Celica svoje okolje zaznava preko receptorjev, ki ob vezavi ustreznega liganda spremenijo delovanje celice, ali pa preko fizikalnih lastnosti okolja, ki vplivajo na delovanje posameznih celičnih komponent ali procesov (npr. temperatura vpliva na fluidnost membran in s tem na mobilnost in delovanje membranskih receptorjev ter na hitrost katalize encimov). Določeni signali iz okolja celice so nujni za pravilno delovanje, kot so npr. signali, ki preprečujejo apoptozo, ali signali iz celičnega matriksa, ki celici sporočajo da je pritrjena, kar je nujno potrebno za druge celične procese. Številni signali so v okolju le občasno in vplivajo na običajni celični metabolizem in gensko izražanje.

Zunajcelični matriks (ECM) je mreža makromolekul, sestavljena iz različnih polisaharidov in proteinov, ki jih izločajo celice. To so večinoma molekule, ki omogočajo vezavo celic, kot so fibronektin, vitronektin in laminin, ter strukturne komponente, kot so kolageni, elastin in proteoglikani. Molekule ECM interagirajo med seboj in s specifičnimi receptorji na celični površini, večinoma integrini.

Hormoni in rastni dejavniki so molekulski obveščevalci, ki se prenašajo počasi in po celem telesu, nanje pa lahko odgovorijo le celice z ustreznimi visokoafinitetnimi receptorji. Receptorji za glikoproteinske in proteinske hormone se nahajajo na površini celice, na celico pa delujejo preko signalne verige. Steroidni in tiroidni hormoni lahko prehajajo celično membrano in se vežejo na receptor v citoplazmi, kar omogoči njegovo migracijo v jedro in direktno regulacijo transkripcije. Hormoni v sodelovanju z živčevjem regulirajo prebavo, metabolizem, rast, zorenje, razvoj, reprodukcijo in homeostazo. To dosežejo preko regulacije encimske aktivnosti, transkripcije, transporta, proliferacije, apoptoze in sekrecije drugih regulacijskih molekul.

Molekulski obveščevalci imunskega sistema so citokini in kemokini. So nizkomolekularni proteini, ki regulirajo rast, vnetja, imunost, diferenciacijo, migracijo in obnovo. Delujejo v kompleksnih interakcijah in končni odgovor celice je odvisen od trenutne kombinacije prisotnih mediatorjev in njihove koncentracije. Vloga kemokinov je predvsem rekrutacija imunskih celic na mesto vnetja.

Celice obdajajo tudi številni metaboliti, aminokisline, sladkorji, maščobne kisline, vitamini, soli in odpadni produkti metabolizma. Njihova vsebnost je regulirana v okviru homeostaze tako, da se vzdržuje pravo elektrolitsko ravnotežje in koncentracija hranil.

Te in vse ostale snovi v okolici celice lahko vplivajo na njeno pravilno delovanje. Ob gojenju celic v *in vitro* pogojih večina teh snovi ni prisotnih, kar lahko vpliva na diferenciacijo celice in delovanje številnih procesov in encimov. Temu je lahko podvrženo tudi delovanje receptorja TLR5, kar smo želeli preveriti s to raziskavo. Celice HEK293 smo gojili v prisotnosti številnih komponent ekstracelularnega matriksa, citokinov, hormonov, rastnih dejavnikov in metabolitov in ugotavljali njihov vpliv na aktivacijo in avtoaktivacijo TLR5.

Vsi poskusi so bili narejeni na človeški celični liniji HEK293, ki že sama izraža funkcionalni receptor TLR5. Po transfekciji ekspresijskega plazmida z geni za dodatne kopije receptorja (pFlag-hTLR5) opazimo višjo aktivnost transkripcijskega faktorja NF-κB, odvisno od TLR5 – najverjetneje gre za avtoaktivacijo TLR5. Celice, transficirane s praznim ekspresijskim vektorjem pFlag, smo uporabili kot kontrolo, v vse celice pa smo transficirali tudi p4xNF-κB-luciferazni reportrski plazmid in pTK-LacZ normalizacijski vektor, s pomočjo katerih smo določili aktivnost NF-κB. Ob aktivaciji receptorja, ki aktivira NF-κB, se del teh transkripcijskih faktorjev veže tudi na promotor na p4xNF-κB-luciferaznem reporterskem plazmidu in sproži sintezo luciferaze – encima kresničke, ki ob katalizi ustreznega substrata sprošča luminiscenco. To je mogoče meriti in je sorazmerna

celokupni NF-κB aktivnosti. Izmerjeno vrednost lumniscence smo popravili za delež β-galaktozidazne aktivnosti, ki smo ga izmerili s pomočjo vektorja pTK-LacZ. Za razliko od encima luciferaze, ki nastaja le ob aktivaciji TLR5, se encim β-galaktozidaza sintetizira neprestano in sorazmerno s številom celic v posamezni luknjici mikrotitrske plošče. Rezultati meritve luminiscence, popravljeni za število celic, so bolj realni in zanesljivi.

Da bi preverili vpliv nekaterih mikrookoljskih dejavnikov, kot so npr. komponente zunajceličnega matriksa, bi ob uporabi klasične metode transfekcije morali celice najprej nacepiti v luknjice, prevlečene s komponentami matriksa, in šele nato opraviti transfekcijo. Ker pa bi prevlečene snovi lahko vplivale na učinkovitost transfekcije, smo razvili novo metodo transfekcije v dveh korakih. Po tej metodi smo celice najprej transficirali, jih po 24 urah tripsinizirali in nacepili v prevlečene luknjice. 24 ur po tripsinizaciji smo celice predhodno inkubirali z izbrano snovjo iz mikrookolja in določene celice po pol ure predhodne inkubacije stimulirali s flagelinom, nestimulirane celice pa uporabili kot kontrolo. 5 ur po stimulaciji smo celice lizirali in izmerili luciferazno in β-galaktozidazno aktivnost. Ker se je nova metoda izkazala za uporabnejšo, natančnejšo in enako učinkovito, smo jo uporabili za vse poskuse v tej raziskavi.

Tako kot stimulacija, tudi avtoaktivacija aktivira transkripcijski faktor NF-κB, ki je pomemben transkripcijski faktor za indukcijo številnih regulacijskih citokinov. Nekateri od teh citokinov, kot npr. TNFα in IL-1β ob vezavi signalizirajo preko NF-κB in ga lahko tako še dodatno aktivirajo, kar bi popačilo naše meritve. Da bi preverili, ali se ob avtoaktivaciji sproži transkripcija genov za citokine, smo naredili qRT-PCR za IL-8 in IL-1β mRNA. Rezultati so pokazali, da se ob aktivaciji in avtoaktivaciji TLR5 IL-1β ne prepisuje, medtem ko se prepis mRNA za IL-8 inducira le ob stimulaciji s flagelinom in ne ob avtoaktivaciji. Da avtoaktivirajoče celice ne izločajo citokinov, ki bi lahko vplivali na NF-κB aktivnost, je pokazal tudi poskus, pri katerem smo kontrolne celice inkubirali v gojišču, v katerem so prej rastle avtoaktivirajoče celice. Njihovo gojišče ni značilno povečalo aktivnost NF-κB kontrolnih celic. Ti poskusi so tudi pokazali, da aktivacija ob stimulaciji s flagelinom in avtoaktivacija nista povsem enaka procesa, saj avtoaktivacija ni dovolj za transkripcijo citokinov, ampak je za to potrebna prisotnost flagelina. Pri avtoaktivaciji najverjetneje ne pride do aktivacije vseh signalnih poti in s tem vseh transkripcijskih faktorjev, potrebnih za začetek transkripcije genov za citokine, zato ne opazimo prisotnosti mRNA za IL-8. Razlog bi lahko bil tudi, da avtoaktivacija aktivira NF-κB po drugi signalni poti, ali pa da aktivacija samo receptorja TLR5 ni dovolj. Dokazali so namreč, da se flagelin lahko veže tudi na druge receptorje, kot je npr. IPAF (interleukin (IL)-1-converting enzyme protease-activation factor), ki ob vezavi flagelina sproži svojo signalno pot in spremeni odziv celice. Podobno se lahko dogaja tudi v našem primeru:

avtoaktivacija aktivira zgolj NF-κB, medtem ko flagelin aktivira tako TLR5, kot tudi tisti dodatni receptor, aktivnost obeh pa je potrebna za popolni odziv celice.

Zaradi opažene razlike med aktivacijo in avtoaktivacijo smo pri vseh nadaljnjih poskusih uporabili oba načina spremljanja vpliva mikrookoljskih dejavnikov na aktivnost NF-κB. Morebitne razlike v odgovoru celic bi lahko pripomogle k razumevanju procesa avtoaktivacije. Z gojenjem celic HEK293 v prisotnosti določenih komponent zunajceličnega matriksa, citokinov, hormonov in rastnih dejavnikov, metabolitov in nekaterih inhibitorjev smo torej preverili njihov vpliv na aktivnost NF-κB, odvisne od TLR5.

Zunajcelični matriks je pomemben del celičnega mikrookolja, saj celicam nudi oporo in omogoča številne medcelične interakcije, ki lahko vplivajo na funkcijo in delovanje celic. Prejšnje raziskave so že pokazale, da nekatere komponente ECM, kot npr. heparan sulfat in receptorji integrini, lahko vplivajo na delovanje TLR. V tej raziskavi smo ugotavljali vpliv mucina, človeškega tenascina, kolagena IV in VI ter poli-lizina (poly-L-lysin, PLL). Celice smo 24 ur pustili rasti v luknjicah, prevlečenih z naštetimi komponentami, in jih opazovali pod mikroskopom. Celice, ki so rasle na mucinu, so bile okrogle in le rahlo pritrjene, a še vedno žive, kar nakazuje na to, da se na mucin ne morejo pritrditi. Celice na drugih komponentah so rasle veliko bolje kot kontrolne celice (100 % konfluentnost), ki so rasle v neprevlečenih luknjicah (85–90 % konfluentnost). Ne glede na boljšo rast pa vse komponente niso imele vpliva na aktivnost NF-κB ne ob stimulaciji in niti ob avtoaktivaciji.

Od vseh testiranih komponent so bili najbolj zanimivi rezultati poskusov z intenzivno rastjo celic na tenascinu. Tenascin se namreč večinoma izraža tekom embrionalnega razvoja, pri odraslih pa se pojavlja v malignih tumorjih in med vnetji in obnovo tkiv. Ker zavira vezavo nekaterih imunskih celic in fibroblastov velja za anti-adhezivni protein. Celice HEK293 so se nanj dobro vezale in se celo delile, kar namiguje na to, da ta celična linija izraža receptorje za tenascin, kar do sedaj še ni bilo potrjeno. Opazili so, da se *in vivo* tenascin pojavlja na mestih z intenzivno celično rastjo, kar namiguje da bi tenascin lahko imel vlogo stimulacije celične proliferacije, kar sovpada z odgovorom celic v našem poskusu. Ekspresijo receptorjev za tenascin lahko razložimo z dejstvom, da so HEK293 embrionalne celice.

Podobno bi lahko zaključili za kolagene, ki so glavna in najbolj pogosta komponenta ECM. Oba tipa kolagena sta sprožila proliferacijo, kar nakazuje na prisotnost vsaj enega od integrinov, ki vežejo kolagen ($\alpha_1\beta_1$ in $\alpha_2\beta_1$). Najverjetneje gre za integrin $\alpha_1\beta_1$, saj so za

celice HEK293 potrdili izražanje obeh podenot. Oba integrina ob vezavi regulirata celični cikel in spodbujata proliferacijo.

Poli-lizin (PLL) sicer ni komponenta ECM, a je presenetljivo nekoliko zmanjšala avtoaktivacijo TLR5. PLL je homopolipeptid iz L-lizinskih ostankov, ki ga zaradi njegovega pozitivnega naboja pogosto uporabljamo kot prevleko za boljše pritrjanje celic. Pritrjanje je nespecifično in temelji na negativnem naboju celičnih membran. Ker celice nimajo receptorjev za PLL, je opaženo zmanjšanje najverjetneje posledica zmanjšane lateralne mobilnosti membranskih proteinov na pritrjeni strani celice, kar lahko zmanjša tudi spontano avtoaktivacijo TLR5. PLL namreč ni vplival na opaženo aktivnost NF-κB ob stimulaciji s flagelinom.

Citokini so topni mediatorji imunskega sistema, s katerimi večinoma med seboj komunicirajo imunske celice, nanje pa se lahko odzovejo tudi neimunske celice, večinoma tako, da se s spremembo transkripcije pripravijo na morebitno srečanje s patogenom. V tej raziskavi smo testirali vpliv IL-4, IL-6, IL-10, IL-12, GM-CSF, TNFα in IL-1β. Večina testiranih citokinov (IL-4, IL-6, IL-10, IL-12 in GM-CSF) ni imela nikakršnega vpliva na aktivnost NF-κB, medtem ko sta TNFα in IL-1β provnetna citokina, ki signalizirata preko NF-κB in ga aktivirata neodvisno od TLR5 ter tako onemogočita opazovanje njunega vpliva na od TLR5 odvisno aktivacijo NF-κB. Naša metoda najverjetneje ni najbolj primerna za opazovanje vpliva citokinov na delovanje TLR5, saj večina regulacijskih mehanizmov, ki jih citokini sprožijo, deluje preko spremembe transkripcije, za kar pa bi potrebovali več kot 5 urni interval, kolikor smo inkubirali v naših poskusih.

Celice so obdane tudi z drugimi celičnimi izločki, kot so hormoni in celični metaboliti, ki so bodisi gostiteljevi ali bakterijskega izvora. Za številne (izločki kvasovk in nekaterih bakterij, piruvat ...) so že pokazali, da lahko vplivajo na delovanje receptorjev TLR, v tej raziskavi pa smo preverili vpliv kratkoverižnih maščobnih kislin butirata, propionata in acetata ter sladkorja glukoze. Iz literature je znano, da butirat in acetat delujeta kot inhibitorja encimov histonskih deacetilaz in tako preprečita odstranjevanje acetilnih skupin s histonov in drugih proteinov, med drugim tudi transkripcijskega faktorja NF-κB. Prisotnost neodstranjenih acetilnih skupin na proteinih poveča ali zmanjša njihovo aktivnost. Inkubacija z butiratom in propionatom je v naših poskusih povečala odziv celic na stimulacijo s flagelinom v kontrolnih celicah (brez dodatnega TLR5), ni pa vplivala na intenzivnost avtoaktivacije, ne glede na stimulacijo teh celic s flagelinom. Acetat in glukoza nista vplivala na aktivacijo NF-κB.

Poleg metabolitov smo preverili tudi vpliv hormonov inzulina in epidermalnega rastnega faktorja. Inzulin je metabolni hormon, ki regulira prevzem in koncentracijo glukoze v telesnih tekočinah, epidermalni rastni faktor (EGF) pa regulira rast, delitev in diferenciacijo epidermalnih celic. Celice smo predhodno inkubirali za 30 min v koncentracijski vrsti hormonov in jih stimulirali s flagelinom. Naraščajoča koncentracija inzulina ni vplivala na aktivnost NF-κB, čeprav je za hormon znano, da signaliziranje TLR poveča rezistenco celic na inzulin, kar lahko vodi celo v nastanek diabetesa. V našem poskusu smo najprej aktivirali signalno kaskado inzulina in šele nato stimulirali s flagelinom, nespremenjena aktivnost NF-κB pa nakazuje, da je interakcija med signalnima kaskadama zgolj enosmerna.

Za razliko od inzulina je EGF povečal aktivnost NF-κB v odvisnosti od koncentracije. Vpliv je bil tem večji, čim višja je bila raven z avtoaktivacijo ali stimulacijo aktiviranega NF-κB, kontrolne celice pa se na hormon niso odzvale. Rezultati namigujejo, da EGF sam od sebe ne aktivira NF-κB, ampak zgolj ojača ali podaljša delovanje že aktivnega transkripcijskega faktorja oz. preko interakcije signalne poti EGF s signaliziranjem receptorja TLR5.

TLR5 je transmembranski receptor, ki se nahaja v lipidnih raftih plazmaleme. Plazmalema ali zunanja celična membrana je dinamična struktura, ki ločuje zunanjost celice od njenega okolja, hkrati pa preko membranskih receptorjev omogoča komunikacijo celice z okoljem in tako prilagajanje delovanja celice glede na razmere v mikrookolju. Za pravilno delovanje vseh teh komunikacijskih sistemov, tudi TLR5, sta pomembni tudi fluidnost membrane in struktura lipidnih raftov. Številne raziskave so pokazale, da le dimerizacija nekaterih tipov TLR ni dovolj za njihovo aktivacijo. TLR4, TLR2 in TLR3 med drugim potrebujejo delovanje nevroaminidaze Neu1, ki z receptorjev odstrani ostanke sialične kisline in s tem omogoči pravilno konformacijo pri dimerizaciji. Da bi preverili vlogo membrane in nekaterih membranskih proteinov, smo celice inkubirali s številnimi inhibitorji in drugimi snovmi, ki so spremenile lastnosti membrane.

Filipin III in metil-β-ciklodekstrin sta kemijski komponenti, ki se s svojo strukturo vežeta na holesterol v membrani in tako vsaka na svoj način razbijeta lipidne rafte, v katerih se običajno nahajajo receptorji TLR. Filipin III je povzročil zmanjšanje aktivnosti NF-κB pri vseh celicah ne glede na stimulacijo in avtoaktivacijo, medtem ko je metil-β-ciklodekstrin zmanjšal aktivacijo le pri s flagelinom stimuliranih celicah, ni pa vplival na stopnjo avtoaktivacije. Ti rezultati namigujejo, da proces avtoaktivacije ni odvisen od lipidnih raftov, medtem ko je odgovor na stimulacijo odvisen od pravilne strukture raftov.

Da bi preverili, ali glikozilacija receptorjev vpliva na njihovo delovanje, smo uporabili inhibitorja metaloproteinaz (MPI), CMP-NANA (citidin-5′-monofosfo-N-acetilnevroaminična kislina, prekurzorska molekula za glikozidne ostanke na receptorjih) in timokinon (TQ, aktivator nevroaminidaze Neu4, ki skupaj z metaloproteinazami sodeluje pri odstranjevanju glikozidnih ostankov z receptorjev in omogočijo aktivacijo in signaliziranje TLR4). V naših poskusih je MPI povzročil zmanjšanje aktivacije NF-κB pri vseh avtoaktivirajočih celicah, medtem ko je imel le malo vpliva na kontrolne celice. To nakazuje, da ima ena od metaloproteinaz, ki jih uporabljeni MPI blokira, vlogo pri avtoaktivaciji. Nasprotno je 19-urna inkubacija s CMP-NANA, tekom katere smo pričakovali vsaj delno zamenjavo receptorjev TLR5 z novimi, bolj glikoziliranimi, le malo zmanjšala aktivacijo NF-κB. Avtoaktivacija tako najverjetneje ni posledica pomanjkanja glikozidnih ostankov na receptorjih, a za zanesljivejše rezultate bi bilo potrebnih več poskusov. Inkubacija s TQ je močno zmanjšala aktivnost NF-κB pri stimuliranih in avtoaktivirajočih celicah v odvisnosti od koncentracije, ni pa imela vpliva na kontrolne nestimulirane celice. Celice so bile pri višjih koncentracijah po 5 h inkubacije okrogle in slabo pritrjene, barvanje z eozinom pa je pokazalo, da TQ v odvisnosti od časa in koncentracije povzroča celično smrt, ki je najverjetneje razlog za opaženi upad aktivnosti NF-κB.

Za zaključek, naša raziskava je pokazala, da je odgovor TLR5 precej neodvisen od razmer v mikrookolju. Če bi bilo delovanje receptorja odvisno od okolja, bi bil imunski odgovor odvisen od naključnega nabora prisotnih molekul v nekem trenutku in ne od tipa infekcije. To se povsem sklada s pomembno vlogo TLR5 v prirojeni imunosti, ki je receptorju pripisana.

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