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IN VITRO HODNOCENÍ NOVÝCH LIGANDŮ TOLL-LIKE RECEPTORŮ I

Diplomová práce

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Hradec Králové 2017 Kristína Hudáková

CHARLES UNIVERSITY

Faculty of pharmacy in Hradec Králové

department of pharmacology and toxicology

IN VITRO EVALUATION OF NOVEL TOLL-LIKE RECEPTOR LIGANDS I

Diploma thesis

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ABSTRAKT

Univerzita Karlova

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Název diplomové práce: In vitro hodnocení nových ligandů Toll-like receptorů I

Vakcinace proti infekčním chorobám, zabrání ročně miliónům úmrtí. Imunogenní vlastnosti vakcín jsou ještě posilněny přítomností imunologických adjuvans. Vývoj imunologických adjunvans vede k lepšímu bezpečnostnímu profilu vakcín a taktéž hraje klíčovou roli ve výzkumu nových vakcín proti patogenům, na které ještě v současnosti vakcíny neexistují. Hlavním cílem této diplomové práce bylo ověřit schopnost racionálně navržených malých ligandů ovlivňovat Toll-like receptory a tím pádem taky potenciál jejich využití jako imunologických adjuvans. Testování bylo provedeno za použití modifikových buněčných linií stabilně exprimujících lidské TLR4 nebo TLR8 receptory, jejichž aktivace vede k produkci sekretované embryonální alkalické fosfatázy. Na základě interakce s TLR receptory byly prověřeny agonistické a stejně tak antagonistické vlastnosti deseti analyzovaných látek označených jako DM 001 – DM010. Imunomodulační aktivita těchto testovaných látek byla určená stanovením množství sekretované alkalické fosfatázy pomocí kolorimetrické enzymatické reakce. Nepodařilo se prokázat významnou agonistickou aktivitu těchto molekul, avšak některé ze vzorek vykazovali na hTLR8 potenciální aktivitu antagonistickou. Mnohem slibnější výsledky byly získány hTLR4 agonistickou analýzou, kde tři analyzované látky, jmenovitě DM 002, DM 005 a DM 008, prokázaly v rámci interakce s receptorem výraznější aktivitu a představují podklad pro další výzkum.

Klíčová slova

TLR; imunomodulátory; in vitro hodnocení; adjuvans; vakcíny

ABSTRACT

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Title of diploma thesis: In vitro evaluation of novel Toll-like receptor ligands I

Vaccination against preventable infections prevents millions of deaths each year. Their immunity enhancing activity is strengthened by the presence of vaccine adjuvants. Development of vaccine adjuvants leads to improved safety profile and also can play a vital role in the research of new vaccines against pathogens against which the vaccines currently do not exist. The main aim of this diploma thesis was to verify the ability of rationally developed small molecule ligands to influence Toll-like receptors and thus their potential to be utilized as vaccine adjuvants. The assay was carried out using modified cell lines continually expressing the human TLR4 or TLR8 whose activation leads to production of secreted embryonic alkaline phosphatase. Ten analyzed substances labelled as DM 001 – DM 010 were examined for their agonistic and also antagonistic properties while interacting with the TLRs. Immunomodulatory activity of these tested samples was then determined by quantification of secreted alkaline phosphatase with the help of a colorimetric enzyme reaction. The results of the analysis did not manage to prove a significant agonistic activity of any of the molecules, but some samples may exhibit potential antagonistic activity on hTLR8. More promising results were obtained with the hTLR4 agonist analysis, where three of the analyzed substances, namely DM 002, DM 005 and DM 008, showed stronger activity within the interaction with the receptor and they represent a foundation for further research.

Keywords

TLR; immunomodulators; in vitro evaluation; adjuvants; vaccine

CONTENT

1 LIST OF ABBREVIATIONS

AP – alkaline phosphatase

AP-1 – activator protein 1

APC – antigen presenting cell

APCs – antigen-presenting cells

ATP – adenosine triphosphate

CD-14 – cluster of differentiation-14 protein

CpG – nonmethylated CpG oligonucleotide

DAMP – danger-associated molecular pattern

DC – dendritic cell

DD – amino-(N)-terminal death domain

DMEM –Dulbecco's modified Eagle Medium

DMSO – Dimethyl sulfoxide

DNA – deoxyribonucelic acid

DPBS – Dulbecco's Phosphate Buffered Saline

dsRNA – double-stranded ribonucleic acid

HIV – human immunodeficiency virus

HSP – heat shock protein

hTLR – human Toll-like receptor

hTLR4 – human Toll-like receptor 4

hTLR8 – human Toll-like receptor 8

IFN-β – interferon β

IKK – IκB-kinase complex

IL – interleukine

IRAK – IL-1R-associated kinase

IRF – interferon regulatory factor

IκB – inhibitor of NF-κB

JAK – Janus activated kinase

JNK – c-Jun N-terminal kinase

LBP – Lipopolysaccharide binding protein

LGP2 – laboratory of genetics and physiology 2

LPS-EB – Lipopolysaccharide from E. coli 0111:B4 strain

LPS-RS – LPS from from *Rhodobacter sphaeroides*

LRR – leucine-rich repeat

MALP-2 – macrophage-activating lipopeptide 2

MAPK – mitogen-activated protein kinase

MD-2 – myeloid differentiation-2 protein

Mda5 – melanoma differentiation associated factor 5

MHC – major histocompatibility complex

MPLA – Monophosphoryl Lipid A

MyD88 – myeloid diffrentiation factor 88

NF – nuclear factor

NK – natural killer cell

PAMP – pathogen-associated molecular pattern

PBMC – peripheral blood mononuclear cell

Poly(I:C) – polyinosinic-polycytidylic acid

PRR – pathogen-recognition receptor

R848 - resiquimod

RIG-I – retinoic-acid inducible gene I

RIP1 – receptor-interacting protein 1

SEAP – secreted embryonic alkaline phosphatase

ssRNA – single-stranded ribonucleic acid

STAT – signal transducer and activator of transcription

TAB – TAK1-binding protein

TAK – TGF-β-activated kinase

TBK1 – TRAF-family-member-associated NF-κB activator-binding kinase 1

TGF – transforming growth factor

TIR – Toll/IL-1R

TIRAP – TIR domain containing adaptor protein

TLR – Toll-like receptor

TNF – tumor-necrosis factor

TOLLIP – Toll-interacting protein

TRAF6 – TNF-receptor-associated factor 6

TRAM – TRIF-related adaptor molecule

TRIF – TIR-domain containing adaptor inducing interferon β

UBC13 – ubiquitin-conjugating enzyme 13

UEV1A – ubiquitin-conjugating enzyme E2 variant 1

2 INTRODUCTION

Over the years, vaccination has helped to save millions of lives from the dangerous and sometimes fatal infectious diseases and their numerous complications and it is also responsible for eradication of small pox. It represents much safer and more convenient way of attaining immunity than overcoming the illness itself. Besides the active ingredient, one of the main components includes vaccine adjuvants. Their importance lies in the ability to enhance the immunogenicity of new and sometimes poorly immunogenic vaccine types, such as recombinant or subunit vaccines. Adjuvants usually lead to reduction in dosage, but most importantly they can influence the quality of immune response and thus enhance either humoral or cellular immunity. Recognition of specific pathogen-associated molecular patterns (PAMP) through pathogen-recognition receptors (PRR), such as TLRs is one of the most researched mechanisms of action of adjuvants. PAMP are typical only for the pathogen and necessary for its survival. When PRRs including TLR group recognize PAMP of a pathogen or a specific ligand, it leads to production of cytokines, activation and maturation of immune cells and subsequent immune response. Therefore, novel molecules activating TLRs are of great importance for designing a better adjuvants and better vaccines.

3 THEORETICAL PART

3.1 Vaccination and its importance

Vaccination is defined as the administration of an antigen for the purpose of activation of the immune system and induction of a specific immune response. That leads to the protection against infections caused by the pathogen carrying that particular antigen or another antigen that is sufficiently similar to the original one (Hořejší and Bartůňková, 2009; Leroux-Roels, 2010; Kocourková et al., 2017).

Vaccination allows the organism to acquire the immunity to a pathogen artificially. This provides a lot safer way of acquiring resistance to numerous infections and diseases comparing to naturally attained immunity from the illness itself. The vaccination helps to avoid the dangers and sometimes deadly complications associated with the manifestation of the disease. Vaccination therefore leads to the reduction in morbidity and mortality of such. Not only these matters reduce the expenses of therapy, because prevention costs less than treatment, but it also shortens the time of communicability by stopping the infection before it has the chance to manifest. This way, the vaccines protect not only the individuals, but also the whole communities. If the majority of people in a certain group are vaccinated, the probability of spreading the illness is much smaller, it is commonly known as herd immunity. It also helps to protect the individuals, who are not able to receive the vaccination (Kocourková et al., 2017; National Institute of Allergy and Infectious Diseases, 2008).

Besides all the benefits of vaccination mentioned above, there are still more and sometimes indirect advantages of immunization that can be mentioned. Some of the examples include economic consequences in elongation of life expectancy and decrease in poverty, since people can avoid the infection and its disabling complications. The finances saved in prevention and not treatment of the disease can be returned back into the health care system and used for other purposes. Immunization keeps the infection spread under control and therefore makes tourism and travelling a lot safer and easier (WHO Regional Office for Europe, 2015). Each year immunization is able to prevent 2

to 3 million deaths, but since it is more difficult to reach and immunize children in remote parts of the world, the number of averted deaths can still be higher (World Health Organization; World Health Organization, 2013).

One of the big issues of vaccination is vaccine hesitancy wide spread across multiple countries. Vaccine hesitancy is a complex set of contextual, individual and group or special determinants concerning vaccination that delay or prevent people from being vaccinated. It usually includes strong media influence or historical conventions. In some countries, especially the third world countries, where health care facility can be too distant or otherwise inaccessible. Individual reasons are usually motivated by negative past experiences with immunization, general knowledge of preventable diseases, vaccines and their safety or religious and personal beliefs (World Health Organization, 2015). People sometimes believe myths that do not tend to be too accurate, reliable or evidence-based, such as belief, that proper sanitation and hygiene are good enough prevention or inutility to vaccinate when the infection does not occur in that particular country (World Health Organization, 2017). Quite frequent cause of hesitance is also the fear of adverse effects of vaccines.

Powerful example of such fear was published study executed by Andrew Wakefield that stated that there might be a connection between measles, mumps and rubella (MMR) vaccine and autism. This implication was the cause of a big scare that shaped the outlook on vaccination and immunization of the whole generation of parents, left a great number of children unprotected against preventable infections and later on also to the outbreaks of such diseases. Despite the repeated experiments and epidemiological studies that found no evidence in this MMR vaccine-autism connection and despite evidences of Wakefield's study being fraudulent, flawed and unethically executed, the fear and beliefs of vaccination are ongoing (Godlee et al., 2011; Poland and Spier, 2010).

These beliefs and opinions that are caused by misinformation and fraud usually strongly supported by media can lead to unnecessary disease outbreaks, complications and deaths even though immunization's global impact is apparent. Vaccination is responsible for eradication of smallpox that caused millions of deaths every year and poliomyelitis is near eradication thanks to worldwide immunization campaigns

(Prymula). The number of deaths the vaccine-preventable infections were responsible has dropped from 0.9 million to 0.4 million. Thanks to ongoing immunization plans and projects, the total number of averted future deaths could reach 24.6 million (World Health Organization, 2013).

3.2 Vaccine components

3.2.1 Active ingredient

Vaccines trigger an effective immune response by mimicking an actual infection. The main difference is in the strength of the pathogen virulence. The natural illness is caused by strong and viable pathogen and therefore is manifested through typical symptoms. On the other hand, pathogens used in vaccine are weakened, attenuated or sometimes not even present in its entirety. Modern subunit vaccines often contain only the most purposeful parts of the pathogen. The vaccine must keep the ability to activate the immunocompetent cells, to elicit an efficient immune response and production of the sufficient supply of the memory T and B cells, but without causing the disease symptoms and complications. These memory cells are responsible for the incomparably faster and stronger immune response when the vaccinated organism encounters the actual infection. Through massive maturation of cytotoxic T cells and production of pathogen-specific antibodies the dangerous agent is eliminated before the illness have the chance to develop (National Institute of Allergy and Infectious Diseases, 2008). These vaccine pathogens, also known as vaccine antigens, are the main ingredients responsible for the desired immune response. The antigens are for the purpose of the vaccines altered from its original form, where their main properties and the character of the alteration distinguish several types of vaccines: (Kocourková et al., 2017; National Center for Immunisation Research & Surveillance, 2013).

Attenuated live vaccines

One of the biggest advantages of live attenuated vaccines is strong immune response and even lifelong immunity achieved in only one or two doses. These vaccines are designated to contain live disease-causing pathogens, either viruses or bacteria, that are specifically treated to be weakened, attenuated and therefore closely mimic the

natural infection but without the symptoms and contagiousness. Although for the same reason, they are more dangerous, because attenuated, but live pathogens can revert to their original virulent form and cause the illness they are supposed to avert and become a potential harm especially to immunocompromised patients unable to cope with the actual infection. Vaccines against tuberculosis, measles or rotavirus are produced by attenuation process (Kocourková et al., 2017; National Center for Immunisation Research & Surveillance, 2013; World Health Organization; The College of Physicians of Philadelphia).

Inactivated vaccines

The second form of vaccines contains microorganisms that are killed and therefore inactivated, usually through physical or chemical processes, for example by heat, radiation or formaldehyde. In comparison to live attenuated vaccine, the inactivated form of pathogen does not induce as strong immune response and more doses or booster shots are needed to acquire long term immunity. Killed virus or bacteria lose their capability to reproduce during the inactivation and therefore have less adverse effects and smaller risk of inducing an actual infection even after administration to people with weakened immunity. Examples of inactivated vaccines are inactivated poliomyelitis or hepatitis A vaccine (The College of Physicians of Philadelphia; National Center for Immunisation Research & Surveillance, 2013; Kocourková et al., 2017; National Institute of Allergy and Infectious Diseases, 2008; World Health Organization).

Subunit vaccines

First step for vaccine production is the identification of pathogen epitopes. Epitopes are specific parts of the microbes, that T cells and antibodies have the ability to recognize and therefore subsequently produce immune response. Subunit vaccines do not contain the whole microbe, but only these specific pathogenic fragments generated by chemical break down or using recombinant technology, where genes coding most important parts of the pathogen are inserted into and produced by another microorganism, most commonly *Saccharomyces cerevisiae*. These vaccines are then known as recombinant subunit vaccines. Advantages of subunit vaccines are the absence of live structures and option of administration to immunocompromised patients,

because the risk of inducing an infection is eliminated. They also produce less adverse effects and are more stable than live attenuated vaccines. For this type of vaccine is very important to locate most immunogenic pathogen fragments and find the best combination of antigenic properties to achieve adequate immune response and also there tend to be no guarantee, that they will produce enough memory cells for future potential infections.

Within this vaccine type, three more subtypes can be differentiated. An example of protein-based subunit vaccine is the vaccine against the hepatitis B virus, where only isolated and purified protein part of the pathogen is used. Although, there is always a probability, that the protein could be denaturized and consequently bind to different antibodies. Some bacteria strains can possess an outer coating composed of polysaccharide molecules and their main purpose is to bypass the immune defense systems. Polysaccharide vaccines are therefore directed against bacterial carbohydrate capsule, but these molecules are usually quite small and they have only small immunogenic activity, especially when administered to children, and they create only short-term immunity with almost no memory cells. For that reason, in most of the cases, these polysaccharides are chemically bound to a carrier protein or protein from a different agent, such as tetanus or diphtheria toxoids, in order to create the last subtype, conjugated subunit vaccine, such as pneumococcal and *Haemophilus influenzae* type b (Hib) vaccine. Conjugated carbohydrate molecules tend to have stronger and more efficient immune response against infection pathogen than plain polysaccharide vaccines especially when administered to infants or if long-term immunity is necessary (Kocourková et al., 2017; National Center for Immunisation Research & Surveillance, 2013; National Institute of Allergy and Infectious Diseases, 2008; World Health Organization; The College of Physicians of Philadelphia).

Toxoid vaccines

Some vaccines do not contain the specific fragments of the pathogen or the whole microbes, but they are comprised of toxins produced by these pathogens that are chemically inactivated using a mixture of formaldehyde and sterile water. Those are known as toxoid vaccines. Even a small amount of bacterial toxin can cause an infection with all the typical symptoms, but its inactivated form, toxoid, is in comparison stable,

safe and does not cause the disease because it cannot revert to its active mode. Toxoid vaccines, for example against tetanus or diphtheria, require administration of multiple doses and employment of a vaccine adjuvant to reach effective immune response and sufficient protection (Kocourková et al., 2017; National Center for Immunisation Research & Surveillance, 2013; World Health Organization; National Institute of Allergy and Infectious Diseases, 2008).

Deoxyribonucleic acid vaccines

The next two vaccine types are still in their experimental phases, but they can present a very promising path of vaccine technology development. The first potential vaccine type is deoxyribonucleic acid (DNA) vaccine. The mechanism of action is based on the DNA plasmids, small parts of pathogenic DNA containing genes for microbial antigens (National Institute of Allergy and Infectious Diseases, 2008). These plasmids are introduced to an organism through intramuscular, subcutaneous, intranasal or oral administration. The DNA plasmids need to be injected in a higher volume, because quite a big portion of the DNA is degraded by nucleases present in the organism (Tregoning and Kinnear, 2014). Once the DNA had entered the nuclei of the cells located at the site of the injection, most likely via nuclear pores, the genetic information is transcribed and translated producing pathogenic antigens. The DNA is either taken up by antigen presenting cell (APC) or by non-antigen-presenting cell like keratinocytes or myocytes (Tregoning and Kinnear, 2014; Kocourková et al., 2017). These cells then mediate the antigen-stimulated antibody response and also display the antigens on their surfaces to trigger the cellular response (National Institute of Allergy and Infectious Diseases, 2008). Usually, APCs display the antigen through major histocompatibility complex I (MHC-I), which leads to activation of cytotoxic T-cells that kill the transfected cells (Tregoning and Kinnear, 2014). Eventually, this step can lead to decrease in antigen expression and therefore lowered immunogenicity. If the DNA is adopted by non-antigen-presenting cells, the antigen is secreted or released after cell death. Antigens are then picked up and presented on MHC-II activating helper T-cells or B cells or the antigen is subsequently taken up by APCs and presented on MHC-I (Kocourková et al., 2017; Tregoning and Kinnear, 2014).

Thanks to the DNA molecule stability, this type of vaccines is highly thermostable in comparison to other vaccine types. Also, its development process is significantly faster, easier and financially less demanding and the vaccines tend to have less adverse effects and lower risk of pathogen reverting back to virulent state. Although, these vaccines lead the immunization to a new technology level, there are still some safety concerns that need to be dealt with. The first concern was the plasmids purity and the risk of transfecting also the genes for antibiotic resistance (Kocourková et al., 2017; Tregoning and Kinnear, 2014; National Institute of Allergy and Infectious Diseases, 2008). The other disadvantage includes scaling of the administered doses and volumes that would need to be much larger than normal vaccine volume which can lead to higher risk of inflammation and other side effects. Secondly, the difficulties with extrapolation of information about immune response and DNA uptake, processing and expression between different species (National Institute of Allergy and Infectious Diseases, 2008; Kocourková et al., 2017; Tregoning and Kinnear, 2014).

Recombinant vector vaccines

Some pathogens or viruses have a specific ability to attach to the surface of a host cell and inject their genetic information into them in order to trigger an infection. For research purposes, this ability can be of a great advantage, where the harmless shell of one pathogen is used as a vector or carrier for the genetic information of target pathogen (the one against the immunity should be elicited). The inserted sequence codes the most important antigen epitopes. The genetic information of the second pathogen is produced by infected cell thus creating mechanism of action analogous to the DNA vaccine. Since, the recombinant vector vaccines mimic a natural infection; they induce strong and effective immune response and can be of a great use in diseases with complicated infection process like human immunodeficiency virus (HIV) or other infections like measles or rabies. Although, vaccines of this type are still in development stage and needs more clinical evaluation, they can considerably contribute to the development of immunization (National Institute of Allergy and Infectious Diseases, 2008; Kocourková et al., 2017).

3.2.2 Adjuvants

Adjuvant is a component that is usually present in the vaccine in small amounts. Its main purpose is to stimulate better and more effective immunogenicity that is achieved by strengthening and lengthening of the immune response to a vaccine. Thus smaller amount of antigen or fewer immunization doses are required. Adjuvants also have the ability to form immune response more rapidly, to create more memory cells and long-term immunity and lead to antibody titers elevation. Most commonly used adjuvants include mineral salts like aluminium hydroxide, aluminium phosphate or potassium aluminium sulphate, liposomes, polysaccharides, TLR agonists, cytokines, emulsions or their combinations (Kocourková et al., 2017; Honegr et al., 2015; National Center for Immunisation Research & Surveillance, 2013; Oxford Vaccine Group).

3.2.3 Preservatives

Preservatives are compounds used as protectors of the vaccines against bacterial and fungal contamination. Preservative employment was carried over from the past as a necessity, because multi-dose vials were used and there occurred many cases of acquired infection after vaccine administration due to microbial contamination. Nowadays, most of the vaccines contain a preservative, most commonly phenol, 2-phenoxyethanol or thiomersal. Although, these compounds are quite controversial, since some of the molecules may cause severe adverse effects, like eczema or nephrotoxicity and neurotoxicity. There a few studies performed indicating, that the concentrations of vaccine preservatives are more harmful to humans than microbes and sometimes, the doses are not high enough to sufficiently kill the microbes. Therefore, vaccines manufactured under aseptic conditions are supposed to be more preferred in the future in order to eliminate the use of preservatives and their risks for patients (Kocourková et al., 2017; Geier et al., 2010; Offit and Jew, 2003).

3.2.4 Stabilizers

Additives or stabilizers are used to protect vaccines from the unfavorable environmental conditions, such as repeated freezing-drying, heating, humidity, light or acidity. They also help to maintain and prolong antigen’s stability and effectiveness. Stabilizers keep individual components and immunogens from adhering to vial walls.

The main representatives of additive include lactose and sucrose, very often used in combination vaccines due to their higher stabilizing activity, amino acids, for example glycine or glutamate, human serum albumin or gelatin, that could be responsible for instant hypersensitivity to vaccines. Big potential for the use as stabilizer have also silk, that confers high temperature stability therefore, it can grant useful advantages to vaccine handling (Offit and Jew, 2003; Kocourková et al., 2017; National Center for Immunisation Research & Surveillance, 2013).

3.2.5 Diluents

Since most of the commonly used vaccines are administered in a form of a solution, diluents, separately added liquids need to be used to dilute all the components of the vaccine in order to prepare appropriate concentrations. Certain types of vaccine, such as live attenuated are stored in lyophilized form and need to be reconstituted using a diluent, sterile saline solution or sterile water, prior to administration to patient (National Center for Immunisation Research & Surveillance, 2013; World Health Organization).

3.2.6 Trace components

Antibiotics

During the manufacturing process of a vaccine, many different compounds are used; those serve as technical substances, meaning that they are used during production process, but do not appear in the finished product or appear only in trace amounts. Examples of a technical component are antibiotics, such as neomycin, polymyxin B, amphotericin B, streptomycin, gentamicin and chlortetracycline, molecules that are supposed to prevent bacterial contamination of the tissue cultures where the viruses and microbes are grown. These components contribute to a higher risk of immediate hypersensitivity reaction like local skin reaction or anaphylaxis. Although, antibiotics most prone to systemic allergic reactions like penicillins, cephalosporines or sulfonamides are not used in vaccine synthesis (Kocourková et al., 2017; National Center for Immunisation Research & Surveillance, 2013; Eldred et al., 2006; World Health Organization).

Inactivating agents

Formaldehyde and glutaraldehyde are in vaccine world known for their inactivation activity that is used in the manufacture process of inactivated antigens and also for purification of bacterial toxins of diphtheria and tetanus vaccines, but without destroying important antigen parts. Even though, formaldehyde is known as potential carcinogen, the amount present in the finished product is very small and quite unlikely to be the cause of cancers in patients. Most of the inactivating agents are removed from the vaccine during the purification process (World Health Organization; National Center for Immunisation Research & Surveillance, 2013; Eldred et al., 2006).

3.2.7 Other components

Other components include trace amounts of tissue cultures the microbes and viruses are grown on. Some vaccine can contain traces of egg protein, since they are cultivated in chicken eggs or chick embryos, other vaccines are grown using yeast cultures or human cell lines. Although, filtering and centrifugation process reduce a great amount of culture tissue cells from remaining in the final vaccine form, but trace quantities may still remain (National Center for Immunisation Research & Surveillance, 2013).

3.3 Vaccine adjuvants

As it was discussed in the subsection 3.1, vaccination has a lot of evident advantages that prove their importance in health care. Although, there are still some adverse effects, toxicity and risks that need to be overcome. One of the promising approaches could be research and employment in the field of vaccine adjuvants. Even though this research is only part of a bigger project, its need and necessity are still shared very closely. Development of vaccine adjuvants can bring improvement and preferable safety profile; they can also play a crucial role in the research of new vaccines against dangerous and lethal infections such as HIV, malaria or tuberculosis and therefore prevent numerous unnecessary deaths (Prymula).

3.3.1 Reasons for use of vaccine adjuvants

An incorporation of adjuvants into vaccines is guided by two main reasons: the qualitative and the quantitative reason.

The segment of quantitative adjuvant use include an induction of more efficient and long-lasting immune response in vaccine types that do not have as strong immunogenic potential than other types. Poorly immunogenic vaccines are e.g. subunit and recombinant vaccines comprised of purified and separated antigens that lack the additional parts of the pathogen that tend to work as adjuvants boosting the immune response to that particular antigen. In this case, new vaccines require the presence of an adjuvant in order to remain safe, but still sufficiently potent. They also evoke stronger immune responses manifested as higher antibody titers and seroconversion rates not only to protect general population, but also immunocompromised patients, elderly and children most desirably after primary immunization. The presence of adjuvant in a vaccine can lead to lower dose of the antigen or also to lower number of immunizations needed to achieve strong protection. This is particularly important trait in the cases of pandemic outbursts, where enormous amounts of antigens are needed in a short period of time and production capabilities are limited, or just to decrease financial expenses of manufacture (Gupta and Siber, 1995; Coffman et al., 2010). Lower antigen doses can be very useful when administering combination of more antigens, so called combination vaccines, where higher pathogen amounts can lead to various complications, for example antigen competition for certain carrier epitopes (Gupta and Siber, 1995). The requirement of multiple dose administration is especially inconvenient disadvantage, because patient compliance can be considerably affected and in some countries of the world, it can cause not only storage, but also logistic difficulties (Coffman et al., 2010).

The qualitative relevance of adjuvants is given through selective modulation of the immune response. With the help of adjuvants, antigen-presenting cells (APCs), and other significant pathways of the innate immune system are activated and their activation subsequently leads to induction of adaptive immune response that can be qualitatively altered in order to generate the most adequate type of immunity against particular pathogen (Gupta and Siber, 1995; Coffman et al., 2010; Leroux-Roels, 2010). Adjuvants can selectively enhance either humoral or cellular immune response or both

of them. Humoral response usually posed by antibodies is further specified by antibody type, subclass or its affinity to antigen. Form of cellular response is modulated by antigen recognition along with the recognition of major histocompatibility complex (MHC), either class I or class II leading to a different type of T lymphocytes production. Intracellular pathogens are usually presented by MHC class I leading to maturation and induction of cytotoxic T lymphocytes, on the other hand, extracellular pathogens like protein antigens and inactivated microorganisms tend to generate higher production of specific antibodies. Adjuvants can also manifest their immunomodulation properties through up and downregulation of certain cytokines and therefore creating different types of T helper cells, Th1 and Th2 cells. Intracellular pathogens, viruses and bacteria and antigens injected with the help of a virus vector promote the production of IL-2, IL-12 and interferon γ (IFNγ), cytokines that accompany delayed-type hypersensitivity and IgG antibody production typical for Th1 mediated response. Elicitation of Th2 cells is associated with IL-4, IL-5, IL-6 and IL-10 cytokines resulting in increased levels of circulating antibodies, IgE and IgG of a different subtype than in previous case. Sources of this type of immunity are inactivated pathogens and protein antigens (Gupta and Siber, 1995; Cox and Coulter, 1997). Vaccine adjuvants have the potency to change not only the qualitative parameter of the response, but thanks to these mechanisms also extensity and speed of the immunity. Therefore, instead of only eliciting the protection against the antigen, they accelerate this response convenient especially at times of pandemic outbreaks. Another practical advantage is enhancement of memory cells generated after overcoming an infection or after successful vaccination with sufficiently immunogenic pathogen. It is a population of cells that create strong and rapid response, when the organism encounters that particular pathogen again (Coffman et al., 2010).

New molecules developed as vaccine adjuvants can also manifest some more potential benefits that might be of great use in therapeutic vaccines as well. Although, there is more research required on this topic to fill in the gaps, but strongly immunogenic and specific adjuvants can become a key component in the development of medication for serious diseases like cancer or human papilloma virus (HPV). Very appreciated ability of new vaccine adjuvants is in increasing the breadth of the induced immune response, especially against pathogens like influenza, HPV or malaria. Problematic characteristic of these pathogens in their broad antigen diversity caused by

antigenic drifts or different strain modifications. The key basis does not lie within increased antibody titers, but within increased diversification of B cells producing wider variety of antigens (Reed et al., 2013).

3.3.2 Characterization of an ideal adjuvant

No vaccine or adjuvant is able to meet the ideal characteristics, but every new molecule or technology tries to approach these goals of improving the risk benefit ratio or administration efficacy. Importance of ideal adjuvant definition is crucial in order to design and produce safer and more potent adjuvant structures. Best way to ensure effectiveness, transferability and consistency in manufacture process is to use precisely defined molecules prepared by chemical synthesis. They tend to be safer with higher sustainability and purity in comparison with natural sources where higher risks of disease transfection and product variability have to be taken into consideration. Also, the production process needs to pose low financial expenses in order to provide vaccine supply for large amount of patients and for remote parts of the world. Important aspects include adjuvant particles and their morphology. Smaller particles have an easier entrance in to lymph nodes and can exclude the necessity of aseptic manufacture. "*Orientation and shape of non-spherical particles affects cell uptake; charge and chemical structure of surface groups are crucial factors in resulting bioactivity; targeting molecules such as mannose may enhance delivery to APCs"* (Reed et al., 2013). Vaccine adjuvant should be characterized by high stability in particle shapes, sizes and chemical structure ensuring low toxicity, high adjuvanticity and long shelf life. They should be biodegradable but without content of dangerous substances produced by chemical side reactions or degrading process. This oxidative deterioration can be prevented by packaging under the control of inert gas guards (Reed et al., 2013). An ideal adjuvant must be compatible with the antigen and also with other vaccine components essential for the effect and not exhibit any interference with antigen activity. Although, some level of association of the antigen structure and adjuvant formulation is desired; this interaction should be closely defined in order to prevent any unwanted influence on immunity (Reed et al., 2013; Gupta and Siber, 1995).

3.3.3 Bioactivity of vaccine adjuvants

Route of the administration plays the crucial role in the biological activity of the vaccine adjuvant. Considering various routes of administration, there are significant differences in mucosal, parenteral, intramuscular or dermal and subcutaneous against the intramuscular route as a result of local toxicity of many adjuvants, the last in the list being more potent in immunity stimulation. The resulting effect can therefore be noticeably influenced by new delivery systems, antigen vector or formulation (Reed et al., 2013; Aguilar and Rodriguez, 2007). The preparation should be able to induce a highly specific immune response most effective against specific pathogen including modulation of cellular response and great antibody affinity, but also broad spectrum of antibody types against different strains. Antibody isotype plays an important role in shaping the immune response. Most preferable is IgG isotype with the ability to pass between extravascular and intravascular compartments and also through placenta membrane into a fetus. Cytotoxicity is in many cases, especially in fighting the intracellular pathogens and tumors, very desirable and antibody IgG isotype activates the complement and co-operate with antigen-dependent cells responsible for cytotoxicity more than other antibody types. IgE antibodies are useful only against certain parasites, but their higher levels are rather inadequate, since they play an important role in allergic reactions. An adjuvant producing IgG antibodies with high affinity to the antigen and all of the properties mentioned above would create persistent response of high quality reducing not only amount of antigen and adjuvant needed but also the number of vaccinations. Good adjuvant formulation should elicit sufficient immunity protection when used with weak antigens like polysaccharide-protein conjugates in elderly, infants or immunocompromised patients. Ideally, a perfect adjuvant would possess all of these properties at the same time and in all cases, but no adjuvant or molecule is able to reach this goal (Reed et al., 2013; Gupta and Siber, 1995; Allison and Byars, 1991).

3.3.4 The safety profile

Considering the mechanism of action of many adjuvants, there is a constant battle between toxicity and safety, because the more potent the adjuvant activity is, the more toxic and more adverse effects the adjuvant tends to exhibit. Local reactions are

often associated with the formation of depot at the site of injection and include acute or chronic pain, local inflammation, swelling with the formation of sterile abscesses, granulomatous reaction, and erythema or tissue necrosis. Systemic reactions vary from one adjuvant to another, but generally involve fever, eosinophilia, drowsiness, nausea and vomiting, allergic or even anaphylactic reactions (Edelman, 1992; Aguilar and Rodriguez, 2007; Petrovsky and Aguilar, 2004; Gupta et al., 1993; Allison and Byars, 1991). Some adjuvants can also, with low incidence, cause carcinogenesis, teratogenesis or cross-reaction with own human antigens leading to glomerulonephritis or different organ specific toxicity (Edelman, 1992). Some adjuvants, for example muramyl dipeptide or lipopolysaccharide, have been replaced with their less dangerous derivatives because of their pyrogenicity, a trait undesirable in any vaccine adjuvant (Gupta et al., 1993). Since some adjuvants are meant to have an immunomodulatory effect usually through interaction with the innate immune system, concerns of inducing autoimmune diseases is understandably in place. Adjuvants can trigger autoimmune anterior uveitis, arthritis, urethritis or amyloidosis (Aguilar and Rodriguez, 2007; Edelman, 1992; Petrovsky and Aguilar, 2004). Immunotoxicity can be manifested not only through autoimmune illnesses, but through release of cytokines and immunosuppression as well (Edelman, 1992; Aguilar and Rodriguez, 2007; Gupta et al., 1993; Petrovsky and Aguilar, 2004; Allison and Byars, 1991). Safety and tolerability or risk benefit ratio still remains one of the biggest challenges of adjuvant research, because most of the vaccines are meant for prophylactic and not therapeutic purposes and because they would be administered to large number of people and infants as well (Reed et al., 2013).

3.4 General mechanisms of action of adjuvants

Although, mechanisms of action usually require a lot of research in order to provide sufficient rational characterization of the process and are also sometimes challenging to understand, they are the part of the highest relevance for systematic adjuvant design and their most suitable employment in the vaccines. One of the most important steps in induction of specific immune response is the activation and maturation of APCs and especially dendritic cells (DCs) (Awate et al., 2013). These are considered to be natural adjuvant of the immune system, because their ability to

recognize, take up and process the antigen is one of strongest (Kwissa et al., 2007; Lambrecht et al., 2009). DCs localized in tissues are usually immature and their main specialization is in antigen recognition and uptake (Lambrecht et al., 2009). After the APCs and DCs have taken up the antigen, it needs to be processed in a specific way; it has to be broken down into small pieces and peptides in order to be presented onto MHC molecules localized on the surface of the APCs (Awate et al., 2013).

Large lipid antigens are placed in so called early phagosomes, vesicles filled with phagocyted material, that enhance the presentation. The size of the antigen plays an important role in the overall effectiveness of the presentation, because small antigens are deposed in small vesicles that tend to form late phagosomes and the presentation process is then protracted (Awate et al., 2013). While the antigen is being processed, the APCs, especially their subclass of DCs migrate to a T cell paracortex of a draining lymph node and in the process they lose their ability to take up other alien molecules and they mature expressing all the costimulatory molecules necessary for the interaction with naïve $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes (Cox and Coulter, 1997; Lambrecht et al., 2009). Their subsequent activation leads to specific immunomodulation. $CD4^+$ T-helper cell activation leads to higher levels of Th1 cells that are responsible for cytotoxic T-lymphocyte production effective against intracellular pathogens. Interaction with B cells then activates their differentiation into plasma cells and the production of potent antibodies of specific subclass. Higher levels of Th2 cells generate eosinophil-mediated response against pathogens (Kwissa et al., 2007; Cox and Coulter, 1997; Lambrecht et al., 2009). From antigen recognition and uptake through foreign material processing to presentation to lymphocytes, APCs create very important bridge between innate and adaptive immunity (Lambrecht et al., 2009). The activation of immune system and induction of immune response can be characterized by three mechanisms.

1. Formation of a depot with a slow release of antigen at the site of injection stimulates antibody production.

2. Another mechanism of action leads to pro-inflammatory environment at the administration site with up-regulation of cytokines and chemokines that are responsible for APCs attraction and recruitment.

3. Activation of APCs or DCs can be achieved through antigen uptake; it is a direct recognition of pathogen-associated molecular patterns (PAMP), molecules like endotoxins or peptidoglycans typical for the pathogen structure. These patterns are recognized with the use of pattern-recognition receptors (PRR) localized either on the cell surface or in the cytosol of APCs. Indirect way of PRR activation is through the interaction with danger-associated molecular patterns (DAMP), like uric acid or adenosine triphosphate (ATP) released by organism's own cells upon cell damage.

The steps of mechanism of action could be simplified into following points that can be manifested in a concerted way (for illustration see: Figure 1) (Awate et al., 2013; Cox and Coulter, 1997; Lambrecht et al., 2009; Kwissa et al., 2007; Honegr et al., 2015).

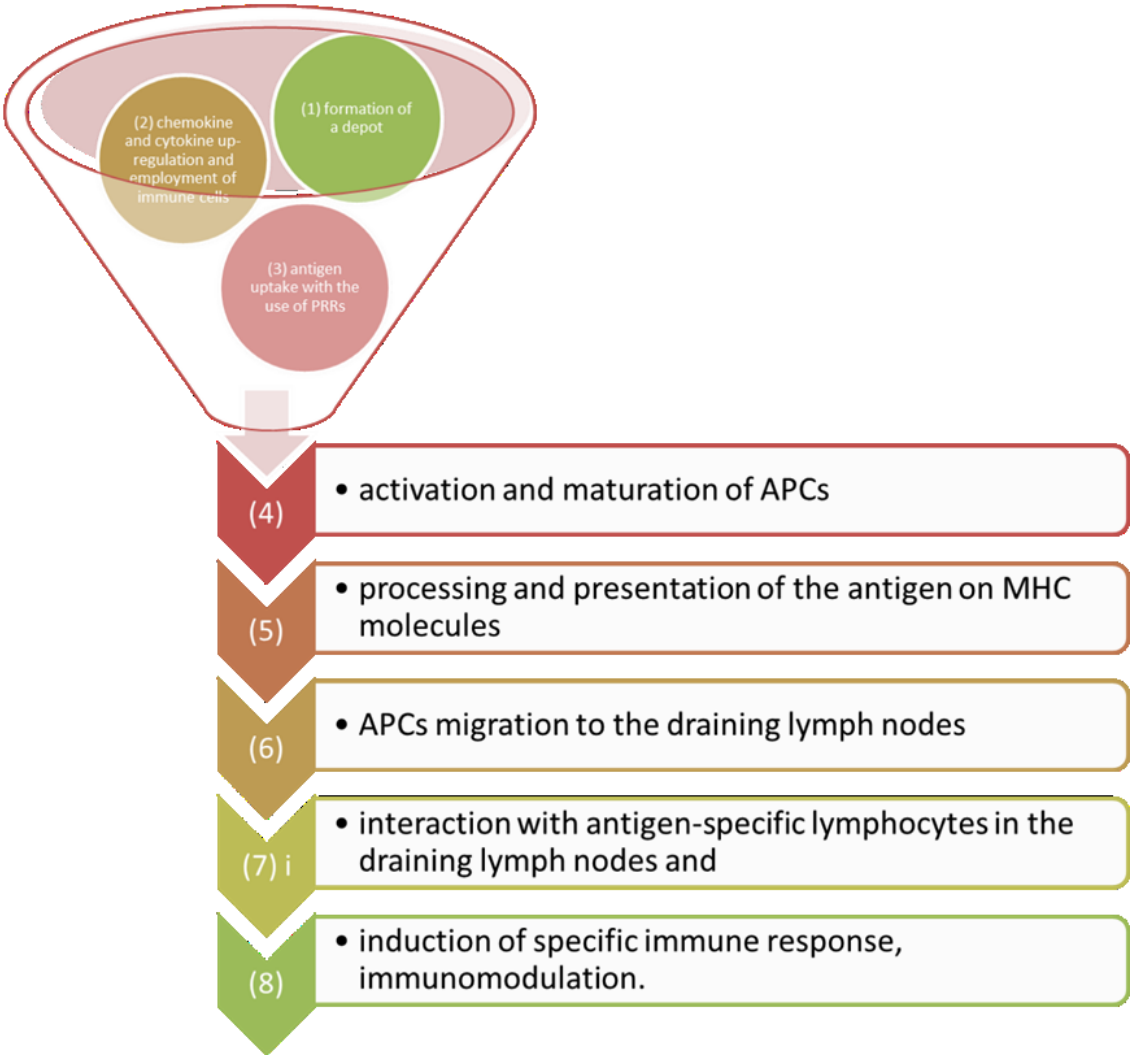


Figure 1 Mechanism of action of adjuvants

3.4.1 Depot formation

Depot formation is considered to be one of the oldest and most classic mechanisms of adjuvant action (Awate et al., 2013). Complex of antigen and adjuvant are trapped at the injection site. This allows consistent slow release and stimulation of the immune system, antigen uptake and especially activation of APCs, like macrophages and dendritic cells (DC), leading to higher levels of antibodies and increased T-cell proliferation as this was shown in a research by Mannhalter et al., 1983 (Mannhalter et al., 1985; Awate et al., 2013) with antigen adsorbed on aluminium adjuvant (Marciani, 2003). Two types of depot can be distinguished, long-term and short-term; the latter being typical for aluminium salts and emulsion adjuvants. Long-term depots are formed using synthetic polymers. They are usually administered as

microspheres that are slowly degraded releasing the antigen in a pulsing manner. Particle sizes of this insoluble form of adjuvant, also known as particulate adjuvant, are crucial for immunogenic activity. Microspheres should be bigger than 10 μm, so they are able to generate a depot at the injection site and they can be degraded at a certain time to produce a pulsed release of the antigen and effective stimulation of APCs (Cox and Coulter, 1997).

3.4.2 Cytokine and chemokine secretion and cell recruitment

Some types of adjuvants do not form a depot as a main mechanism of action, but instead create a specific environment at the injection site and subsequently lead to the activation of immune system. Adjuvants of this type include for example alum, nonmethylated CpG oligonucleotide (CpG) and squalene-based oil in water emulsion, known as MF59 (Awate et al., 2013; Mosca et al., 2008). The mechanism is based on the generation of specific immunocompetent surroundings at the injection site. This kind of adjuvant has the ability to enhance transcription of specific genes encoding the cytokines, such as IL-1β and IL-2, chemokines, adhesion molecules and immune receptors (Lambrecht et al., 2009). Released cytokines are then responsible for the recruitment of the APCs and their migration to the injection site. This mechanism of action was closely observed in the research executed by Mosca et al. in 2008 (Mosca et al., 2008)**,** where the three mentioned adjuvants were analyzed and compared in their adjuvanticity (Awate et al., 2013).

Although it could be considered that simple administration of an injection is able to cause some inflammation at the injection site and also modulate expression of genes encoding cytokines and chemokines as well. According to this research (Mosca et al., 2008), the injection itself does regulate expression of some genes, but adjuvants have the ability to activate and modulate much larger number of the genes besides those affected by the administration process and PBS in this case. These genes are considered to be adjuvant-responsive (Mosca et al., 2008). Some of these adjuvant-responsive genes were up-regulated not by only one or two tested adjuvants, but by all three of them, which makes this gene group encoding chemokines and cytokines, also known as adjuvant core response genes, particularly important (Mosca et al., 2008; Awate et al., 2013).

Immunocompetent environment created by up-regulated gene expression and higher release of chemoattractants and cytokines enhance the recruitment and migration of innate immune cells, including monocytes, DCs, eosinophils, natural killer cells and many other types to the administration site. Thanks to this adjuvant activity, higher levels of immune cells then ensure higher level of antigen uptake and consecutive antigen presentation to the naïve T cells. Such mechanism of action is therefore able to create stronger and more efficient immune response (Awate et al., 2013; Lambrecht et al., 2009).

3.4.3 Antigen uptake through pathogen-recognition receptors

Pathogen-recognition receptors play an important role in detecting the presence of an infectious agent in host organisms. It is a property that was scientifically unknown for very long time and that is also very crucial for eradication of that particular pathogen but without damaging host's own tissues. Over the centuries, host organisms' immune recognition systems like PRRs have evolved in order to fight pathogens with high mutation and replication rates and great molecular diversity. Therefore, PRRs recognize PAMPs comprised of microbial metabolism products, gene products or components of microbial metabolic pathways that are necessary for pathogen's sustainability and survival and not products and patterns characteristic for their virulence.

Although, it might seem more logical and efficient to recognize pathogens on the base of the virulence factors, this system would not help the host effectively fight and eradicate the parasite, because virulence factors are coded through mobile DNA that can be easily turned on or off during different phases of infection and also undergo frequent mutations. Virulence factors have the tendency to change according to conditions the pathogen has in the host organism and they are usually very specific for each microbial strain or specie.

On the other hand, PAMPs possess some properties ideal for innate immune targeting; they are necessary for pathogen survival, if they undergo any change, it usually leads to death or reduced potency and vitality of the microbe. PAMPs are also produced only by the microorganism and not by the host itself; therefore they are distinguishable for the innate immune cells.

Lastly, PAMPs are quite stable and typical for the whole class of microorganisms and therefore, with only a few patterns encoded by PRRs, the immunity is able to recognize tremendous number of pathogens within that class (Medzhitov, 2001).

According to recent development in knowledge of immune response mechanisms, there are several types of PRRs families that can be expressed either on the cell surface and cell membranes, like Toll-like receptor (TLR) family and C-type Lectine Receptor (CLR) or they are localized in the cytosol or intracellular compartments, for example RIG-I-like receptors (RLRs) (Kocourková et al., 2017). RLRs comprise of three receptors*," retinoic-acid inducible gene 1 (RIG-I), melanoma differentiation associated factor 5 (Mda5) and laboratory of genetics and physiology 2 (LGP2)"* (Loo and Gale, 2011) and their main purpose is to identify RNA viruses. The second cytosolic group of PRRs are NOD-like receptor family (NLRs) consisting of more than 20 members. Upon recognition of PAMP and cellular stress molecules, they trigger pro-inflammatory mechanisms and secrete IL-1β (Kawai and Akira, 2010).

3.5 Toll-like receptors

3.5.1 Structure of Toll-like receptors

In order to understand the specific receptor ligand interaction and subsequent signaling, it is crucial to comprehend details of its structure. TLRs are recognized as type I transmembrane glycoprotein structures that are composed of three main structures, leucine-rich repeat (LRR) modules, transmembrane α-helical signaling part and intracellular domain structurally homologous to human interleukin-1 receptor, also known as Toll/IL-1R (TIR) domain (Chang, 2010; Akira and Takeda, 2004).

Extracellular domain

Firstly, extracellular domain of LRR modules, where these LRR motifs are not typical only for TLRs, but they are also found in much larger number of proteins, also known as LRR family proteins that maintain many different physiological functions, such as enzyme or immune regulation, or signal transduction mostly through interaction with other adaptor or signaling molecules (Jin and Lee, 2008). These specific

ectodomains of TLRs are composed of 16-28 LRR sections where each LRR section is composed of 24-29 amino acids forming two distinguishable parts, the leucine-rich "LxxLxLxxN" motif with x representing any amino acid and another more variable sequence composed of large number of hydrophobic amino acids (Jin and Lee, 2008; Akira and Takeda, 2004).

The whole region of LRR involves a secondary structure of α-helical and β-strand parts that are connected by loops (Chang, 2010). Spatially, the structure of LRRs forms a horseshoe-like shape where the inner concave region of the middle part is mostly formed by parallel β-strand structures and mostly contains leucines. The other convex part is more variable, because it contains mainly hydrophobic residues, which have the tendency to form more spacious α-helices and loops and less β-strands. The inner concave region also plays a crucial role in ligand binding since it is supposed to recognize and bind to various pathogen-associated molecules and thus transmit the signal downstream. The constitution of the end parts of the horseshoe is comprised of N and C terminals and since they do not contain LRR modules, their main purpose is to protect the hydrophobic middle part from exposure to solvent and this way stabilizing the whole structure. For most parts, they include cysteines shaping disulfide bridges (Jin and Lee, 2008). Although, the whole LRR sequence involved in the PAMP recognition tends to be very conservative, it still enables recognition of structurally various pathogen-associated molecules which may be also facilitated through different layout of particular TLRs. TLR1, TLR2 and TLR4 have their location on the cell surface and others, like TLR 3, TLR7, TLR8 or TLR 9 are located on the intracellular structures since they are specialized to recognize pathogenic nucleic acids (Akira and Takeda, 2004; Chang, 2010).

Considering the deflections in the structure of different TLRs, two main subgroups can be distinguished. TLR1, TLR2 and TLR4 show a number of deviations from the typical structure of other TLRs and their LRR region (Jin and Lee, 2008).

Firstly, the overall horseshoe structure is also stabilized by so called asparagine ladders, structures forming hydrogen bonds that connect oxygens of backbone carbonyls of the β strands located next to each other. Asparagine amino acid can be replaced with other amino acids capable of forming hydrogen bonds, but in TLRs 1, 2 and 4, these

asparagine ladders are broken and therefore responsible for their unusual horseshoe form (Jin and Lee, 2008; Werling et al., 2009). Secondly, the LRR motifs of these receptors do not tend to have the typical number of residues and therefore they do not have the typical length of the LRR regions distorting the final spacious structure. The horseshoe is then characterized by a smaller or bigger radius depending on the lengths of the LRR regions. Longer LRR modules contain bigger and more spacious α helix structures in the convex parts and smaller LRRs have much less spacious loops instead. The last deviation includes random insertion of α helices in the convex area causing changes in the curvature of the structure (Jin and Lee, 2008).

Intracellular part

The second part of the TLR structure is the transmembrane region and it is thought to be formed only by a single α-helix. The last part, the intracellular region is the TIR domain consisting of approximately 200 amino acids that form a secondary structure of five-stranded β sheets in the middle of the molecule and five α-helices on each side of this center section. B-sheets and α-helices are then connected by the formation of loops that were given their names according to the secondary structures they connected. β-sheet marked as B is therefore connected to α-helix also marked as B forming the BB loop. This structure in particular plays a crucial role in downstream signaling pathways (Jin and Lee, 2008; Akira and Takeda, 2004).

This whole secondary arrangement of the TIR domain ensures three different types of interaction. The first interaction occurs upon the association of a ligand with receptor extracellular part and leads to oligomerization and formation of complexes of the receptor TIR domains. The interface responsible for this interaction is also referred to as R face. Amino acid sequences and residues at this R interface do not exhibit a high degree of conservation and therefore are characterized by a significant diversity. On the other side, this trait allows a high degree of specificity in the signaling pathways of different receptors. Hence, R faces of different TIR domains, for example TIR domains of different receptors, would not be able to form complexes, oligomers and then subsequently activate particular signaling pathway as it was documented in research experiment (Xu et al., 2000).

The second interface, also known as A face is also responsible for formation of oligomers of the TIR domains, but in this case on the site of the adaptor molecule that lies downstream of the receptor. The last part of the TIR domain, the S face, plays a very important role in the formation of a connection between the TIR domain of a receptor and the TIR domain of an adaptor molecule. Surface of this part of the TIR domain is mostly comprised of BB loop structure that has highly conserved amino acid sequence and composition. The experiments show that mutation of amino acid residues in different positions leads to serious signaling activity decline and therefore leading to aggravated signal transduction. Another reason for this structure conservation is the need for interaction between the TIR domain of many different Toll-like receptors and TIR domains of only a few adaptor molecules. This S face surface therefore needs to provide highly specific and conserved composition for interaction with the adaptor molecule so it would not interrupt the signaling pathway (Xu et al., 2000).

The extracellular part of the TLR plays an important role in the interaction with different kinds of ligands, the specific intracellular TIR domain region with its affinity for oligomer formation that affected by ligand association and also by receptor overexpression is necessary for construction of the signaling complex that is able to communicate the signal further down the signaling pathway and in the end induce an immune response (Xu et al., 2000; Jin and Lee, 2008).

3.6 Toll-like receptor signaling pathways

Stimulation of PRRs leads to efficient immune response and innate cell activation. One of the most important PRR families is TLR receptor family, but their molecular mechanisms of action remained unclear until now, when the research of innate and adaptive immunity enlightened TLR signaling pathways in gene expression and subsequent cytokine production (Takeda and Akira, 2005; Akira and Takeda, 2004).

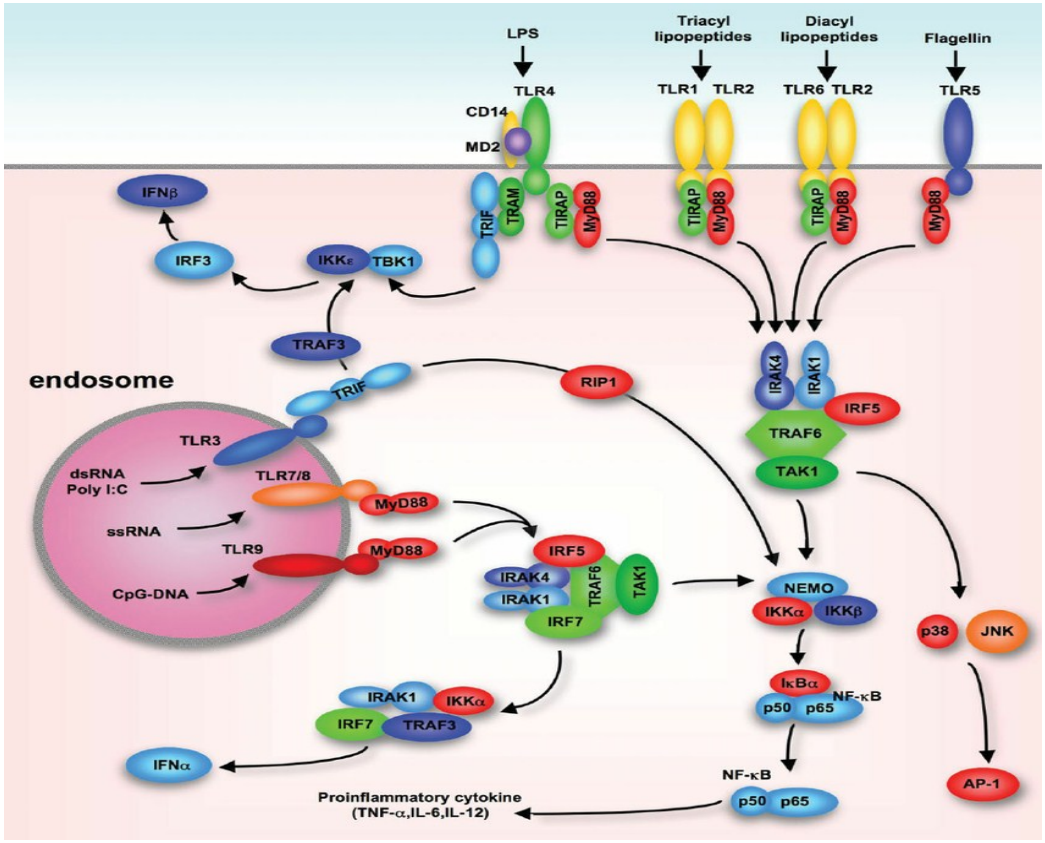


Figure 2 Scheme of Toll-like receptor signaling pathways

Adopted from: (Seki and Brenner, 2008).

An overview of the TLR signaling pathways is schematically described in Figure 2. The molecular mechanisms of TLR activation start with their cytosolic structure, the TIR domain comprised highly conserved surface regions that are crucial for connection with other adaptor molecules or other molecules crucial for downstream signaling (Takeda and Akira, 2005; Akira and Takeda, 2004). Adaptor molecules associated with TLR activation include myeloid differentiation factor 88 (MyD88), TIR-domain containing adaptor protein (TIRAP), TIR-domain containing adaptor inducing interferon (IFN)-β (TRIF) and the last one identified only recently, TRIF-related adaptor molecule (TRAM) (Seki and Brenner, 2008). Although these molecules are necessary for activation of activator protein 1 (AP-1), nuclear factor (NF)-κB and production of interferon regulatory factors (IRFs), not all adaptor molecules are involved in the activation of all of these molecules at the ends of the signaling

pathways, as it is apparent from the Figure 2 Therefore two types of pathways can be distinguished, MyD88-dependent, also referred to as shared signaling pathway, because MyD88 is common to all TLRs and the second one, MyD88-independent, also known as specific, since it is used only by a certain TLRs (Medzhitov, 2001).

3.6.1 MyD88-dependent signaling pathway

After the ligand binds to the receptor, it leads to the conformational change of the TIR domain necessary for the interaction with the downstream MyD88 molecule. This adaptor molecule is comprised of an amino (N)-terminal death domain (DD) that is connected with its carboxy (C)-terminal TIR domain through short linker sequence (Akira and Takeda, 2004). TIR domain of MyD88 is connected to TLR TIR domain, whereas DD is associated with DD of the following molecule called IL-1R-associated kinase (IRAK) (Medzhitov, 2001; Akira and Takeda, 2004). There are four types of IRAK, but only two of them; IRAK1 and IRAK4 exhibit intrinsic kinase activity that is why MyD88 forms a complex with IRAK4 facilitating IRAK4-mediated phosphorylation of IRAK1 (Akira and Takeda, 2004; Takeda and Akira, 2005). Activated IRAK1 then undergoes a process of auto phosphorylation of its own N-terminal domain and enables tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) to attach to the formed complex (Takeda and Akira, 2005; Medzhitov, 2001). Toll-interacting protein (TOLLIP) is another molecule associated with the formation of TLR-IRAK complex. Although, it does not contain a TIR domain, TOLLIP uses its two C2 domains for interaction with membrane lipids and for the attachment of IRAK to the receptor complex (Medzhitov, 2001).

After the auto phosphorylation of IRAK1 and attachment of TRAF6, this formed receptor complex is detached from the receptor and at the plasma membrane interacts with other preformed complex consisting of transforming growth factor-β (TGF-β)-activated kinase (TAK1) and two TAK1-binding proteins 1 and 2 (TAB1 and TAB2) (Akira and Takeda, 2004). TAB1 functions as TAK1 activator through its ability to potentiate the kinase properties of TAK1, TAB2, on the other hand, operate as link between TAK1 and TRAF6 subsequently promoting activation of TAK1 (Akira and Takeda, 2004). IRAK1 is degraded at the plasma membrane and therefore the remaining complex of TAK1, TAB1, TAB2 and TRAF6 then binds two other molecules,

ubiquitinating factors ubiquitin-conjugating enzyme E2 variant 1 (UEV1A) and ubiquitin-conjugating enzyme 13 (UBC13) (Akira and Takeda, 2004; Seki and Brenner, 2008). TRAF6 is able to connect to UBC13 and function as an ubiquitin-ligase attaching polyubiquitin chain to lysine residue and thus activating the TAK1 (Akira and Takeda, 2004; Kawai and Akira, 2010; Seki and Brenner, 2008).

Activated TAK1 can then trigger two different pathways. First pathway includes activation of inhibitor of NF-κB (IκB)-kinase complex (IKK) consisting of three parts, IKK-α, IKK-β and a regulatory part IKK-γ, also referred to as NF-κB essential modulator (Akira and Takeda, 2004). This IKK complex plays a crucial role, because it induces phosphorylation followed by polyubiquitilation of IκB. Attachment of ubiquitin marks this molecule for sequential proteasome-mediated degradation allowing the NF-κB to be translocated to nucleus where it induces expression of specific genes necessary for immune response enhancement and modulation (Akira and Takeda, 2004; Kawai and Akira, 2010). NF-κB is a group of transcription factors including p65, p50, p52 that are able to activate gene transcription and thus the production of pro-inflammatory cytokines and chemokines, such as TNF, IL-6 and IL-12 (Akira and Takeda, 2004; Takeda and Akira, 2005; Seki and Brenner, 2008). Second pathway using activated TAK1 leads to activation of mitogen-activated protein kinases (MAPKs) such as p38 and c-Jun N-terminal kinase (JNK) through their phosphorylation, not necessarily their ubiquitination (Kawai and Akira, 2010; Seki and Brenner, 2008). This activation then leads to production of transcription factors, such as AP-1 and induction of immune response (Seki and Brenner, 2008; Medzhitov, 2001; Takeda and Akira, 2005).

In the MyD88-dependent signaling pathway, there is also involved a second adaptor molecule, TIRAP, structurally related to the MyD88. Although, it was at first thought, that TIRAP adaptor molecule was associated with MyD88-independent pathway, it was discovered that TIRAP plays a key role in MyD88-dependent signaling pathway and only with the association with TLR2 and TLR4 (Akira and Takeda, 2004; Takeda and Akira, 2005). According to various experiments held on specifically deficient mice, the TIRAP seems to be acting upstream of MyD88, even though it does not possess DD (Akira and Takeda, 2004).

3.6.2 MyD88-independent signaling pathway

The production of inflammatory cytokines is mediated by MyD88-dependent signaling pathway, but according to research carried out on MyD88-deficient mice, there is another signaling pathway, MyD88-independent pathway that activates the transcription factor IRF-3(Akira and Takeda, 2004; Takeda and Akira, 2005). Although cytokine production is suppressed in the MyD88-deficient mice, the activation of NF-κB still occurs within TLR4 signaling but with delayed kinetics and also in the MyD88-independent manner (Medzhitov, 2001; Akira and Takeda, 2004). Besides TLR4, IRF-3 activation was also monitored within TLR3-mediated signaling pathway (Takeda and Akira, 2005). This transcription factor subsequently leads to generation of IFN-β, which then activates the expression of numerous IFN-inducible genes, such as immunoresponsive gene 1 and glucocorticoid-attenuated response gene 16 (Akira and Takeda, 2004). These findings also led to an assumption, that there could be another TIR-domain containing molecule that facilitates MyD88-independent signaling. The hypothesis was confirmed by identification of a third adaptor molecule known as TIR-domain containing adaptor inducing IFN-β or TRIF and also the experiments proved, that TRIF plays a crucial role in TLR4 and TLR3 MyD88-independent signaling pathways (Takeda and Akira, 2005). Soon after this discovery, the fourth adaptor molecule, TRIF-related adaptor molecule, TRAM, was identified, but this one is involved only in TLR4 MyD88-independent signaling pathway, located upstream of TRIF molecule (Takeda and Akira, 2005; Akira and Takeda, 2004). Although, the interesting thing within TLR4 signaling is, that in TRAM/TRIF-deficient mice, not only the IFN-β production is impaired, but also the inflammatory cytokine production based on NF-κB activation does not occur as well, even though, the MyD88-dependent pathway was not defective. Hence the TLR4 based production of proinflammatory cytokines seems to be dependent on activation of both, the MyD88-dependent and also MyD88-independent/TRIF-dependent signaling paths (Takeda and Akira, 2005).

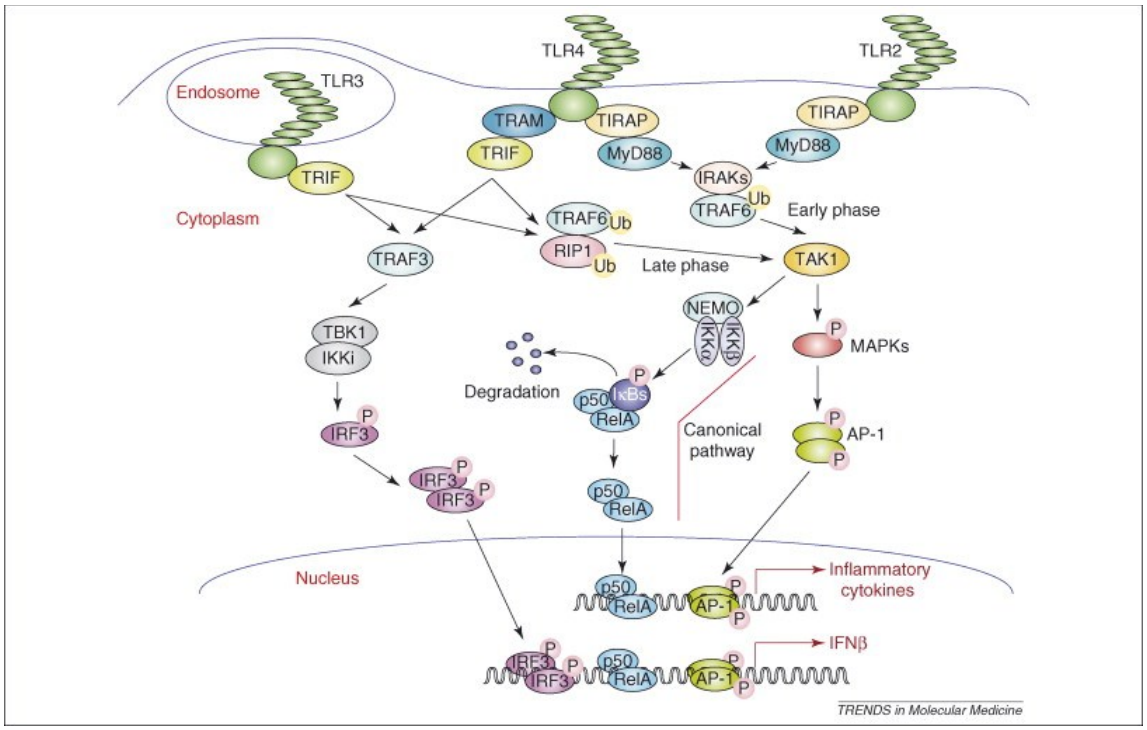


Figure 3 TLR2-, TLR3- and TLR4-mediated signaling pathways

Adopted from: (Kawai and Akira, 2007).

The mechanism by which TRIF mediates the activation of IRF-3 is determined by the structure of this molecule composed of C-terminal and N-terminal with the TIR domain in the middle. Both of these terminal fragments are involved in activation of NF-κB-dependent promoter (Takeda and Akira, 2005). N-terminal of TRIF communicates with TRAF molecule as it demonstrated in Figure 3 in TLR3 signaling pathway and leads to activation of NF-κB and also with TBK1, as it will be explained further in text, activates the IRF-3 molecule crucial for IFN-β production. TLR3 signaling pathway also demonstrates the use of C-terminal of TRIF molecule in the activation of NF-κB mediated by another molecule, receptor-interacting protein 1 (RIP1) (Takeda and Akira, 2005; Akira and Takeda, 2004; Anonym, 2004). It is important to realize that proinflammatory cytokine generation is dependent on NF-κB activation, but including both the early and late phase using MyD88 and TRIF as necessary mediators (Akira and Takeda, 2004).

On the other hand, IFN-β promotor is activated with the help of only N-terminal part of the TRIF molecule, but leads to the activation of both, IRF-3 and NF-κB as well (Takeda and Akira, 2005; Kawai and Akira, 2010). After the activation of TRIF, the next step is mediated by two IKK-ε proteins and TRAF-family-member-associated NF-κB activator-binding kinase 1 (TBK1). These molecules are then responsible for the phosphorylation process of IRF-3 that leads to its activation as a response to viral infection or stimulation of TLR3 or TLR4 (Akira and Takeda, 2004). These two kinases, IKK-ε and TBK1, ensure the phosphorylation of C-portion of IRF-3, which then induces the formation of IRF-3 dimers and IRF-3 is subsequently translocated to the nucleus in order to activate the transcription of particular genes. Usually viral infections induce the production of IRF-3 and then generation of IFNs that mediate the transcription of IFN-inducible genes necessary for appropriate response using Janus activated kinase (JAK)-signal transducer and activator of transcription (STAT) signaling. Furthermore, IRF-3 induces production of IFNs and their presence then leads to activation and production of IRF-7 also using JAK-STAT signaling pathway (Akira and Takeda, 2004). This transcription factor is employed in the signaling pathways of TLR7, TLR8 and TLR9. Together, both of these transcription factors, IRF-3 and IRF-7, are responsible for the formation of late types of IFNs, such as IFN-α (Akira and Takeda, 2004; Seki and Brenner, 2008).

3.7 Toll-like receptors and their role in recognition of microbial ligands

In the past century, it was believed that innate immune system recognizes foreign pathogen only by a nonspecific way. This concept radically changed after a discovery of Toll receptor in *Drosophila* that is characterized to only have innate immune system. The research showed that this Toll receptor was crucial in recognizing and defending the organism against fungal pathogens and thus proving the exerted theory not entirely right. Homologous structures with the ability to induce immune response to different pathogens were also identified in mammalian organisms and they were named Toll-like receptors, thereby making the innate immunity research rapidly progressive. To date, 13 TLRs have been identified, but not all of them are functional in human organism. TLR1-TLR9 have been found in humans and mice and all of them are proved to be functional in both species as well. The research does not provide a lot of

information about TLR10, but it is supposed to be functional in human organism, but mouse TLR10 gene seems to be not functional due to presence of a non-productive gene sequence. On the other side, TLR11, TLR12 and TLR 13 have probably been deleted from human genetic information (Kawai and Akira, 2010; Takeda and Akira, 2005).

3.7.1 Toll-like receptor 1, 2 and 6

TLR2 is able to recognize wide range of microbial ligands including peptidoglycans and lipoteichoic acid typical for Gram-positive bacteria, mycobacterial lipoarabinomannan or also the whole mycobacteria, glycoinositolphospholipids characteristic for *Trypanosoma cruzi* and also atypical lipopolysaccharides of *Leptospira interrogans* or *Helicobacter pylori.* It also reacts to fungal pathogen zymosan and cell wall components of yeast, but most importantly, they recognize lipoproteins, protein structure that contains covalently attached lipid chains to the cysteines of a NH_2-terminus. Lipoproteins are common for variety of Gram-positive, Gram-negative microbes, as well as for mycoplasmas but with apparent differences in their lipoprotein molecules. One of the main questions that arise considering the TLR2 is how can it recognize and react to such wide range of microbial pathogens? There are two explanations that need to be taken into consideration. First, an ability of TLR2 to recognize slight differences between various lipoprotein structures is mediated by formation of heterodimers with other molecules, especially with TLR1 and TLR6 that are structurally similar to each other and to TLR2 as well. Lipoproteins exhibit a strong immunomodulatory effect that is determined by the presence of lipoylated cysteine at its N-terminus, but differ by the number of acyl groups attached to the cysteine residue.

Dimerization and formation of a complex consisting of cytoplasmic TIR domains of TLR2 and TLR1 enables the recognition of triacylated lipopeptides typical for Gram-negative bacteria and mycobacteria and also response to soluble factor of *Neisseria meningitidis.* On the other hand, TLR2 association with TLR6 leads to recognition of diacylated lipoprotein molecules that originate from Gram-positive bacteria and mycobacteria such as macrophage-activating lipopeptide 2 (MALP-2). The presence of either TLR1 or TLR6 allows differentiation between diacylated and triacylated cysteine residues and between lipopeptides of bacterial and mycobacterial origin, which is given by their spatial structures. Both heterodimers form an

arrangement similar to m-shape, but the triacylated ligand interacts with TLR2 through two of its fatty acid chains and the third bound to TLR1 and its hydrophobic channel. TLR6, on the other hand, does not possess this hydrophobic channel, thus making it hard for the third acyl to bind and therefore leading to lipoprotein differentiation (Kawai and Akira, 2010; Takeda and Akira, 2005; Akira et al., 2001; Takeda et al., 2003; Takeuchi et al., 2002).

The second mechanism explaining the wide range of pathogen recognition of TLR2 includes the ability to functionally cooperate with other receptor structures located on the cell surface. CD36 assist the TLR2-TLR6 complex in recognition of some TLR2 agonists. Other collaborating structures include dectin-1 and C-type lectin, both of them belonging to the lectin family of receptors that are able to bind fungal-derived β-glucan and promote its internalization into the APC (Kawai and Akira, 2010; Takeda and Akira, 2005).

3.7.2 Toll-like receptor 3

The ligand recognition of TLR3 was initially identified using polyinosinic-polycytidylic acid, known as poly (I:C), which is a synthetic surrogate of double-stranded RNA (dsRNA). Later, it was proved that TLR3 is able to bind and distinguish dsRNA essential for reproduction of many viruses either in the form of an intermediate in viral RNA formation or in the form of a byproduct within viral genome transcription process. TLR3 also recognizes specific virus types, such as respiratory syncytial virus, West Nile virus or virus causing encephalomyocarditis. The recognition of these ligands is mediated through specific structure of the receptor that is slightly different from the structure of other TLRs. The extracellular domain is responsible for the ligand recognition and resembles the horseshoe-like shape with LRR that is shared by all TLR ectodomains. The TLR3 ectodomain is characterized by higher degree of glycosylation than other receptors, but on the other hand, it also contains higher number of glycan-free regions with positively charged residues, especially on the lateral sides of the horseshoe structure. Another specification of this structure is the presence of two sulfate molecules in some of the LRR modules. These sulfate molecules and the phosphate molecules typical for dsRNA share the same ion arrangement. Therefore, it is suggested that these sulfate molecules and glycan-free regions mediate binding and recognition of

dsRNA and subsequently lead to homodimerization of the receptor, that through TRIF adaptor molecule triggers the signaling cascade leading to strong antiviral response and formation of type I IFN and proinflammatory cytokines essential for virus eradication (Kawai and Akira, 2010; Takeda et al., 2003; Bell et al., 2006).

3.7.3 Toll-like receptor 4

TLR4 was the first Toll-like receptor that was identified in mammals. Its main purpose is to recognize, bind and respond to bacterial lipopolysaccharide (LPS). LPS, as an important element of the Gram-negative bacterial outer membrane, has remarkably strong immuno-activating potential, able to activate the TLR4 even by a small amount of the substance present. In order to induce a sufficient immune response, a complex of LPS and lipopolysaccharide-binding protein needs to be formed and with the help of CD14 molecule transported to the complex of TLR4 and molecule associated with the receptor, MD2. This whole aggregate is then able to activate an adaptor molecule and trigger the signaling cascade (Takeda et al., 2003; Akira et al., 2001; Kawai and Akira, 2010; Takeda and Akira, 2005).

TLR4 also responds to other pathogen molecules including taxol, a plant derived diterpen with antitumor activity that has a mechanism of receptor activation similar to LPS, fusion protein from respiratory syncytial virus or pneumolysin of *Streptococcus pneumoniae.* Furthermore, according to research, TLR4 might be involved in identification of numerous endogenous ligands involved in many processes. Some of them are heat shock proteins (HSP) that are part of so called danger signals. These are the structures and molecules that are released upon cell damage, abnormal cell death or under the stress conditions such as heat shock, viral or bacterial invasion or ultraviolet radiation and their main purpose lies within protein folding, protein chaperoning and transport. One example can be production of HSP 60 upon the cell stress caused by *Chlamydia pneumonia* chronic infection, which causes formation of atherosclerotic lesions. Released HSP 60 then attracts and activates the APCs and dendritic cells through TLR4 and leads to production of proinflammatory cytokines and inflammation at the atherosclerotic site initiating the immune response. Other endogenous ligands generated at the site of injury and involved in the tissue reparation, remodeling and healing processes including polysaccharide components of heparan sulfate,

oligosaccharides of hyaluronic acid or type III repeat extra domain A of fibronectin interact with the TLR4 and this way mediate the maturation or activation of dendritic cells. Although, there is a high chance of immune activation based on the interaction of these endogenous ligands with the TLR4, but in contrast with the LPS, high concentrations of these substances are required for the receptor to response (Takeda et al., 2003; Takeda and Akira, 2005; Gallucci and Matzinger, 2001).

3.7.4 Toll-like receptor 5

Some Gram-positive and Gram-negative bacteria possess flagella, highly specific rod-like structures that form extensions from the bacterial outer membranes. Besides their main purpose in movement of bacteria around the aqueous environment, they also help them to attach to host cells and to invade the host organism, thus enhancing and promoting the virulence of the pathogen. The main component of this flagellar structure is flagellin, a ligand with strong immunostimulatory activity that binds to another TLR, namely TLR5. Flagellin has some properties that differentiate it from other PAMPs recognized by other TLRs. It is a protein that does not undergo the classical process of posttranslational modification, which would make it difficult to distinguish this structure from organism's own proteins, but on the other side, the flagellin amino- and carboxy-termini are important in the formation of a hydrophobic central part of the flagella that are extremely conserved in their structure. The structure conservation and the relevance of flagellum for survival of bacteria elicit the importance of this structure as a TLR ligand. Another form of discrimination between pathogenic and non-pathogenic is established by a location of TLR5 expression that is centered on the basolateral side of the intestinal epithelial cells and on the DCs located in the lamina propria of the small intestine. This position is crucial because the pathogenic bacteria have the ability to cross the plasma membrane whereas commensal and non-pathogenic bacteria cannot. According to further research, the presence of flagellin also activates the lung epithelial cells thus promoting the importance of TLR5 in mucosal pathogen recognition and subsequent production of inflammatory cytokines, NF-κB activation or maturation of naïve B-cells into plasma cells producing immunoglobulin A (Medzhitov, 2001; Akira et al., 2001; Takeda et al., 2003; Takeda and Akira, 2005; Kawai and Akira, 2010).

3.7.5 Toll-like receptor 7 and 8

TLR7 and TLR8 are structurally very similar receptors that tend to recognize similar synthetic compounds and ligands. Both receptors are included in the TLR9 subfamily that is supposed to recognize and distinguish between different pathogenic nucleic acid-like structures and components, which are analogous to the TLR2 subfamily receptors. This subfamily including TLR1, TLR2 and TLR6 discriminate between different types of lipoproteins.

Only recently has been recognized that synthetic compounds, such as imidazoquinoline compounds like imiquimod or resiquimod and guanosine analogues with antitumor and antiviral properties also have the ability to activate TLR7 and TLR8. These compounds were used to treat genital warts caused by viral infection or possess anti-tumor and anti-viral properties. Strong structural similarity of these compounds to single-stranded ribonucleic acid (ssRNA) and guanosine nucleotides predicted importance of these two receptors in recognition of viral nucleic acids.

This assumption was confirmed by the research focused on the activation of TLR7 and TLR8 using uridine- and guanosine-rich ssRNA derived from different viruses including human immunodeficiency virus, influenza virus or vesicular stomatitis virus. Although, ssRNA is quite abundant in the host organism, there is a way of distinguishing between own and pathogenic nucleic acid. Since the TLR7 and TLR8 signaling is dependent on the acidification, these receptors are expressed in the endosomes and lysosomes that are characterized by acidic environment. The fact that host own ssRNA is not delivered to these organelles, in contrast with the pathogenic nucleic structures that are delivered and internalized into the endosomes or lysosomes through receptor-mediated uptake or through the fusion with another budding virus and secondly, they are recognized by the TLRs, which leads to antiviral immune responses, such as production of IFN-α and other cytokines and activation and maturation of DCs.

As these receptors can recognize not only the parasitic structures and ligands, but also the useful synthetic molecules, they may also pose a great potential in identification of TLR-activating compounds that might be beneficial in clinical treatment of cancer or infections (Takeda and Akira, 2005; Takeda et al., 2003; Kawai and Akira, 2010; Heil et al., 2004).

3.7.6 Toll-like receptor 9

Pathogenic DNA has a string ability to activate the immune cells as it was mentioned in the previous chapters, but this DNA pattern has yet another specification, that allows the TLRs distinguish between self and non-self. Microbial genetic information contains a large number of unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine) (CpG) motifs. Cysteine residues of these CpG motifs are not attached to methyl group, which is typical for bacterial and viral DNA, but not for the vertebrate DNA, since in vertebrates, the number of CpG motifs is much lower and the cysteine residues tend to be highly methylated. Even a small change in the pathogenic motif, such as methylation of the cytosine or substitution with another nucleotide leads to loss of immunostimulatory activity. Research shows that there are more different types of CpG motifs. The first one identified, B/K-type, also known as conventional, mediates the production of inflammatory cytokines, mainly TNF-α and IL-12. The other type, A/D-type, is responsible for generation of IFN-α that is produced by plasmacytoid DCs and has a potent anti-viral properties, therefore proving that TLR9 is not only involved in recognition of bacterial, but also viral pathogens (Takeda and Akira, 2005; Kawai and Akira, 2010; Medzhitov, 2001; Hemmi et al., 2000).

Pathophysiological processes involved in the pathogenesis of some autoimmune diseases can be mediated through TLR9. The main keystone lies within the recognition of chromatin, the structure that includes hypomethylated CpG motifs. One example could be rheumatoid arthrosis, where specific immunoglobulin antibody, IgG2a forms a complex with chromatin. Subsequently, the immunoglobulin interacts with B cell receptor and this way the complex is internalized by the B cell, where TLR9 is able to interact and identify the hypomethylated part of the chromatin and thus induce the production of rheumatoid factor. The similar mechanism is also involved in the pathogenesis of systemic lupus erythematosus, another autoimmune disease (Takeda and Akira, 2005).

3.8 Analyzed substances

The analyzed substances were acquired as a result of virtual high throughput screening consisting of 10 000 compounds. Small number of these compounds, exactly

100, was synthesized in the laboratory according to information based on the published papers, 200 substances were chosen from the our own laboratory database and the residual 9700 compounds were obtained from the Zinc database as a selection from over 2 000 000 commercially available molecules. All of the 10 000 compounds then underwent the process of docking into the active place of TLR4 and the bonding energy for each compound was determined, The 60 structures with the highest binding energy were chosen for further analysis, where 10 lead compounds with the best modification potential were examined to determine their intrinsic activity on the cell lines expressing TLR4. Based on their most convenient physic-chemical properties, two lead compounds were chosen and synthesized, as well as 10 derivatives of the best lead molecule and 12 derivatives of the second best candidate following fundamental medicinal chemistry principles. Subsequently, the immunomodulatory activity of the two lead compounds and their derivatives was analyzed on the cell line.

4 AIM OF THE WORK

The main objective of this diploma thesis was to identify new substances that exhibit potential immunomodulatory activity when interacting with TLR and evaluate their potential for utilization as new vaccine adjuvants. Vaccine adjuvants with their immunomodulatory activity have the ability to enhance and qualitatively change the immunity ensured by vaccine active ingredient. Adjuvants can also decrease the number of doses needed and the amount of pathogen needed for administration. The analyzed substances were acquired as a result of virtual high throughput screening, where 10 000 potential adjuvants were docked into the active spot of a toll-like receptor and substances with the highest binding energy and structure most convenient for further modification were chosen. These lead compounds and their derivatives were synthetized and then tested in vitro for determination of their immunomodulatory activity.

5 EXPERIMENTAL PART

5.1 Materials

5.1.1 Cell Model

- Human embryonic kidney cells HEK-Blue™ human TLR8 cells (hTLR8) – Secreted Embryonic Alkaline Phosphatase (SEAP) Reporter 293 cells expressing the gene for the human TLR8, Invivogen
- Human embryonic kidney cells HEK-Blue™ human TLR4 cells (hTLR4) – SEAP reporter293 cells expressing the gene for human TLR4, Invivogen

5.1.2 Instruments

- Laminar flow cabinet Safeflow 1.2 (BioAir), EuroClone
- Laboratory incubator CO_2 FORM Direct Heat 311, Thermo Scientific
- Multi-Detection Microplate Reader Synergy HT, BioTek
- Centrifuge Universal 320 R (Hettich), Schoeller
- Analytical scale CPA 225, Sartorius Stedim Biotech
- Laboratory water bath, PolyScience
- Microscope, Meropta
- Pipette Controler, accu-jet® pro
- Automatic pipettes Research Plus, Eppendorf
- Ultrasonic bath – Bandelin Sonorex

5.1.3 Chemicals

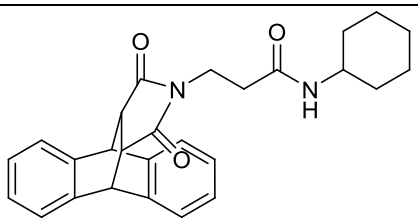
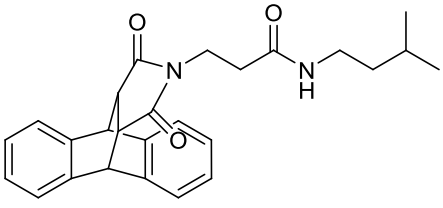
- Culture medium Dulbecco's Modified Eagle's Medium with glucose 4,5 g/l and L-glutamine 2mM, Sigma-Aldrich
- Fetal Bovine Serum (FBS) 10%, Sigma-Aldrich
- Penicillin 50 U/ml, Sigma-Aldrich
- Streptomycin 50ug/ml, Sigma-Aldrich
- Blasticidin 10mg/ml, Invivogen

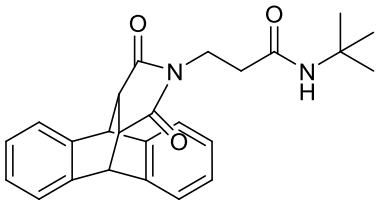
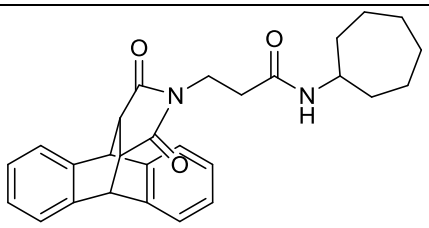
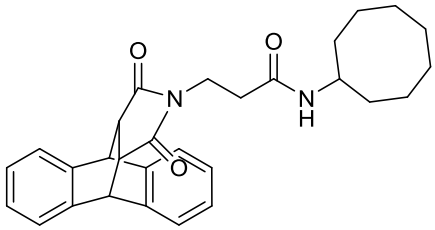
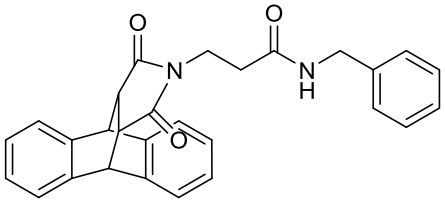
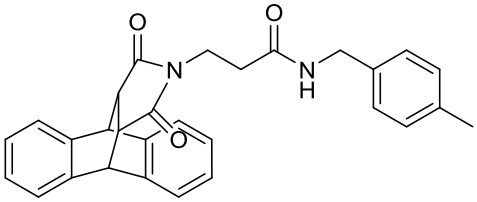
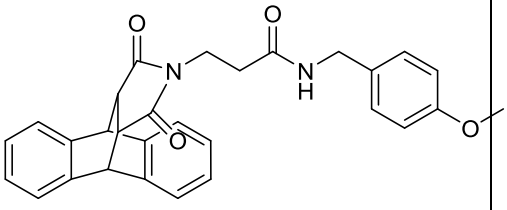
- Normocin™ 50mg/ml, Invivogen
- Zeocin™ 100mg/ml, Invivogen
- Dimethyl sulfoxide for molecular biology (DMSO), Penta
- Dulbecco's Phosphate Buffered Saline (DPBS), Sigma-Aldrich
- Detection medium Quanti-Blue™, Invivogen
- Lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS) – TLR4 antagonist, Sigma-Aldrich
- Ultrapure lipopolysaccharide from *E. coli* 0111:B4 (LPS-EB) – TLR4 agonist, Sigma-Aldrich
- Resiquimod (R848) – ligand of TLR7/8, Sigma-Aldrich
- Synthetic Monophosphoryl Lipid A (MPLA) – TLR4 ligand, Invivogen

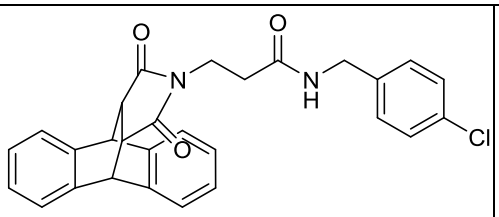
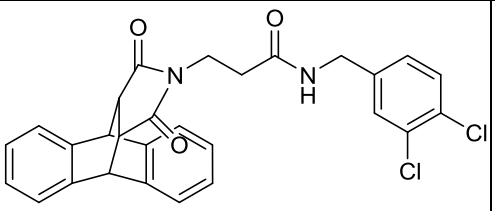
5.1.4 Analyzed substances

The tested substances with their molecular structures and main characteristics are described in Table 1.

Table 1 Analyzed substances

Structure	Designator	Melting point(°C)	Calculated Molar Mass(g.mol^{-1})
	**DM 001**	226,4 - 228,4	428,53
	**DM 002**	179,8 – 181,8	416,52

	**DM 003**	204,7 – 206,7	402,49
	**DM 004**	242,2 – 244,2	442,56
	**DM 005**	239,5 – 241,5	456,59
	**DM 006**	202,3 – 204,3	436,51
	**DM 007**	195,1 – 197,1	450,54
	**DM 008**	194,2 – 196,2	466,54

	**DM 009**	175,0 – 177,0	470,95
	**DM 010**	160,5 – 162,5	505,40

5.2 Methods

5.2.1 Cell culture preparation and maintenance

Human embryonic kidney cells expressing human TLR8 gene used in this experiment were derived from human embryonic kidney 293 cells at specialized laboratory, where adenovirus 5 DNA was used for this transformation process. These hTLR8 cells were transported frozen in a freezing medium and stored under conditions of liquid nitrogen. Therefore, thawing process and first handling were crucial for proper reproduction and stock generation of the cells. Vial with the frozen cell line was placed in a 37^{o}C water bath without immersing the vial cap in the water. The thawing process was accelerated by gentle agitation of the vial. Next steps were carried out under the aseptic conditions of the laminar flow cabinet. In order to prevent contamination of the vial and the cell line, the whole vial was sprayed with 70% ethanol prior to placement in the cabinet. Subsequently, the cells were transported into a 15 ml vial with 13 ml of pre-heated growth medium. This growth medium did not yet contain any of the selective antibiotics. The vial with the cell line was centrifuged for 5 min at 1000-1200 rotations per minute (RPM). After the centrifugation process, supernatant fluid over the cell disk at the bottom was carefully removed. The growth medium containing all the selective antibiotics was then added to the cell disk. This second growth medium created more appropriate environment not only for cell growth and proliferation, but also for cell

handling and passaging and preventing contamination of the culture. The vial contents were then transferred to prepared culture flask. In order to prevent cell decrement in the transfer process, part of the cell-medium suspension was used for careful rinse of the vial and moved back to the flask. In the end, the tissue culture flask was incubated at 37^{o}C in 5% CO_2 for further multiplication and proliferation of the cells.

For the following assays and experiments, it was crucial to maintain and subculture the cells and ensure their fast and efficient division, so we could have a sufficient amount of viable cells not only for the experimental procedure, but also for cell passaging and further proliferation. Optimal confluency for the cells to be passaged was 70-80%. Although, at the beginning of the research, it was very difficult to reach the desired confluency, because the cell division and growth were really slow. The main reason was probably the freeze-thaw process, which could have caused the cells to proliferate in much slower manner. On the other hand, we have to take into consideration, that the experiment is performed with the use of independent living entities, the cells. Therefore, even if the procedure is performed according to protocols, the results may still vary. The maintenance of the subculture was based on the renewing of the growth medium twice a week or every time the culture was passaged or part of the culture was used to perform the assay.

When the desired confluency was reached and there was no assay performed at the time, it was necessary to passage the cells before they approached 100% confluency. The old growth medium from the culture flask was removed into the waste beaker. The cells were detached from the flask bottom using stronger flow of the fresh growth medium controlled by a pipette controller. The volume of the new growth medium used for passaging was 2ml. This small amount then contained all the cells acquired from the flask bottom. The cell suspension was subsequently divided into two culture flasks for further proliferation and sub culturing in the incubator at 37^{o}C in 5% CO_2 (Invivogen, 2016a).

5.2.2 Preparation of solutions

Growth media

Dulbecco's Modified Eagle's Medium (DMEM) was used as the main base for the growth media for hTLR8 cells and also for hTLR4 cells as well. This medium already contained required concentration of glucose, 4.5g/l and 2mM concentration of L-glutamine, although, other components were needed to be added to prepare the medium with all the required properties. According to our calculations, 50ml of 10% (v/v) fetal bovine serum (FBS) was added to the medium as a growth supplement. FBS contains high concentrations of appropriate growth factors and other components that are able to satisfy metabolic requirements of the cells. To prevent the culture from contamination, selective and non-selective antibiotics were mixed in the media. Penicillin 50 U/ml and streptomycin 50 μg/ml were already premixed together and 2.5 ml were added to the growth medium. Formulation of three antibiotics known as Normocin™ was supposed to protect the medium from mycoplasmas, fungi and bacteria. Amount of 50 mg was sufficient to provide this protective barrier for the whole volume of DMEM. Selective antibiotics, 1.5 mg of blasticidin and 50 mg of Zeocin™ were required to maintain cell line stability and to prevent genetic instability that reduces cell responsiveness to performed assays and experiments and reduces the accuracy of results. Blasticidin prevented changes in plasmid coding for hTLR8 gene and Zeocin™ conserved plasmid sequence necessary for secretion of embryonic alkaline phosphatase and hence cell responsiveness to analyzed substances. DMEM was distributed in 500 ml bottles to prepare sufficient stock of the growth media. Therefore, in order to maintain the required concentrations of all of the components, it was necessary to replace 54.5 ml of the medium and add 50 ml of FBS, 2.5 ml of penicillin/streptomycin mixture, 1 ml of Normocin™, 0.5 ml of Zeocin™ and 0.150 ml of blasticidin. The complete growth medium was then stored in the fridge at 5^{o}C and pre-warmed in the 37 ^{o}C water bath before use.

To perform the experiment with assurance of exact and reliable results, it was not possible to use the growth medium prepared for cell cultivation. The main reason for this complication is the FBS. Fetal bovine serum sometimes contains a certain amount of alkaline phosphatase that can interfere with the assays' results, where

quantity of SEAP is determined for each analyzed substance. Therefore, is recommended to replace FBS with heat-inactivated FBS. Because of financial reasons, FBS was inactivated by 30 min incubation at 56 ^{o}C instead of using commercially available FBS. This method was perfectly sufficient for the experiment, because alkaline phosphatases are thermosensitive and therefore are destroyed during the process (Invivogen, 2016a).

Detection Medium

Detection medium was prepared using prepackaged pouches that were commercially available. Content of one pouch was emptied into a 250 ml beaker and 100 ml of endotoxin-free water was added. The beaker was then swirled and placed into the 37^{o}C for 30 minutes until the powder was completely dissolved. The detection medium was used immediately for performing the analysis or stored in the fridge and again heated up to 37^{o}C right before use. The powder of the detection medium is composed of a colorimetric enzyme that has the ability to detect and quantify activity of any alkaline phosphatase that is secreted by the cell in to the supernatant during the assay; in this case it detected the SEAP produced by the hTLR8 or hTLR4 cells (Invivogen, 2016e).

Analyzed substances

The analyzed substances were synthetized in a laboratory for synthesis of Biomedical Research Center, but for the experimental purposes, it was necessary to transfer these substances in the solution form of a certain concentration. The desired concentration was 10mM in DMSO, which allowed us to conveniently prepare the same concentration series of every tested substance and therefore conveniently compare results and immunomodulatory activity of every sample. Dimethyl sulfoxide was used as a dissolution reagent, because it has good dissolution properties and it does not disintegrate the structure of the tested compounds. The stock volume of the solution of each substance needed to perform the whole experiment was 2 ml. The last information necessary to determine calculated mass was the molecular weight of each tested structure, which was provided by the synthetic laboratory. After the calculations, the substances were weighted out on the analytical scale and real weights were recorded

down. Consequently, these were used to calculate the exact volume of DMSO needed to reach the required 10mM concentration of the final sample solution.

TLR Ligands

Resiquimod (R848) is an imidazoquinoline compound, that possess antiviral activity and according to research is able to interact with the human TLR8 using MyD88-dependent signaling pathway leading to immune cell activation. Therefore, this hTLR8 ligand functioned as a standard to which immunomodulatory activity of tested structures was compared to in the experiment (Jurk et al., 2002). The initial concentration of resiquimod was 10 mg per 1ml, but for the experiment purposes and easier handling, it was more convenient to prepare a stock of 100 μg/ml solution by dissolution in endotoxin-free water. This solution was then divided into 30 1 ml safe-lock tubes and stored in a freezer. When the assay was performed, two tubes of resiquimod were thawed and a concentration series was prepared using physiological saline solution as dissolution reagent. The concentration series was created using a binary dilution method and consisted of five concentrations, 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml and 6.25 μg/ml that corresponded to concentration series of analyzed substances.

In the next phase of the experiment, the hTLR4 cells were used to determine immunomodulatory activity of the most promising structures on a different Toll-like receptor. Therefore, different ligands were needed to be applied in order to function as comparative standards. Lipopolysaccharide derived from *E. coli* 0111:B4 strain (LPS-EB) served as a TLR4 agonist. LPS-EB was then dissolved in endotoxin-free water to create dilution with concentration of 10 ng/ml and serves as positive control. On the other hand, lipopolysaccharide derived for *Rhodobacter sphaeroides* (LPS-RS) matched the characteristics of TLR4 antagonist, negative control. LPS-RS was added to endotoxin-free water to reach 100 ng/ml concentration needed for the experiment. Monophosphoryl Lipid A (MPLA) was used as a hTLR4 standard, ligand that has the ability to activate the receptor and is also used as a vaccine adjuvant. MPLA solution was prepared by dissolution of 1mg of this standard in 1ml of endotoxin-free water to create a 1 mg/ml concentration and then the solution of 10 μM concentration was formed and used in the analysis (Invivogen, 2016d).

5.2.3 Human TLR8 agonistic and antagonistic assay

First part of the assay was determination of solubility of each analyzed compound. It enabled us to prepare the concentration sequence that would be used to quantify and compare immunomodulatory activity of all tested samples. It was also important to work with clear solutions without any debris of undissolved substance in order to prevent inaccurate results. Solubility test started with the placement of 20 µl of the substance solution into a test tube. This volume was replenished with physiological saline solution up to 2 ml. If the sample compound was not dissolving fast enough, the process was usually accelerated by placing the test tube in an ultrasonic bath for about 10-15 minutes. After the sample was dissolved, the serial dilutions were prepared through binary dilution method. The 2 ml solution with the concentration of 100 µl was then relocated from the test tube to a 2 ml safe-lock micro tube. This concentration was a starting point for the rest of the concentration sequence, which continued through 50 µM, 25 µM, and 12.5 µM to 6.25 µM concentrations in separate safe-lock micro tubes with correspondent labelling. Serial dilutions of the standard, resiquimod, were prepared as described in the previous chapter.

Culture growth medium and heat-deactivated growth medium assigned for analysis stored in the fridge were placed in a water bath to preheat to a 37 ^{o}C and then placed in the laminar flow closet.

In the next step, culture flask with the hTLR8 cell line was sprayed with disinfecting 70% ethanol solution and then located in a prepared laminar flow cabinet. Old growth medium was drained out into the waste beaker. The cells attached to the bottom of the flask were detached using a strong flow of 3 ml of the heat-deactivated growth medium controlled by the pipette controller. The main aim was to gently scrape all the cells from the base into the medium in order to create a concentrated cell suspension which was then transferred to a 50 ml test tube. Exactly 200 µl of the cell suspension was moved into another smaller test tube and 1800 µl of Dulbecco's Phosphate Buffered Saline was added. This solution was then pipetted into a Buerker counting chamber to determine the number of cells in 1 ml of the concentrated cell suspension. The information was necessary for preparation of cell suspension that was pipetted on the assay microplates. The calculations were based on the assumption, that

there is the total volume of 23 ml of cell suspension sufficient for one microplate with the cell concentration of 140 000 cells per 1 ml. After the concentrated cell suspension and heat-deactivated growth medium were mixed together in another 50 ml test tube to achieve the required cell concentration, the rest of the unutilized concentrated cell suspension was placed back in the tissue culture flask with the non-deactivated growth medium for further cultivation in the incubator.

The cell suspension was then pipetted with a multichannel pipette on a 96-well microplate with the volume of 180 µl in each well. Subsequently, standard solutions and tested substance solutions in particular concentrations were added to the microplate as described in the Table 2.

Table 2 Pipetting scheme of the human Toll-like receptor 8 agonist analysis

	1	2	3	4	5	6	7	8	9	10	11	12	
A		100	50	25	12.5	6.25	100	50	25	12.5	6.25		A
B		100	50	25	12.5	6.25	100	50	25	12.5	6.25		B
C		100	50	25	12.5	**6.25**	100	50	25	12.5	6.25		C
D		100	50	25	12.5	6.25	100	50	25	12.5	6.25		D
E		100	50	25	12.5	6.25	100	50	25	12.5	6.25		E
F		100	50	25	12.5	6.25	100	50	25	12.5	6.25		F
G		100	50	25	12.5	6.25	100	50	25	12.5	6.25		G
H		100	50	25	12.5	6.25	100	50	25	12.5	6.25		H
	1	2	3	4	5	6	7	8	9	10	11	12	

Legend
analyzed substance [µM]
standard Resiquimod [µg/ml]

The volume of the correspondent solution added to each well was 20 µl, so the tenfold dilution of the sample and the standard was created.

After the solution pipetting, the microplate was left for 30 min in the laminar flow cabinet and then placed at 37 ^{o}C in a 5% CO_2 incubator for 16 to 24 h incubation.

The incubation times for hTLR8 agonist testing sequence were within the range of 16. 5 to 20 h. Upon expiry of the incubation period, the prepared and preheated detection medium was pipetted 180 µl per well on the new microplate. Subsequently, 20 µl of cell supernatant was transferred with multichannel pipette from the incubated microplate containing samples and standards to the microplate with the detection medium. The detection microplate was then incubated at 37 ^{o}C for another hour. The SEAP levels were then determined for each sample using the multi-detection microplate reader Synergy HT at wave-length of 630 nm. After the first analysis, the microplate was incubated for another hour and the second analysis was then performed two hours from the sample pipetting to the detection medium.

Next part of the experiment, hTLR8 antagonism testing sequence, had the same working procedure, but there were a few differences, that needed to be acknowledged. In the first place, the concentration sequences of the analyzed substances were almost the same, only the last concentration was 0 µM instead of 6.25 µM concentrations in the hTLR8 agonist analysis. The same situation appeared in the case of the standard, resiquimod, where the last concentration of the serial dilutions was 0 µg/ml instead of 6.25 µg/ml and therefore corresponding to the concentration sequences of the tested samples. Secondly, the calculations used for the preparation of the cell suspension created by mixing concentrated cell suspension and heat-deactivated growth medium was not based on the 140 000 cells/ml, but on the 156 000 cells/ml, and that is why cell cultivation and proliferation were desired to be high and stable. Since, this was an antagonism analysis, it is quite clear, that the pipetting pattern was different from the previous experiment as shown in Table 3 below:

Table 3 Pipetting scheme of the human Toll-like receptor 8 antagonist analysis

	1	2	3	4	5	6	7	8	9	10	11	12	
A		100	50	25	12.5	0	100	50	25	12.5	0		A
B		100 0	50 0	25 0	12.5 0	0 0	100 0	50 0	25 0	12.5 0	0 0		B
C		100 12.5	50 12.5	25 12.5	12.5 12.5	0 12.5	100 12.5	50 12.5	25 12.5	12.5 12.5	0 12.5		C
D		100 25	50 25	25 25	12.5 25	0 25	100 25	50 25	25 25	12.5 25	0 25		D
E		100 50	50 50	25 50	12.5 50	0 50	100 50	50 50	25 50	12.5 50	0 50		E
F		100 100	50 100	25 100	12.5 100	0 100	100 100	50 100	25 100	12.5 100	0 100		F
G		100	50	25	12.5	0	100	50	25	12.5	0		G
H		100	50	25	12.5	0	100	50	25	12.5	0		H
	1	2	3	4	5	6	7	8	9	10	11	12	

Legend
analyzed substance [µM]
standard Resiquimod [µg/ml]

As indicated in Figure 3, 20 µl of each, the sample solution and the standard solution are put together in one well. Therefore in order to maintain the same dilution factor for both of the substances, it is necessary to pipette only 160 µl of the cell suspension in one well and not 180 µl as it is described in the previous table, where there is either 20 µl of the analyzed compound or 20 µl of resiquimod added to cell suspension in one well. Throughout this experiment sequence, the incubation times stayed within 16 to 17.5 h range.

5.2.4 Principle of the hTLR8 analysis

For the hTLR8 agonist, as well as antagonist testing, HEK-Blue™ hTLR8 cells were used. These cells originated from HEK293 cells, which were modified by transfection of the gene for human Toll-like receptor 8, reporter gene for secreted embryonic alkaline phosphatase (SEAP) and transcription factor NF-κB luciferase reporter plasmid. Easy transfection of these cells, high efficiency of gene and plasmid transfection and higher ability of SEAP reporter gene expression are some of the

advantages of this type of cells that are suggestive of its suitability for this analysis (Jardin et al., 2008).

Resiquimod, imidazoquinoline compound, used as a standard in this analysis has the ability to bind to hTLR8 and activate this receptor by enhancing the production of transcription factor NF-κB. This transcription factor then plays an important role in dose-dependent production of the SEAP, because its reporter gene is controlled by IFN-β promoter, that is activated by the attachment of five NF-κB and alkaline phosphatase (AP) binding sites. Subsequently, the SEAP reporter gene activation then leads to production and higher levels of SEAP that were detected in the assay. The same mechanism of hTLR8 activation is then employed by the analyzed substances and therefore, it allows quantifying and comparing measured results between the samples and between the samples and the standard (Jurk et al., 2002; Invivogen, 2016a; Invivogen, 2016f).

Levels of SEAP are then quantified from the incubated cell supernatant using the detection reagent, QUANTI-Blue™, a colorimetric enzyme that has the ability to determine any alkaline phosphatase activity. The detection medium changes color from purple to blue based on the presence and quantity of the AP. The color change is then analyzed on the microplate reader Synergy HT at 630 nm (Invivogen, 2016e; Invivogen, 2016a).

5.2.5 Principle of the hTLR4 analysis

TLR4 is proven to be responsible for activation of the immune response against gram-negative bacteria. This activation is based on the recognition of lipopolysaccharide that is an important structural part of a bacterial wall. However, there have been a few researches showing that the pathogen recognizing extracellular domain structures of TLR4 and the receptor itself are not sufficient in conferment of LPS responsiveness. Other molecules are required to participate in this process in order to achieve higher induction of immune response against bacterial LPS. Bacterial endotoxin, LPS, is attached to LPS-binding protein (LBP). Subsequently, this aggregation enables the CD-14 protein, cluster of differentiation-14 protein (CD-14), a glycosylphosphatidylinositol-anchored glycoprotein, to extract the endotoxin and create

a CD-14: endotoxin complex that is soluble. The soluble form of endotoxin can then attach to MD-2 protein to form a complex that is able to activate TLR4 and produce adequate immune response even at small amounts of endotoxin present on site. Myeloid differentiation-2 (MD-2) is a protein associated with TLR4 on the cell surface and its presence plays an indispensable role in LPS signaling (Teghanemt et al., 2005; Shuto et al., 2005).

The cells used in this experiment were therefore created by co-transfection of the MD-2 and CD-14 expressing genes, hTLR4 gene and SEAP reporter gene into the same original cell type as the one used for hTLR8 cell formation. The whole complex of TLR4, MD-2, CD-14 and LBP was then able to recognize and bind LPS or other structurally similar hTLR4 ligands and thus induce the production of transcription factor NF-κB, which then leads to production of numerous proinflammatory cytokines and to the production of SEAP. SEAP reporter gene is controlled by IL-12 p40 promoter and for its activation, five molecules of NF-κB and AP-1 binding sites need to be attached to the promoter. The levels of SEAP are then determined the same way using QUANTI-Blue™ as detection medium (Shuto et al., 2005; Shimazu et al., 1999; Invivogen, 2016b).

In this assay, two types of hTLR4 ligands were employed, LPS-RS and LPS-EB. LPS have a specific polysaccharide portion, called Lipid A that is embodied in the bacterial membrane. It also contains dephosphorylated β-1, 6-linked D-glucosamine disaccharide that is connected to hydroxy fatty acids substituted with nonhydroxylated fatty acids. The number of these fatty acids is the main determinant of immunogenic potency of this endotoxin. LPS containing six fatty acyl groups displays agonistic properties and leads to strong immune response, in this analysis presented by LPS EB. According to numerous researches, underacylated forms of endotoxin appear to have either reduced ability to initiate TLR4-dependent signaling or antagonistic activity. The second endotoxin, LPS-RS, is composed of pentaacylated lipid A containing short-chain fatty acids. LPS-RS binds to the same binding sites of the MD-2 protein as hexacylated endotoxin and therefore leads with its antagonistic activity to a dose-dependent inhibition of the agonist or other TLR4 ligand with agonistic properties (Invivogen, 2016c; Teghanemt et al., 2005; Qureshi et al., 1999).

5.2.6 Human TLR4 agonistic assay

The hTLR4 agonistic analysis was performed analogously to the hTLR8 agonistic assay, but since different standards, controls and sample concentrations were used, there was also a different pipetting scheme as it is described in the Table 4. The MPLA, positive and negative control presented by LPS-RS and LPS-EB and different samples were placed in the well in the amount of 20 µl with the 180 µl of the prepared cell suspension of HEK-Blue hTLR4 cells. The sample and MPLA concentrations were the same, 10 µM, so it was made possible to compare the activity of the analyzed substances with the TLR4 ligand, MPLA. The pipetted plate was incubated at 37°C and 5% CO_2 for 16 h. After the incubation was complete, 20 µl of the supernatant from each well was transported onto the new plate with the detection medium. After another incubation period, the microplate reader determined the amount of SEAP produced by each sample and standard and the results were evaluated.

Table 4 Pipetting scheme of the human Toll-like receptor 4 agonist analysis

	1	2	3	4	5	6	7	8	9	10	11	12	
A													A
B		DM 001	DM 002	DM 003	DM 004	DM 005	DM 006	DM 007	DM 008	DM 009	DM 010		B
C		DM 001	DM 002	DM 003	DM 004	DM 005	DM 006	DM 007	DM 008	DM 009	DM 010		C
D		DM 001	DM 002	DM 003	DM 004	DM 005	DM 006	DM 007	DM 008	DM 009	DM 010		D
E		MPLA	MPLA	MPLA	MPLA	MPLA	LPS-EB	LPS-EB	LPS-EB	LPS-EB	LPS-EB		E
F		LPS-RS	LPS-RS	LPS-RS	LPS-RS	LPS-RS	LPS-RS	LPS-RS	LPS-RS	LPS-RS	LPS-RS		F
G													G
H													H
	1	2	3	4	5	6	7	8	9	10	11	12	

Legend
Analyzed substances DM 001-DM 010 [10 µM]
MPLA [10 µM]
standard LPS-EB [10 ng/ml]
standard LPS-RS [100 ng/ml]

6 RESULTS

6.1 Human TLR4 agonistic assay

In the agonistic screening of the analyzed substances on TLR4 receptors was performed as comparison between the activities of each sample with the activity of TLR4 ligand, monophosphoryl lipid A. The final results are presented in the Figure 4.

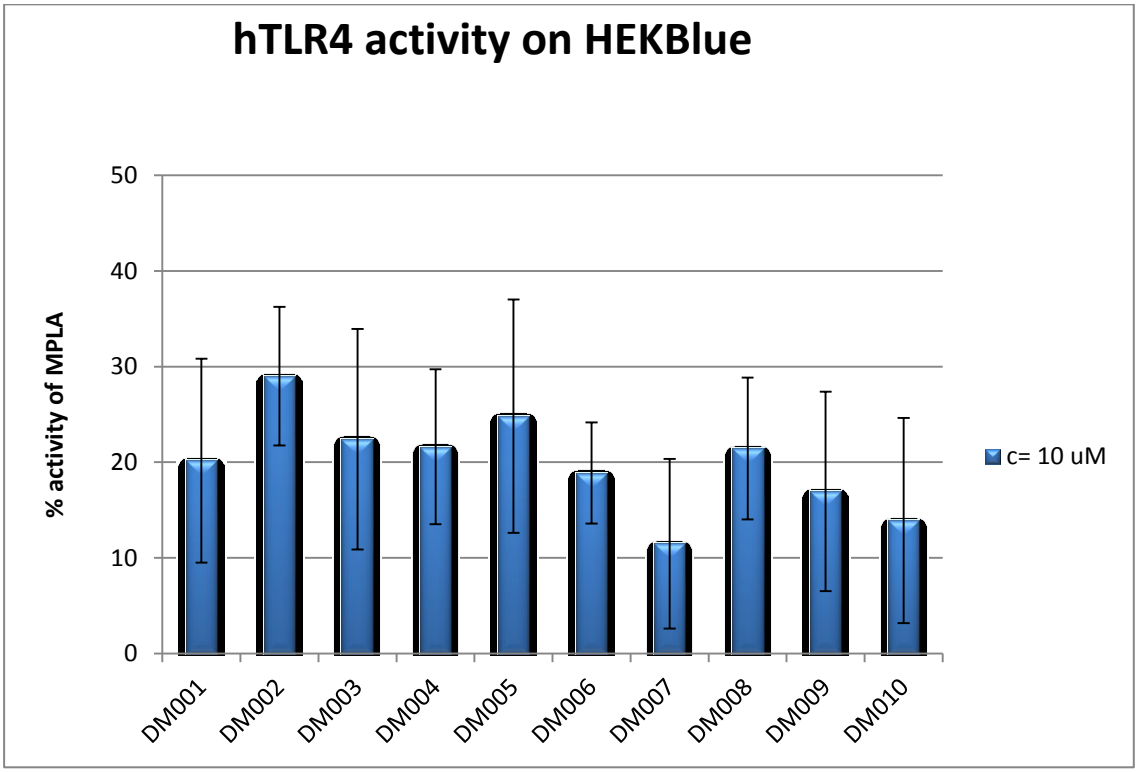


Figure 4 Agonistic screening of DM 001-DM010 on hTLR4 (expressed as % of MPLA activity)

6.2 Human TLR8 agonistic assay

At first, the agonistic testing of all of the samples was performed on the hTLR8 cell line and the results entrancing functionality between sample production of SEAP and sample concentrations are summarized in Figure 5, Figure 6 and Figure 7.

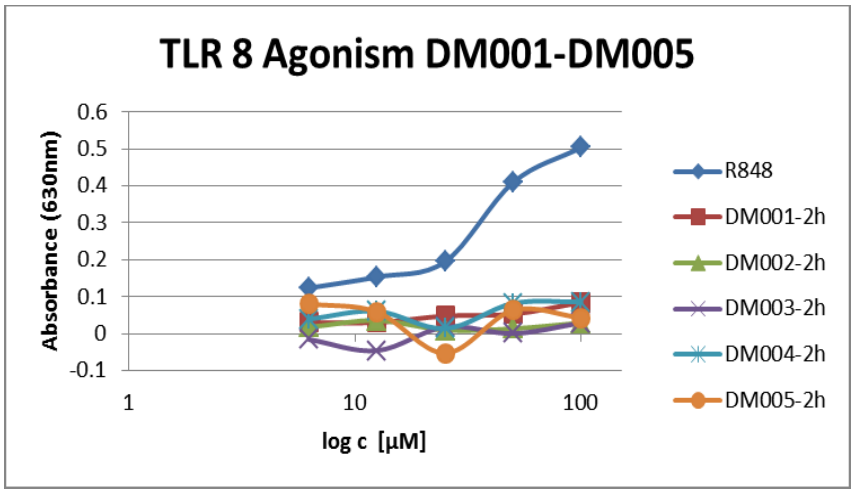


Figure 5 Agonistic assay of DM 001-DM 005 on hTLR8

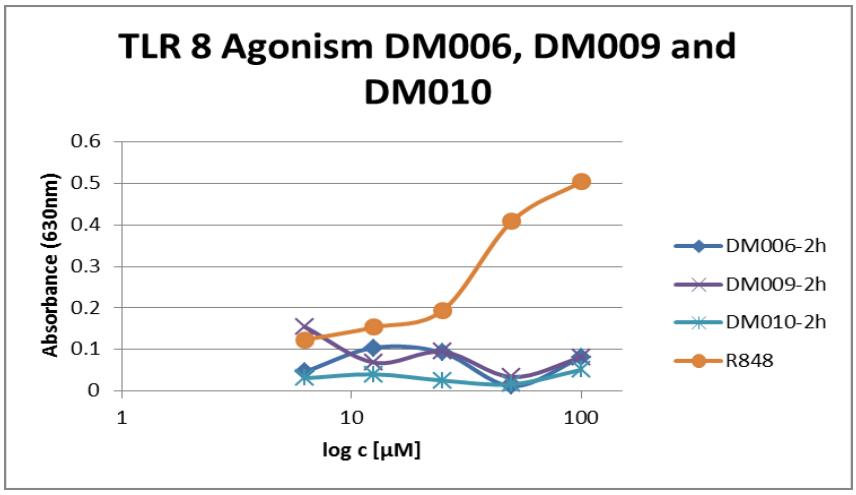


Figure 6 Agonistic assay of DM 006, DM 009 and DM 010 on hTLR8

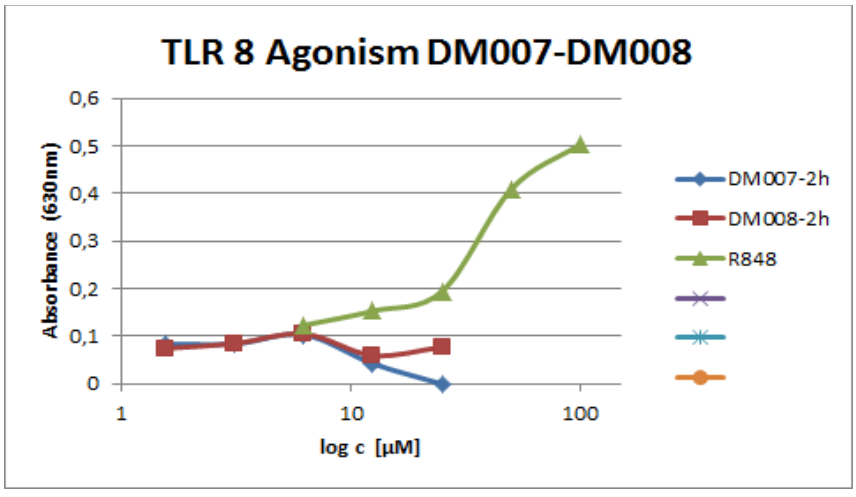


Figure 7 Agonistic assay of DM 007 and DM 008 on hTLR8

6.3 Human TLR8 antagonistic assay

The next part of the experiment was the determination of antagonistic activity of each of the sample substances according to methods described above. The responses of the analyzed substances with the respect to decreasing standard concentrations are presented in the Figures 8-17.

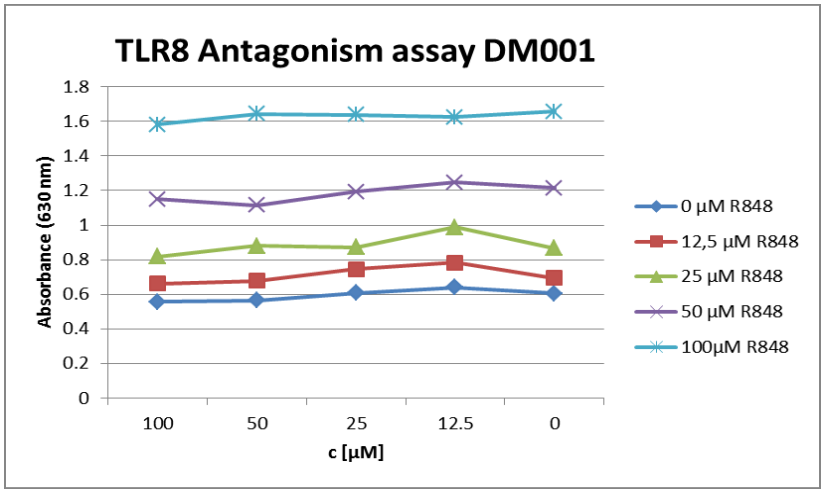


Figure 8 Antagonistic assay of DM 001 on hTLR8

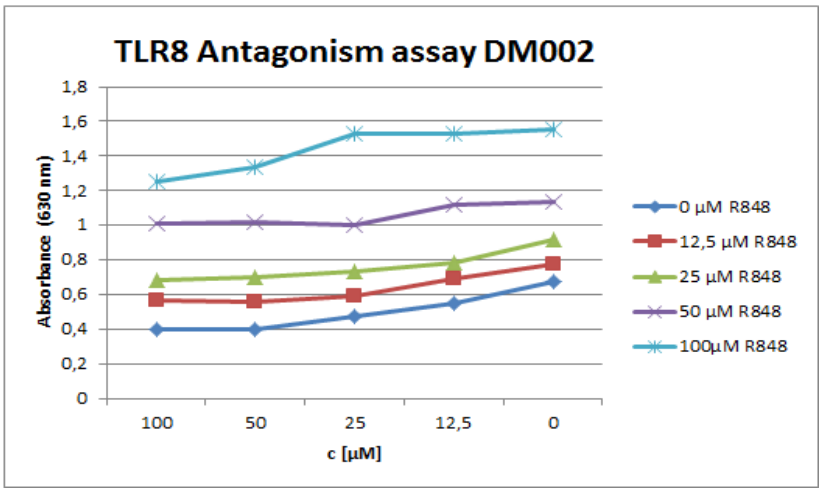


Figure 9 Antagonistic assay of DM 002 on hTLR8

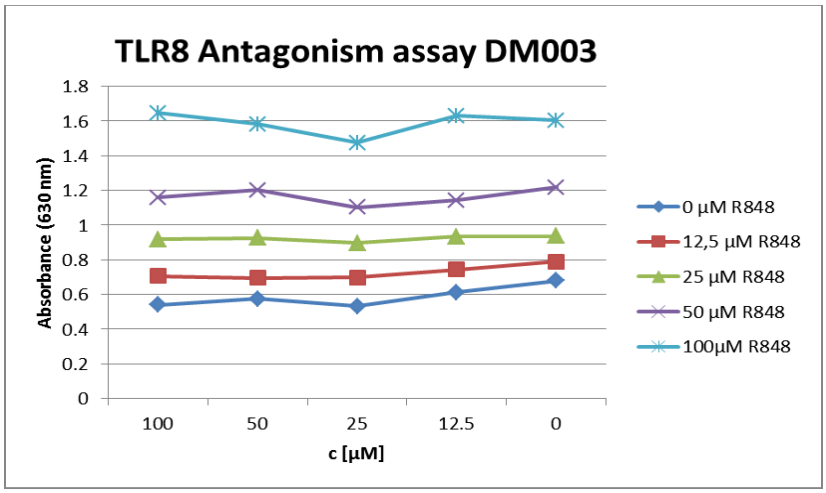


Figure 10 Antagonistic assay of DM 003 on hTLR8

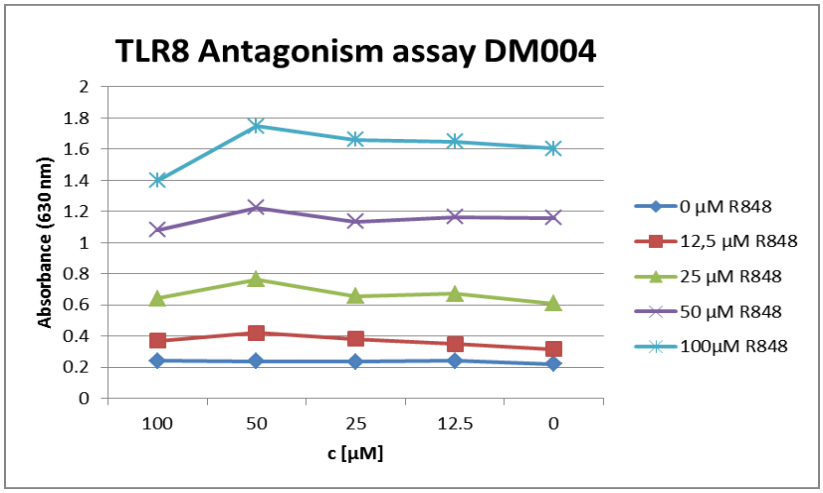


Figure 11 Antagonistic assay of DM 004 on hTLR8

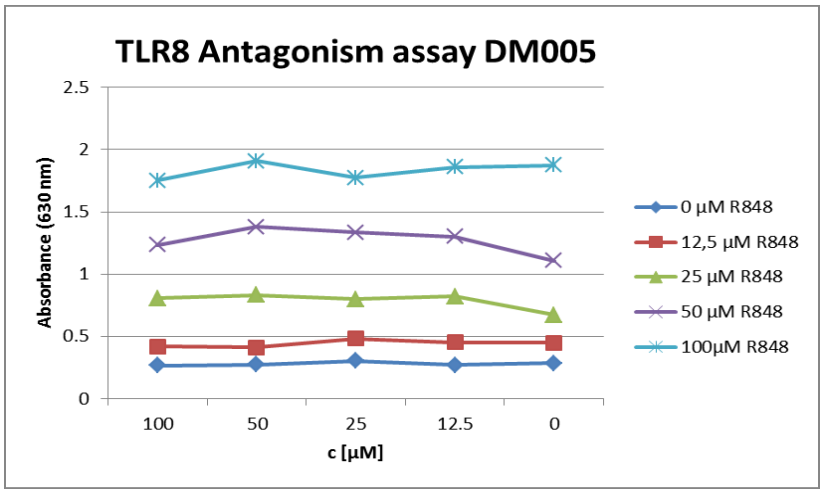


Figure 12 Antagonistic assay of DM 005 on hTLR8

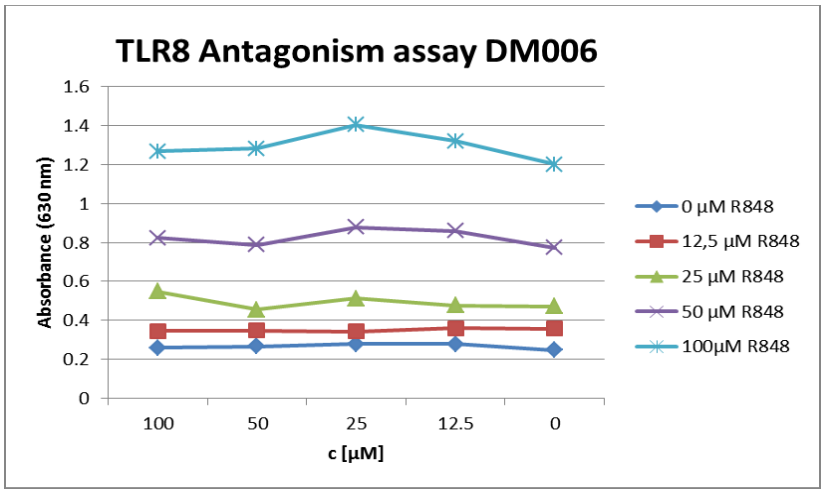


Figure 13 Antagonistic assay of DM 006 on hTLR8

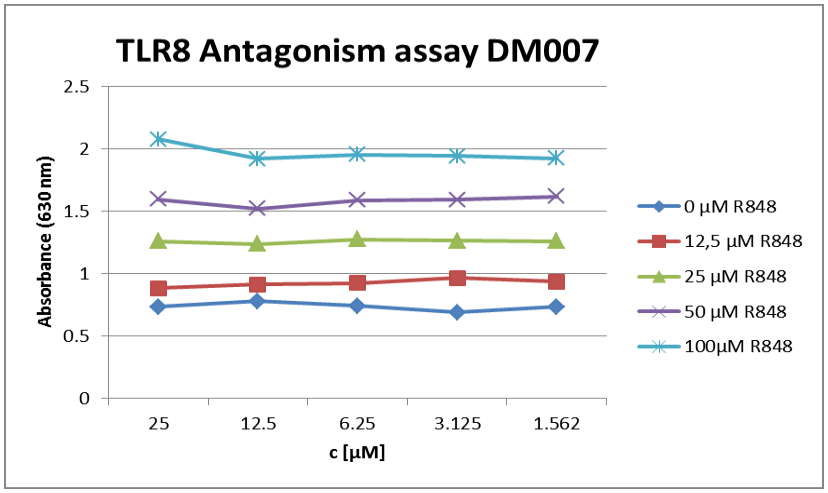


Figure 14 Antagonistic assay of DM 007 on hTLR8

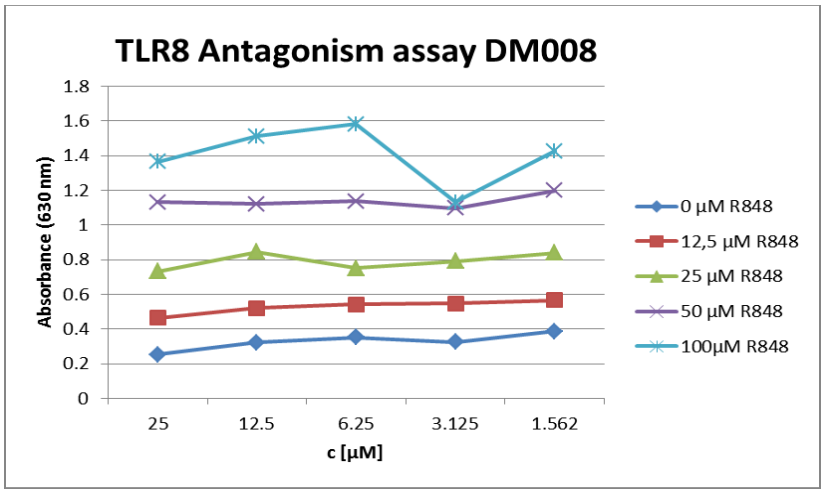


Figure 15 Antagonistic assay of DM 008 on hTLR8

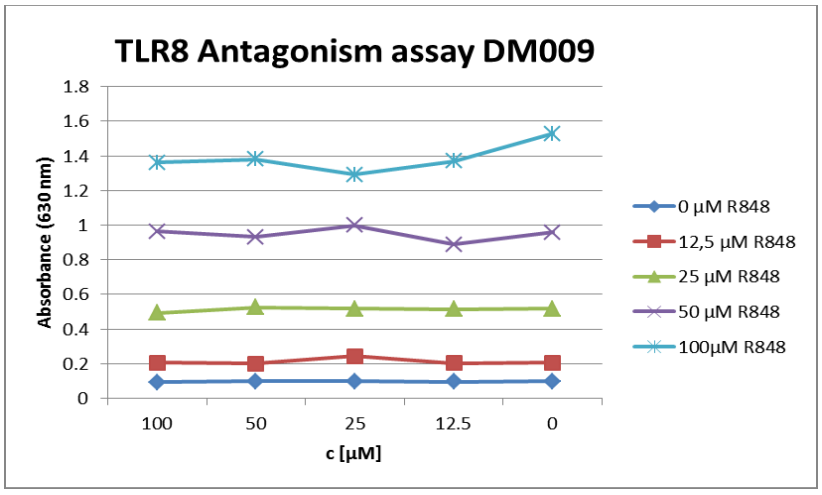


Figure 16 Antagonistic assay of DM 009 on hTLR8

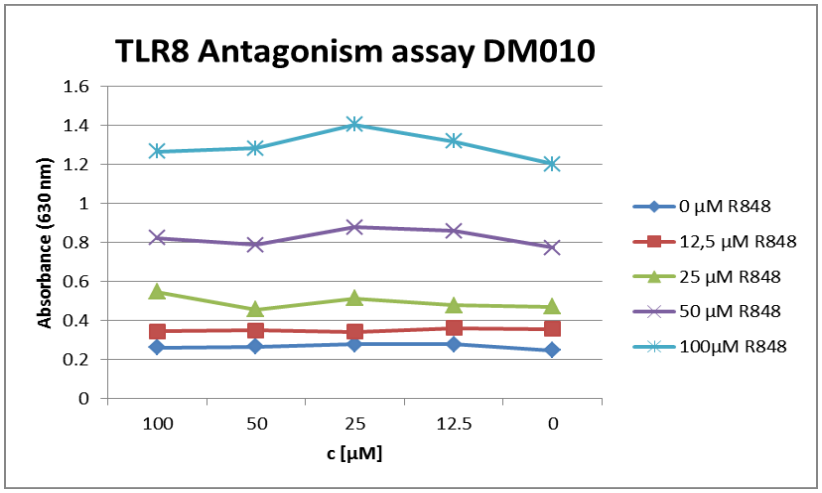


Figure 17 Antagonistic assay of DM 010 on hTLR8

7 DISCUSSION

The first analysis was performed using the hTLR4, where the activity of each sample, DM 001 – DM 010 was evaluated and compared to the activity of commonly used TLR4 ligand, MPLA. For each DM sample, there were three wells with the same concentration of 10 μM, which corresponded with the concentration of the MPLA standard. Considering the results and responses of the samples, the DM 002 exhibit 29% of the activity of MPLA and DM 005 and DM 008 displayed activity over 20% of the MPLA activity. Even though, their responses do not exceed or closely approach the activity of the standard, they still present a great basis for further research and potential for utilization as vaccine adjuvants .Currently used MPLA has much more complex structure and therefore more complicated synthesis in comparison with the rationally designed small-molecules that can be synthesized and used in practice more easily.

Resiquimod as a standard used in hTLR8 analysis has the ability to interact with the TLR8 receptor and enhance the production of NF-κB, nuclear factor that is essential for production of SEAP in a dose-dependent manner and this mechanism also applies to the analyzed substances while interacting with hTLR8 cells and the receptor. Quantification of produced amount of SEAP is determined with the help of colorimetric enzyme reaction where change color occurs in the presence of SEAP and this change can be measured on a microplate reader.

The microplate reader response for each sample and the standard is presented on the vertical axis, while the concentrations are marked on the horizontal axis. According to the results presented in the graphs for TLR8 agonistic analysis, the tested substances did not show any considerable SEAP production and therefore any meaningful immune response in comparison to the standard, resiquimod, which showed anticipated response. The main reason why the analyzed samples did not exhibit any significant results was due to testing on the TLR8 receptors instead of TLR4 receptors where they were supposed to be analyzed primarily. The TLR4 analysis was not possible for further execution since it was impossible to maintain cell lines viability after the restoration

from a frozen stock and due to time press. However, the experiment had its foundation in the in-silico screening, which predicted a promising interaction of DM 001 – DM 010 samples with the TLR8 receptors.

Since two of the sample substances, DM 007 and DM 008 did not show a good solubility, their solution concentrations needed to be reduced in order to obtain solutions suitable for the analysis. The results did not show any significant responses while interacting with the TLR8 as well.

In the antagonism assay, the vertical axis also represents the instruments response and the horizontal axis marks the concentrations of the analyzed samples. The continuous lines then express the responses of particular standard concentrations. In the ideal case, the response of resiquimod at the highest sample concentration should be the weakest and at the lowest sample concentration should be observed the strongest response. Most of the samples did not produce any important or conclusive results, but some of them might have exhibit antagonistic activity to some extent.

According to the graph, DM 002 shows the most potential antagonistic activity, since the resiquimod responses show ascending tendency with the descending sample concentrations, as it would have been expected. Thanks to these results, it is logical to assume antagonistic activity of this specific sample.

DM 008 sample was similarly as the DM 007 sample poorly soluble, therefore in order to prepare the proper solution for the analysis, the concentrations needed to be decreased. The responses exhibit an estimated pattern with the response of resiquimod increasing with descending substance concentrations in most continuous lines. Although the continuous line corresponding with the 100μM concentration of resiquimod shows a strong deflection at the 3.125 μM concentration, cause of this deviation presumably includes multiple influences and is difficult to be accurately determined.

The last sample with promising antagonistic properties was DM 009. The continuous lines corresponding with the lower concentrations of the agonist did not produce any significant responses. The higher concentrations of resiquimod did show some stronger response to the lower sample concentrations even though the continuous lines exhibit some deflections, especially within the substance concentration of 25 μM.

Three of the samples, DM 002, DM 008 and DM 009 display promising potential in antagonistic activity, although for more accurate results and confirmation of this property, more research is needed. One way could be the repetition of this assay in more precise concentrations and further revision of the obtained results. Another way of testing immunogenicity of these samples could be the assay using peripheral blood mononuclear cell (PBMC) that is used in vaccine development and in various models of autoimmune and infectious diseases or cancer (Currier et al., 2002).

For the most samples, it is apparent that the results do not correlate with the ideal conclusion. It is important to take into consideration that cells used in the analysis are living organisms and their growth within the microplate wells cannot be entirely controlled or influenced and therefore, this can affect the final results. Their growth can be also influenced by the environment conditions during analysis execution. Uneven airflow in the incubator can cause unequal temperatures in the microplate wells and therefore affect the cells. Although, this risk could be reduced by putting the highest and the lowest sample concentrations next to each other as it is demonstrated in the pipetting schemes. Another factor influencing the final results is solubility of the tested substances. DM 007 and DM 008 were samples that were difficult to dissolve and therefore their concentrations presented in the graphs are different from the rest of the samples. Solubility affects the response especially in the highest sample concentrations.

There is always a risk of inaccurately executed experiment or unintentional errors caused by the human factor. It is an inaccuracy that is always present to some extent, although the aim is to reduce this risk as much as possible by repeating the assays that produced imprecise or unclear results or multiple wells dedicated to one sample concentration in order to reduce the variability of the results. However, the most presumable explanation of these inconclusive results is the inadequate production of SEAP and insufficient response of the analyzed substances while interacting with the TLR8.

8 CONCLUSION

Vaccine adjuvants represent one of the most important parts of the vaccine that significantly enhances immunogenic potential of a vaccine and also helps modulate qualitative and quantitative response of the immune system. Rationally designed small molecule ligands that are able to interact and activate the TLRs and also induce strong immune response, represent a promising field for adjuvant and subsequently vaccine development.

In this diploma thesis, the main aim was focused on the verification of immunomodulatory activity of the novel small molecule ligands that have the ability to interact with TLRs. According to acquired experiment results, most of the tested samples did not express any distinctive agonistic activity, only the standard created an ideal response. On the other hand, three samples, DM 002, DM 008 and DM 009 are presumed to have antagonistic effect on the TLR8. Although, based on these results, more precise and more advanced assay using peripheral blood mononuclear cells (PBMC) is needed for unambiguous confirmation of their antagonistic properties. Agonistic assay brought more promising results, where DM 002, DM 005 and DM 008 exhibit immunomodulatory activity that might bring more promising and potentially applicable results with further research.

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