SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Expression and modulation of certain Nod-like receptors and their co-acting partners in macrophages and corneal epithelial cells

by

Aliz Varga

Supervisor: Szilvia Benkő PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

DEBRECEN, 2014

EXPRESSION AND MODULATION OF CERTAIN NOD-LIKE RECEPTORS AND THEIR CO-ACTING PARTNERS IN MACROPHAGES AND CORNEAL EPITHELIAL CELLS

By Varga Aliz MSc

Supervisor: Dr. Szilvia Benkő PhD

Doctoral School of Molecular Cell and Immune Biology,
University of Debrecen

Head of the **Examination Committee**:
Prof. Dr. László Fésüs MD, PhD, DSc, MHAS

Members of the Examination Committee:
Dr. Imre Kacskovics DVM, PhD, DSc
Dr. Judit Szabó MD, PhD

The Examination takes place in room 3.506, Department of Biochemistry and Molecular Biology, Faculty of Debrecen, University of Debrecen
at 11 AM, 6th of November, 2014.

Head of the **Defense Committee**:
Prof. Dr. László Fésüs MD, PhD, DSc, MHAS

Reviewers:
Prof. Dr. Gabriella Sármay PhD, DSc
Dr. Viktória Jeney PhD

Members of the Defense Committee:
Dr. Imre Kacskovics DVM, PhD, DSc
Dr. Judit Szabó MD, PhD

The PhD Defense takes place at the Lecture Hall of Building "A", Department of Internal Medicine, Faculty of Medicine, University of Debrecen
at 1 PM, 6th of November, 2014.

1. Introduction

Part 1

Nod-like receptors (NLRs) are intracellular pattern recognition receptors (PRRs) that play a crucial role in the regulation of immune responses. Out of the NLR family NLRP3 protein is one of the most studied member that forms multiprotein complexes, which are called inflammasomes. The NLRP3 inflammasome is activated by pathogen- and danger-associated molecular patterns (PAMPs and DAMPs). It is unlikely that these various kinds of activator agents are directly detected by NLRP3 inflammasomes. It is hypothesized that most of the NLRP3 inflammasome activators are able to induce the generation of reactive oxygen species (ROS) and the produced ROS are required for the signaling mechanisms of inflammasome activation. The activation of the NLRP3 inflammasome leads to the secretion of IL-1β, which has an important role in various diseases, such as allergic rhinitis that is mainly caused by the ragweed pollen. It has been described that ragweed pollen grains and their extracts have intrinsic NADPH oxidase activity, which generates ROS. In our work we aimed to study whether ragweed pollen influences the function of NLRP3 inflammasome and the IL-1β production in lipopolysaccharide (LPS)-activated macrophages.

Part 2

Corneal epithelial cells are non-keratinized cells that act as passive physical barrier, but they also have active immunological roles with the secretion of cytokines and chemokines. At the time of our studies there was no information about the expression of NLR family in these cell types. Therefore we aimed to show the mRNA expression pattern of NLRs and key inflammasome components in primary human corneal epithelial cells and to find a cell line which can be a good model for NLR studies in corneal epithelial cells. We also investigated the effect of UV-B irradiation - as one of the most abundant environmental stress signals for the eyes - on the expression of NLRs.

2. Theoretical background

2.1 Pattern-recognition receptors

The innate immune system is the first line of defense and the rapid recognition of danger signals by the innate immune system is critical for the generation of proper adaptive immune responses. Danger signals are recognized by PRRs situated on the surface or in the cytosolic compartments of host cells, furthermore, PRRs may also be secreted by the producer cells.

2.1.1. Soluble pattern-recognition receptors

Soluble PRRs are secreted by the cells that produce them, such as complement receptors and pentraxins.

2.1.2. Membrane-bound pattern recognition receptors

A wide range of PRR families have been described that remain associated with the producing cells. These are located either on the cellular surface or in endosomal compartments.

a, Toll-like receptors

Toll-like receptors (TLRs) are key molecular sensors of the innate immune system in recognizing various pathogen-derived molecular patterns such as glycolipids, proteins and nucleic acids. Most of the TLRs are expressed on immune cells, but they have been detected on non-immune cells. TLR1, 2, 4, 5 and 6 are expressed on the cell surface, TLR3, 7, 8 and 9 are expressed in the endosomas within the cytosol.

b, Dectins

Dectins are predominantly expressed in macrophages and dendritic cells. Dectin-1 recognizes β-glucans, whereas Dectin-2 recognizes α-mannans.

2.1.3. Intracellular pattern recognition receptors

Intracellular pattern recognition receptors play substantial roles in the sensing of not only intracellular, but also extracellular danger-signals. One group of the intracellular pattern recognition receptors is the Nod-like receptor family, another well studied group is the RIG-like helicases. RIG-I belongs to the RIG-like helicases, because it

has a helicase domain that contains a caspase recruitment domain (CARD) as an essential regulator for dsRNA-induced signaling. Another member of RIG-like helicases is MDA5, which is also specialized in detecting viral RNAs in infected cells.

2.2. Nod-like receptors

Almost 15 years ago a new PRR family was identified in vertebrates with tripartite domain structure. The C-terminal leucine-reach repeat (LRR) domain senses danger signals. The centrally located nucleotid-binding domain (NBD) is thought to be involved in self-oligomerization and activation. Based on the N-terminal effector domain, the NLR family members are classified into subfamilies such as the NLRP and NLRC subfamilies.

2.2.1. NLRC subfamily

a, NOD1, NOD2

NOD1 senses the iE-DAP dipeptide and Tri-DAP, which are found in peptidoglycan (PGN) of bacterial cell wall of all Gram-negative and certain Gram-positive bacteria. NOD2 recognizes MDP, which is the minimal bioactive PGN motif common to all bacteria. Upon activation, the oligomerization of the NBD initiates the binding of RIP2 adaptor protein via the CARD domain. This protein complex is referred to as NODosome in some articles. After the assembly of NODosome, RIP2 has an important role in the activation of NFκB, MAPK, ERK and JNK signaling pathways, which upregulate the expression of proinflammatory cytokines.

b, NLRC5, NLRX1

NLRC5 is the largest member of the NLR family. NLRC5 is a cytosolic protein, but after activation it was found to shuttle between the cytosol and the nucleus. Conflicting reports have been presented about the function of NLRC5. It has been described that NLRC5 expression is strongly induced by IFNγ and more modestly by TLR-ligand LPS. Another protein of the NLR family belonging to the NLRC subfamily is NLRX1. The literature is discrepant about the function of this protein, but it may play an important role in the host defense against viral infections.

2.2.2. Inflammasomes

Upon activation, a subgroup of NLRs and some non-NLR family member proteins form multiprotein complexes. These complexes are called inflammasomes. The inflammasomes consist of an NLR sensor, an adaptor ASC protein and an effector caspase-1 enzyme. The active caspase-1 cleaves pro-IL-1β and pro-IL-18 into mature cytokines. Based on the sensor protein of inflammasomes, different inflammasome complexes can be distinguished.

a, NLRP1 inflammasomes

NLRP1 is a 1473 amino acid protein that contains both an N-terminal PYD domain and a C-terminal CARD domain. NLRP1 inflammasome in humans consisted of NLRP1, caspase-1, caspase-5 enzymes, and ASC adaptor protein. One of the most known ligands of NLRP1 inflammasome is the anthrax lethal toxin. The gene polymorphisms of NLRP1 are implicated in the enhanced activity of the inflammasomes, leading to the risk of several autoimmune diseases.

b, NLRP2 inflammasomes

The 1062 amino acid NLRP2 protein contains a PYD domain, followed by an NBD domain and 8 LRRs. Although NLRP2 is an NLR family member known for a long time, the physiological role of this protein remains poorly understood. It has been described that NLRP2 has an inhibitory effect on NFκB activation induced by different kinds of proinflammatory stimuli.

c, NLRP6 inflammasomes

The 892 amino acid NLRP6 protein has a PYD domain, a central NBD domain, and at least 5 LRR motifs. It is supposed that NLRP6 is an important player in the maintenance of intestinal homeostasis.

d, NLRP7 inflammasomes

NLRP7 is a 980 amino acid containing protein with PYD domain, 9 LRR repeats and an NBD domain. Conflicting reports described NLRP7 as either inflammasome activator or as an inhibitor of the secretion of IL-1β. Mutations in NLRP7 gene are associated with *Hydatidiform mole*, which is a rare autosomal recessive condition.

e, NLRP12 inflammasomes

NLRP12 is a 1061 amino acid protein that has a PYD domain, a central NBD domain, and a C-terminal domain with at least 12 LRR motifs. Both inflammatory and anti-inflammatory functions of NLRP12 have been assumed. It is known that NLRP12 is an important player in the maintenance of intestinal homeostasis. Mutations of NLRP12 are linked to hereditary inflammatory disease and atopic dermatitis.

f, NLRC4 inflammasomes

NLRC4 is a member of the NLRC subfamily of NLRs. In contrast to NLRP proteins it interacts directly with pro-caspase-1 through a CARD–CARD interaction. NLRC4 has been shown to be responsible for caspase-1 activation in response to bacterial flagellin.

g, AIM2 inflamamsomes

Although AIM2 is structurally different from NLRs, it functions as a cytosolic PRR and forms inflammasomes. In AIM2 inflammasomes the HIN200 domain of AIM2 binds to cytosolic DNA of engulfing apoptotic cells, whereas the PYD associates with the ASC leading to the activation of caspase-1 and consequently the cleavage of pro-IL-1β and pro-IL-18 to active cytokines.

2.3 NLRP3 inflammasomes

NLRP3 is a 1036 amino acid protein protein that contains a PYD domain, an NBD domain and 9 LRRs. The NLRP3 inflammasome complex contains NLRP3 protein as the sensor of the complex, an ASC adaptor, and caspase-1 effector enzyme. The gain of function mutations of NLRP3 gene and the improper control of NLRP3 inflammasome activation lead to the enhanced NLRP3 inflammasome activity, and consequently to the increased IL-1β secretion, which has been associated with a variety of maladies including chronic inflammatory diseases, autoinflammatory diseases, cancer predisposition and metabolic diseases. Therefore this is evident that the proper regulation of NLRP3 inflammasome activation is of high importance.

2.3.1. The regulation of IL-1β and IL-18 expression via the activation of NLRP3 inflammasomes

2.3.1.1. IL-1β as proinflammatory cytokine

IL-1β is synthesized as an inactive 31 kDa precursor protein (pro-IL-1β) and processed into mature 17 kDa cytokine by enzymatic cleavage. Unlike other secreted proteins, pro-IL-1β lacks a hydrophobic leader sequence and is not found in organelles composing the classical secretory pathway. IL-1β is a potent proinflammatory cytokine, which is involved in many inflammatory conditions including autoinflammatory and allergic disorders. Blood monocytes, tissue macrophages and dendritic cells are the primary sources of IL-1β.

2.3.1.2. The activation of NLRP3 inflammasome and the regulation of IL-1β production

Based on the current dogma the secretion of IL-1β requires two signals. The first signal is necessary for the induction of pro-IL-1β (and inflammasome members) expression in a NFκB-mediated process through TLRs. It has been described that most PAMPs, which are known NLRP3 activators appear to be required only for the priming step. The second signal, most predominantly DAMP, is required for inflammasome assembly and a subsequent cleavage of precursor into mature IL-1β by caspase-1. This second signal is frequently associated with the production of ROS or endosomal rupture, changes in intracellular and extracellular calcium concentration and potassium efflux.

2.4. The role of reactive oxigen species in the activation of NLRP3 inflammasomes

Recent findings suggest that most identified NLRP3 activator agents also induce the production of ROS and the produced ROS are required in the signaling mechanisms of inflammasome activation. Thioredoxin (TRX)-interacting protein (TXNIP) may directly activate the NLRP3 inflammasome upon oxidative stress. It has been described that TXNIP interacts with TRX, therefore it is unable to activate NLRP3. Upon oxidative stress TXNIP is released from oxidized TRX and directly binds NLRP3, leading to the inflammasome assembly. Most of the NLRP3 inflammasome

activators induce the dissociation of TXNIP from TRX in an ROS-sensitive manner and allow the subsequent binding of NLRP3.

2.4.1. Antioxidants

The imbalance between ROS production and the ability to neutralize these oxidizing radicals generate oxidative stress. One of the most studied antioxidants is a general ROS scavenger N-acetyl cisteine (NAC). Among the mitochondria-targeted antioxidants, MitoTempo is one of the most studied molecule. Diphenyleneiodonium (DPI) is widely used as an inhibitor of NADPH oxidase and it also inhibits the production of ROS by mitochondria.

2.4.2. The role of ROS and the NLRP3 inflammasome in allergic rhinitis and asthma

Allergic rhinitis is mainly caused by ragweed pollen. Ragweed pollen grains and their extracts contain NADPH oxidases. After inhalation, ragweed pollen induces ROS generation in airway epithelial cells. In these patients the high level of ROS is involved in the exacerbating of symptoms.

The role of NRLP3 inflammasomes in the pathomechanism of allergic rhinitis is unclear and controversial. It has been demonstrated that NLRP3 does not have a role in the development of allergic airway disease induced by either acute or chronic house dust mite exposure. Another research group has shown that patients suffering from allergic rhinitis present lower NLRP3 mRNA levels than both controls and patients during pollen season. In contrast to these findings it has also been described that allergic airway inflammation depends on NLRP3 inflammasome activation. It is known that environmental pollutants increase ROS production in lungs and exacerbate the symptoms of allergic asthma. It has been described that human airway epithelial cells contains functional NLRP3 inflammasomes that respond to urban particular matter exposure with caspase-1 cleavage and IL-1β production.

2.5. Macrophages

Macrophages are critical cells of the innate and adaptive immune responses, contributing to the immediate and robust defense against danger signals. They detect these signals using PRR such as TLRs and NLRs. Tschopp et al. discovered the functional inflammasome, which was first identified in THP-1 macrophage cell line.

Dysregulation of the NLR function in macrophages and consequently the improper secretion of IL-1β have been described in case of a variety of maladies, including chronic inflammation, autoimmunity and cancer predisposition.

2.6. Corneal epithelial cells

The major cell types of the corneal immune system are corneal epithelial cells. They present a physical barrier and also actively secrete cytokines, chemokines, antimicrobial peptides to activate microbial defense. It has been described that corneal epithelial cells express different kinds of PRRs for the recognition of pathogen and environmental-derived danger signals (such as UV-radiation). At the time of our studies, the expression of several TLR family proteins was studied in corneal epithelial cells and in human immortalized cell lines, but there was no information about the expression of various NLR molecules and key inflammasome components.

2.7. Aims of the studies

- To analyze the effects of ragweed pollen extract on the IL-1β secretion of untreated and LPS-activated macrophage cells.
- To study the contribution of ROS to the IL-1β secretion of macrophages upon ragweed pollen extract and/or LPS activation.
- To examine whether or not the NLRP3 inflammasome is involved in the IL-1β secretion of LPS-activated and ragweed pollen extract-treated macrophage cells.
- To clarify which signaling pathways are involved in the IL-1β secretion of ragweed pollen extract-treated and/or LPS-activated macrophages.
- To find a cell line which can be a good model for NLR studies in corneal epithelial cells.
- To identify the expression pattern of NLRs in human corneal epithelial cells.
- To examine whether UV-B irradiation can alter the expression of NLRs.

3. Methods

3.1. Cell culture of THP-1 macrophages

THP-1 monocyte cell line (ATCC TIB-202) was a generous gift of Prof. Laszlo Nagy. THP-1 cells were cultured in RPMI 1640 (Gibco) containing 10% heat-inactivated FBS, penicillin-streptomycin and L-glutamine, and maintained at 37°C under 5% CO_2. The cells were differentiated into macrophages in tissue culture dishes with 0.5 μM phorbol myristate acetate (PMA, Invivogen) for 3 hours, then washed three times with PBS and plated at $5x10^5$ cells/ml for ELISA or 10^6 cells/ml for real-time PCR and Western blot methods. The cells were left to adhere overnight, then they were treated with 100 ng/ml ultrapure LPS (InvivoGen), 10 μg/ml ragweed pollen extract (Greer Laboratories, 100 μM NADPH (Sigma-Aldrich) or 0.3 mM H_2O_2 (Sigma-Aldrich). The endotoxin content of pollen extract was 16.31 pg/g protein, negligible compared to the used LPS concentration. N-acetyl-cysteine (NAC, 30 mM, Sigma-Aldrich), MitoTEMPO [2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2 oxoethyl] triphenylphosphonium chloride monohydrate) (300 μM, Santa Cruz Biotechnology), diphenyleneiodonium (DPI, 10 μM, Sigma-Aldrich) or caspase-1 inhibitor (Z-YVAD-fmk, 20 μM, BioVison) were added to the cells 1 hour before treatments.

3.2. Macrophage and dendritic cell generation

For monocyte separation local Ethical Committee approval was received for the studies and the informed consent of all participating subjects was obtained. CD14+ monocytes were separated with anti-CD14–conjugated microbeads (VarioMACS Separation System; Miltenyi Biotec) from leukocyte-enriched buffy coats from healthy donors and plated in RPMI 1640. Cells were plated in 12-well culture dishes at a density of 1.5×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS, 500 U/ml penicillin-streptomycin (Life Technologies), and 2 mM L-glutamine (Life Technologies). For macrophage and dendritic cell (DC) differentiation cells were treated with 80 ng/ml GM-CSF (Leucomax; Gentaur Molecular Products) or 80 ng/ml GM-CSF and 100 ng/ml IL-4 (PeproTech), respectively. IL-4 and GM-CSF were replenished on day 3. The macrophages and DCs were challenged with 500 ng/ml LPS, 100 μg/ml RWE and 100 μM NADPH for 24 hours at day 5 of culturing.

3.3. Cell culture of corneal epithelial cells

The SV-40 immortalized human corneal epithelial cell line (HCE-T) was generously provided by Kaoru Araki-Sasaki (Osaka University, School of Medicine, Osaka, Japan) through the Riken Cell Bank. Human corneal epithelial cells were taken from the eyes of healthy individuals by epithelial ablation for photorefractive keratectomy (PRK). Corneal epithelial cells were cultured in DMEM/F12 cell culture medium containing 200 U/ml penicillin and streptomycin, 5% FBS (Gibco, San Diego, CA), 5% glutamine, 5 μg/ml insulin, 0.5% dimethyl sulfoxide (all from Sigma-Aldrich, St Louis, MO), and 10 ng/ml human epidermal growth factor (Invitrogen). For peptidoglycan treatment, we used 4 μg/ml soluble sonicated ultrapure peptidoglycan from *Escherichia coli* K12 (PGN-ECndss; Invivogen).

3.4. Viability assay

THP-1 cell viability was measured using LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen). PMA-treated THP-1 cells were left to adhere overnight in 96-well plate and then were treated with 100 ng/ml LPS, 10 ug/ml RWE and 100 uM NADPH in the presence or absence of DPI and NAC. Methanol was used as a positive control. After 24 hours cells were washed with PBS then were incubated in Live/Dead Viability/Cytotoxicity Solution for 45 minutes. Calcein acetoxymethyl (Calcein AM)-stained healthy cells have green and ethidium homodimer-1 (EthD-1)-stained nuclei of dead cells have bright red fluorescent signal. Fluorescence was detected by BioTek spectrophotometer using the appropriate excitation and emission filter.

For HCE-T cells, the viability was determined after UV-B exposure using MTT (3-(4.5-dimethylthiazol-2-yl)-2.5 diphenyl-tetrazoliumbromide) colorimetric method.

3.5. Ultraviolet-B treatment of HCE-T cells

At approximately 85%–90% confluency, the growth medium was removed and collected, then cells were irradiated with 30 mJ/cm^2 dose of UV-B. The UV-B source consisted of a FG15T8 bulb, which produced maximal output in the UV-B range. After irradiation, the cells were cultured further in their conditioned medium for 24 hours. Control cells were treated in the same manner except that they were not irradiated. Control and irradiated cells were harvested 6 hours and 24 hours after UV-B treatment.

3.6. Reactive oxygen species measurement

10^6 THP-1 cells were loaded with 50 M 2'-7'-dihydro-dichlorofluorescein diacetate (H2DCFDA, Invitrogen) at 37 ºC for 20 min and treated with the indicated compounds. At the indicated times, cells were resuspended and analyzed by flow cytometry using FACSCalibur (Becton Dickinson). FlowJo software was used for analysis. Relative ROS levels are given in arbitrary units of mean intensity of fluorescence with respect to untreated controls.

3.7. siRNA transfection

Differentiated THP-1 cells were electroporated with 2.5 μM NLRP3-specific or scrambled siRNA (Silencer Select Pre-Designed and Validated, Ambion), then plated. After 48 hours, cells were treated with the indicated compounds and 24 hours later the supernatants were collected for ELISA, while cells were used for real-time PCR and/or Western blot.

3.8. RNA preparation and RT-PCR

Total RNA was extracted with TriReagent (Molecular Research Center) and isolated according to the manufacturer's instructions. The concentration and homogeneity of RNA preparations were determined by a spectrophotometer (NanoDrop ND1000; Promega Biosciences). Standardized amounts of RNA were then digested with DNase (Ambion), and subjected to reverse transcription using Super Script II RNase H – Reverse Transcriptase and Random Primers (Invitrogen).

3.9. Real-time quantitative PCR

Real time analyses were performed in 96 or 384-well optical reaction plates in ABI Prism 7900HT Sequence Detector System (Applied Biosystems). Oligo mixes were purchased from Applied Biosystems. Taq DNA Polymerase (Fermentas) was used for amplification, and Rox Reference Dye (Invitrogen) was used for normalization of the fluorescent reporter signal. Amplification was conducted in 25 μl or 12.5 reaction mixture containing 125 ng of cDNA. Real-time PCR data were analyzed by using Sequence Detector System version 2.1 software (Applied Biosystems). The expression levels were calculated by the ΔCt method and normalized to that of the human housekeeping gene cyclophilin or *36B4*.

3.10. Western blot analysis

The treated and non-treated cells were washed with ice-cold PBS and suspended in a lysis buffer containing 30 mM Tris (pH 7.6), 140 mM NaCl, 5 mM EDTA, 50 mM NaF, 2 mM Na-pyrphosphate, 50 μM phenylasine-oxide, 1% Triton-X and 1 mM Na3VO4 with freshly added protease inhibitors (1 μg/ml aprotinin, 0.5 μg/ml pepstatin, 1.25 μg/ml leupeptin, 1 mM PMSF). The total protein concentration of the samples was determined using BCA protein assay reagent kit (Pierce). 30 μg of total proteins were heated with SDS sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS, 0.025% bromephenol blue). Lysates were separated on SDS-PAGE gel, and transferred onto nitrocellulose membranes using wet electro-blotting. Membranes were blocked in Tween-TBS containing 5% nonfat milk and stained with antibodies recognizing NLRP3 and NLRP1 (mouse monoclonal, Alexis Biochemicals), cleaved IL-1β and caspase-1 (rabbit polyclonal, Cell Signaling Technology), pro-caspase-1 (rabbit polyclonal, Santa Cruz Biotechnology), phospho-p38 MAPK, phospho-SAPK/JNK (rabbit polyclonal, Cell Signaling Biotechnology), phospho-p38 and p38, phospho-SAPK/JNK and SAPK/JNK, phospho-c-Jun (Ser63 and Ser73) and c-Jun, phospho-c-Fos and c-Fos, p-IκB-α (mouse monoclonal; Santa Cruz Biotechnology or Cell Signaling Biotechnology) overnight at 4 °C. Primary antibodies were detected using HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit, Amersham) for 1 hour at room temperature. Proteins were visualized by Supersignal West- Pico peroxide/luminol enhancer solution (Pierce). Equal amount of protein sample loading was verified by detecting β-actin (rabbit polyclonal, Sigma-Aldrich) protein expression.

3.11. Measurement of caspase-1 activity

Caspase-1 activity in cell lysates was determined using the acetylated and AMC-conjugated fluorometric peptide substrate Acetyl-Tyr-Glu-Val-Asp-7-amino-4-methyl-coumarin (Anaspec). Lysis of the cells was performed on ice for 30 min in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5 mM EDTA, 0.5 % NP-40, 1 mM PMSF, 1 μg/ml aprotinin, 0.5 μg/ml pepstatin, 1.25 μg/ml leupeptin and 1 mM DTT. After centrifugation (10000 g, 10 min at 4 °C), 30 μg protein lysate supernatants were incubated in 100 μl lysis buffer with 40 μM substrate (final concentration) in microtiter plate wells at room temperature, and the increase of fluorescence due to the release of AMC was detected at 460 nm, using 355 nm

excitation wavelength in a Wallac 1420 Victor2 fluorimeter-luminometer (Wallac Oy).

3.12. Determination of secreted cytokine concentrations

The concentrations of secreted cytokines in the cell culture supernatants after the indicated times of treatments were measured by ELISA according to the manufacturer's instruction for IL-1β, IL-6, IL-8, IL-10, IL-12 (BD Biosciences) and IL-18 (R&D System). The detection limit of the assays was 10 pg/ml for IL-1β, IL-10, IL-12, and TNF-α, 5 pg/ml for IL-6 and IL-8, and 30 pg/ml for IL-18.

3.13. Statistical analysis

Significance of the differences between mean values was evaluated using a Student's t-test. Data presented as mean ±SD values.

4. Results

4.1. Ragweed pollen extract intensifies LPS-induced priming of NLRP3 inflammasome in human macrophages

4.1.1. RWE enhances LPS-induced IL-1β secretion of human macrophages

We studied the effect of RWE on the IL-1β production in THP-1 macrophages. To avoid the activating effect of the direct contact of pollen grains, we used the extract of the ragweed pollen. Since the pollen extract does not contain NADPH, which is the substrate of NADPH oxidase, therefore we exogenously added NADPH in our experiments. To determine the effect of RWE on IL-1β production, THP-1 macrophages were treated with RWE in the presence or absence of NADPH, or in combination with LPS, and IL-1β content of the culture medium was determined. We found that RWE in the absence or presence of NADPH or NADPH alone did not trigger the secretion of IL-1β. However, RWE in the presence of NADPH significantly enhanced the LPS-induced IL-1β production. In case of the monocyte-derived macrophages and dendritic cells the results are similar to those found with the THP-1 cell line.

4.1.2. RWE induces ROS production, ROS inhibitors abolish RWE-enhanced IL-1β production in LPS-treated THP-1 macrophages

It has been described that pollen NADPH oxidases increase the level of ROS in lung epithelial cells, but no information is available if pollen extract could induce ROS production in THP-1 macrophages. Therefore we measured ROS production of THP-1 cells after the treatments. Whereas RWE alone induced some ROS production (but not NADPH), their combined effect yielded a continuously increasing ROS level. LPS alone did not cause ROS production detectable by this method, in good agreement with previous findings, and also did not enhance the RWE induced ROS production in the presence of NADPH. To determine whether the RWE-dependent enhancement of LPS-induced IL-1β production is mediated by ROS, THP-1 macrophages were pretreated with ROS-scavenger NAC. Our results show that NAC completely abolished IL-1β secretion, indicating that ROS play a crucial role in LPS-induced and RWE-enhanced IL-1β production. To verify the source of ROS involved in the IL-1β secretion, cells were treated with MitoTEMPO, which inhibits ROS

production of mitochondria. We also treated the cells with DPI that inhibits ROS production of NADPH oxidase and mitochondria. While MitoTEMPO only moderately decreased IL-1β secretion, DPI treatment completely cancelled it. Our results suggest that the majority of the ROS involved in the RWE-dependent enhancement of LPS-induced IL-1β production is generated by pollen-derived NADPH oxidases.

4.1.3. Caspase-1 inhibition and NLRP3 silencing abolish RWE-enhanced LPS-induced IL-1β production

To verify the involvement of caspase-1 in RWE-enhanced IL-1β production, THP-1 cells were pretreated with the specific caspase-1 inhibitor Z-YVAD-FMK before LPS and RWE treatment, and secreted IL-1β was measured by ELISA. We demonstrated that the caspase-1 inhibitor significantly reduced the LPS-induced RWE-enhanced IL-1β production. We have also studied the effect of silencing of NLRP3 expression using siRNA on the IL-1β secretion of THP-1 cells. We have shown that the NLRP3 silencing completely inhibited the IL-1β secretion of stimulated THP-1 cells.

4.1.4. RWE enhances LPS-induced priming step for NLRP3-inflammasome function

For NLRP3 inflammasome activation the elevated expression of inflammasome components and pro-IL-1β are required. THP-1 cells were treated with RWE and NADPH in the absence or the presence of LPS, and the expression of NLRP3 and caspase-1 was measured. We demonstrated that LPS significantly induced the expression of NLRP3, pro-caspase-1 and caspase-1 p20. While RWE in the presence of NADPH did not influence the expression of these molecules, it further enhanced the LPS-induced pro-caspase-1 and cleaved caspase-1 expression. Although we found an enhanced transcription of NLRP3, but the increase was not significant in the protein level. Similar results have been published that demonstrate the different expression of NLRP3mRNA from the protein expression after stimulation, but the exact mechanism is unclear. To see whether the increased presence of caspase-1 is accompanied with increased caspase-1 activity, we measured the caspase-1 activity in THP-1 cells. We found that while LPS significantly induced the caspase-1 enzyme activity, treatment of the LPS-primed cells with RWE did not result in further pro-

caspase-1 activation. These results appear to be contradictory, but the western blot technique detects the processed form independent of its activity, and it has been demonstrated that caspase-1 is rapidly inactivated in THP-1 cells leading to the accumulation of processed but inactive caspase-1. We also investigated how RWE treatment by itself or in combination with LPS affects the expression of pro-IL-1β. Our results show that the LPS-induced expression and secretion of IL-1β were further enhanced by RWE treatment.

4.1.5. Induction of key inflammasome components and pro-IL-1β by RWE is NADPH-dependent, and is suppressed by an inhibitor of ROS production, DPI

To demonstrate that the RWE-enhanced expression of pro-IL-1β and key inflammasome components is due to the presence of active NADPH oxidase in the pollen extract, we studied the RWE-induced transcription of these genes. We found that all of the corresponding gene inductions by RWE appeared to be NADPH-dependent. To further explore the role of ROS on the expression of pro-IL-1β as well as the NLRP3 inflammasome components, we studied the effect of ROS production inhibitor DPI on the mRNA level of these proteins. Our results show that DPI substantially inhibited pro-IL-1β and NLRP3 gene expression in the LPS- or RWE-treated cells, as well as in those treated with a combination of these.

4.1.6. RWE triggers p38 MAPK and JNK signaling in an NADPH-dependent manner and results in increased p38 MAPK and JNK and AP-1 phosphorylation in LPS-treated cells

To see whether the major signaling pathways that are involved in the expression of inflammasome members and IL-1β are affected by RWE, we studied the phosphorylation of p38 MAPK, JNK, and IκBα in response to treatment by various combinations of compounds. We found that unlike the phosphorylation of IκBα, RWE induced the phosphorylation of p38 MAPK and JNK, furthermore, the phosphorylation appeared to be NADPH-dependent. We also found that RWE in the presence of NADPH substantially enhanced the LPS-induced p38 MAPK and JNK phosphorylation. We further investigated the phosphorylation of AP-1 complex members c-Jun and c-Fos, which are also important participants of signaling pathways involved in IL-1β expression. These data show that in the presence of NADPH, RWE

triggered the phosphorylation of the AP-1 complex members p-c-Jun and p-c-Fos, moreover it was able to enhance the LPS-induced phosphorylation of these proteins.

4.2. Constitutive and UV-B modulated transcription of NOD-like receptors and their functional partners in human corneal epithelial cells

4.2.1. Transcription of NLRPs and inflammasome components in immortalized and primary corneal epithelial cells

At the time of our studies the expression of several TLR family proteins was studied in primary corneal epithelial cells and cell lines, but there was no information about the expression of various NLR molecules and key inflammasome components. Therefore we measured the transcription of NLRP proteins (NLRP1, NLRP2, NLRP3, NLRP4, NLRP6, NLRP7, NLRP10, NLRP12) and inflammasome components in immortalized and primary human corneal epithelial cells. The relative expression of NLRP1 mRNA was comparable in the cell line and primary cells. The mRNA expression of NLRP3 was more than 20-fold in HCE-T cells than in the primary cells. NLRP7 mRNA expression was only detectable at a very low level in the immortalized cell line, whereas NLRP10 mRNA expression was dramatically higher in HCE-T cells than in primary cells. We could not detect the mRNA expression of NLRP4, NLRP6 or NLRP12, neither in the cell line nor in the primary cells. We also detected a shorter isoform of NLRP1, but we could not detect the protein expression of NLRP3, unlike NLRP3 mRNA that we detected.

We also found that the mRNA expression of ASC and pro-caspase-1 was dramatically lower in HCE-T cells as compared to that of the primary cells. While we clearly detected the protein expression of pro-caspase-1 in HCE-T cells, the activated form was not detectable.

4.2.2. Expression of NLRC subfamily members in immortalized and primary corneal epithelial cells

Besides examining the NLRP subfamily members we also measured the relative mRNA expression of the members of NLRC subfamily (including NOD1, NOD2, NLRX1 and NLRC5) in HCE-T cell line and in primary cells. NOD1, NOD2 and NLRX1 mRNA were expressed at similar levels in both cell types with comparable

expression level in the primary corneal cells. The NLRC5 mRNA expression was under the detection limit in primary cells and it was at a very low level in the cell line. Since we demonstrated that the expression level of NOD1 and NOD2 in the HCE-T cell line was similar to that of the primary cells, we aimed to study whether HCE-T cells are able to recognize and respond to a NOD1/NOD2-specific ligand. For this experiment we treated the HCE-T cell line with ultrapure PGN, which is a specific ligand of NOD1 and NOD2 that acts via NOD1 or NOD2 without the activation of TLR2. It is also known that triggering NOD1 or NOD2 activation by their specific ligands leads to the activation of NFκB, accordingly the phosphorylation of IκB is a marker of NFκB activation. We detected the time-dependent appearance of the phosphorylated IκB after the treatments. The phosphorylation peak was observed in the 4 hours-treated sample. These results show that NOD1 and/or NOD2 present in HCE-T cells are functionally active. Therefore we demonstrated that HCE-T cell line is a good model for NOD1 and NOD2 studies in corneal epithelial cells.

4.2.3. Effect of UV-B irradiation on the transcription of NLRP proteins in HCE-T cells

One of the major environmental stresses for cornea is the UV-B radiation. To study the effect of UV-B irradiation on the expression of various NLRPs in HCE-T cells, we irradiated the cells for 6 hours and 24 hours and the expression levels were compared to the non-exposed control cell samples. Our results show that the mRNA expression of all of the studied NLRPs was downregulated 6 hours after UV-B exposure with the exception of NLRP2 and NLRP7. Interestingly, 24 hours after UV-B irradiation the mRNA levels of NLRPs recovered. Moreover, the expression of NLRP3 and NLRP10 were significantly increased compared to the expression level of non-treated samples.

We also studied the effect of UV-B irradiation on the protein expression of NLRP1 and NLRP3. We observed decreased protein expression of NLRP1 6 hours after UV-B exposure, but 24 hours after treatment protein level recovered nearly up to the protein level of the non-treated control sample. Although the mRNA level of NLRP3 was significantly increased 24 hours after UV-B irradiation, we could not detect the protein expression of NLRP3.

4.2.4. Expression of inflammasome adaptor and enzyme in HCE-T cells after UV-B radiation

We also studied the effect of UV-B irradiation on the expression of ASC and caspase-1. Our results show that the mRNA expression of these proteins was decreased 6 hours after UV-B exposure and 24 hours after irradiation it remained at lower levels. The decrease of caspase-1 mRNA expression became significant compared to the mRNA expression level of non-irradiated cells.

4.2.5. Effect of UV-B irradiation on the expression of NLRC subtypes in HCE-T cells

We also aimed to study the effect of UV-B on the expression of NLRC subfamily members. We found that UV-B significantly decreased the mRNA expression of all studied NLRCs (NOD1, NOD2, NLRC5 and NLRX1) 6 hours after UV-B treatment. However, 24 hours after treatment the expression levels of NOD1 and NOD2 increased compared to the 6 hours treated cells, the mRNA levels of all studied NLRCs remained low compared to the non-treated cells. Our results show that in contrast to the NLRPs, NLRCs are downregulated early and long-term after UV-B treatment.

4.2.6. Cytokine secretion of HCE-T cells after UV-B irradiation

We studied how UV-B radiation effects the cytokine secretion of HCE-T cells. We measured the concentration of IL-1β, IL-6, IL-10, IL-12, IL-18 and TNFα in non-treated and UV-B-exposed cell supernatants. We found that only IL-6 secretion was detectable by ELISA. The secretion of this cytokine is continuous; moreover, UV-B irradiation increased the production of IL-6 (twofold) 24 hours after UV-B exposure. These results show that the observed changes of NLR expression are accompanied by the increased secretion of proinflammatory IL-6.

5. Discussion

Part 1

The main causer of seasonal allergic rhinitis is the ragweed pollen. One of the most important proinflammatory cytokines produced in allergic rhinitis is IL-1β. The production of IL-1β is due to the cleavage of caspase-1 enzyme in association with NLRP3 inflammasome activation. It has been suggested that intracellular ROS production contributes to the activation of NLRP3 inflammasome. It has also been described that ragweed pollen grains have intrinsic NADPH oxidase activity, which is able to generate ROS. The main sources of IL-1β are considered to be inflammatory cells, such as macrophages. Macrophages collected from asthma patients generate higher levels of ROS than in healthy controls. Based on the previous findings, we studied the effect of ragweed pollen on the NLRP3 inflammasome system on the THP-1 human monocytic cell line. It is known that direct contacts between the antigen presenting cells and pollen grains influence the activation of immune cells, which could be responsible for the adjuvant effects of intact pollen. Therefore, to determine the direct molecular effects of ragweed pollen components on macrophages we have used the extract of ragweed pollen to avoid the activating effect induced by the direct contact of pollen grains. Ragweed pollen extract have been shown to contain endogenous NADPH, which is the natural substrate of its NADPH oxidase. The pollen extract does not contain NADPH, therefore the use of pollen extract requires the exogenous addition of NADPH in our experiments to study the effect of pollen NADPH oxidase. As bacterial LPS is usually present naturally on the surface of pollen grains and it is also known that LPS is a robust activator of NLRP3 inflammasome, we treated cells with LPS as well. . It has been demonstrated that there is no release of active IL-1β when THP-1 cells are stimulated with LPS alone. However, when primed with PMA, THP-1 cells release significant amounts of IL-1β. In our experiments we differentiated THP-1 cells into macrophages by PMA, which offered the priming step for the IL-1β secretion. In good agreement with previous findings, our results show that LPS alone strongly induces the secretion of IL-1β in the PMA-primed cells, while neither RWE (in the absence or presence of NADPH) nor NADPH alone triggers the secretion of this cytokine. However, we found that in the presence of NADPH, RWE strongly induces the LPS-induced IL-1β secretion of macrophages and also of dendritic cells. It is in line with the observations that in the

presence of an endotoxin trigger pollen grains substantially exacerbate allergic symptoms.

Although various inflammasome complexes have been associated with IL-1β production, such as AIM2, NLRC4, NLRP1 or NLRP3 inflammasomes, in our previous studies we have shown that RWE alone and in the presence of NADPH and LPS has no effect on the expression of other inflammasome sensors.

It has been described that pollen induces ROS production in various epithelial cells and dendritic cells as well. However, there was no information about whether RWE is able to induce ROS generation in THP-1 macrophages. We found that RWE alone is able to cause a sustained exposure of THP-1 macrophages to ROS, in a good agreement with other studies that had shown long-term intracellular ROS production in pollen-treated alveolar epithelial cells. Still, in the case of LPS-treatment – in line with previous studies – we could not detect ROS generation. To determine whether the RWE-dependent enhancement of LPS-induced IL-1β production is mediated by the produced ROS, we pretreated THP-1 cells with the ROS-scavenger (NAC) or inhibitors of ROS production (MitoTempo, DPI). MitoTEMPO inhibits ROS production of the mitochondria, DPI inhibits ROS production of NADPH oxidase and also the mitochondria. Our results show that NAC completely abolished IL-1β secretion. These results indicate that ROS play a crucial role in LPS-induced and RWE-enhanced IL-1β production. Despite the fact that the main source of ROS in LPS-induced IL-1β production is thought to be of mitochondrial origin, our results show that while MitoTEMPO only moderately decreased IL-1β secretion, DPI treatment completely abolished it. Our findings suggest that the majority of the ROS involved in the RWE-dependent enhancement of LPS-induced IL-1β production is generated by pollen-derived NADPH oxidases. These results altogether show that ROS is required for the LPS-induced RWE-enhanced IL-1β production of THP-1 macrophages.

Recent studies have demonstrated that ROS do not directly induce the activation of inflammasome, but they are required for the priming step, which leads to the induction of the expression of inflammasome components and pro-IL-1β. In our system RWE-induced ROS may act as an additional signal for the secretion of mature IL-1β. Our findings verify this hypothesis, that RWE alone is not able to influence the expression of neither the key inflammasome members, nor pro-IL-1β. However, in the

presence of LPS, RWE strongly increases the LPS-induced expression of these proteins.

As it is known, caspase-1 is responsible for the secretion of IL-1β. Therefore we studied the role of caspase-1 in the RWE-enhanced LPS-induced IL-1β secretion. Using caspase-1 inhibitor we totally inhibited the secretion of IL-1β. This result suggests that caspase-1 has a crucial role in RWE-enhanced IL-1β production. We also found that RWE enhanced the LPS-induced pro- and cleaved caspase-1 protein expression. However, we found that while LPS significantly induced the activity of caspase-1, treatment of the LPS-primed cells with RWE did not result in further caspase-1 enzyme activity. These results appear to be contradictory, but the western blot technique detects the processed form independent of its activity, and it has been demonstrated that caspase-1 is rapidly inactivated in THP-1 cells leading to the accumulation of processed but inactive caspase-1.

Various signaling pathways are associated with LPS-mediated expression of NLRP3 inflammasome components and IL-1β. While RWE does not increase the expression of IL-1β, NLRP3 and pro-caspase-1 by itself, we found that RWE (in the presence of NADPH) induces the phosphorylation of p38 MAPK, JNK and AP-1 complex members c-Fos and c-Jun. However, co-treatment of LPS with RWE resulted in a markedly more intensive phosphorylation of these proteins. These results suggest that ROS-dependent enhancement of LPS-induced IL-1β production by RWE might be associated with p38 MAPK, JNK and AP-1 signaling pathways.

In summary, we described for the first time that in the presence of NADPH, RWE significantly increased LPS-induced IL-1β production of THP-1 macrophages as well as human primary macrophages and dendritic cells. We also demonstrated that elevated IL-1β production is mediated via NLRP3 inflammasome in THP-1 cells. We reported that RWE elevates cytosolic ROS generation in these cells, and ROS inhibitors abolish IL-1β production. Furthermore, we have shown that RWE enhances LPS-induced gene expression of pro-IL-1β and key components of the inflammasome, by a ROS-dependent mechanism produced by the NADPH oxidase of RWE. The observed cooperation of RWE and LPS suggests that bacterial endotoxin contamination has a crucial role in the ragweed pollen-induced seasonal allergic reactions. This should be taken into account when designing treatments for allergic airway inflammations. So far our study is the only one which demonstrates

associations between the NLRP3 inflammasome activation and ragweed pollen-induced ROS generation and the subsequent elevated IL-1β secretion, which is an important characteristic of allergic rhinitis.

Part 2

Besides macrophages, epithelial cells also have an important role on the immunological processes. Epithelial cells cover surfaces of the body, such as skin, airways, intestinal tract or the eyes and provide an important link between the outside environment and the interior body. While the ocular immune system is different from the others as the eye is an immunologically privileged site, it still actively protects the eye from infection and dangerous environmental effects. The major cell type of corneal immune system is the corneal epithelial cell type, which plays its role not only as a passive physical barrier, but it also actively secretes cytokines, antimicrobial peptides to activate microbial defense as first line of defense mediated by innate immunity. At the time of our studies the expression of several TLR family proteins were studied in human corneal epithelial cells and in human immortalized cell line, but there was no information about the expression or the possible roles of the NLR family in these cell types.

We demonstrated for the first time the mRNA expression pattern of various NLR molecules and the expression of key inflammasome components in human corneal epithelial cells. We also aimed to find a cell line which can be good model for NLR studies in corneal epithelial cells, therefore we demonstrated the differences of the gene expression pattern of NLRs between primary cells and epithelial cell line (HCE-T).

We found that the relative expression of NLRP1 is comparable in the HCE-T cell line and primary cells. In 2013, another research group also demonstrated the expression of NLRP1 in corneal epithelial cells. The expression of the NLRP3 gene was more than 20 times higher in the immortalized cell line than in the primary cells. In 2011, the mRNA expression of NLRP3 has also been described in corneal epithelial cells. It has been shown that fungi induce the expression of mRNA NLRP3.

We also observed that NLRP7 is only detectable at a very low level in HCE-T. We further found that the gene expression of NLRP10 is substantially higher in HCE-T cells than in the primary samples. We could not detect the gene expression of NLRP4, NLRP6 or NLRP12 neither in HCE-T nor in primary corneal cells, although the

corresponding gene expression assays were verified by using other cell types that expressed these genes.

We also aimed to detect the expression of NLRP1 and NLRP3 proteins in the HCE-T cells. We found a shorter NLRP1 isoform in HCE-T cell line. Furthermore, we could not detect the NLRP3 protein in HCE-T cells. Therefore HCE-T cells may not serve as a good model for NLRP3 or NLRP1 inflammasome studies.

Corneal epithelial cells play an important role in the host defense of retina. One of the major environmental stresses for cornea is the UV-B radiation. Cornea absorbs a large amount of UV-B radiation. UV-B radiation can cause photokeratitis, which is a painful inflammatory condition that leads to the damage of the cornea. The symptoms typically appear 6–12 hours after the exposure and resolve within 48 hours. In the long term, UV-B irradiation may increase the susceptibility to pathogens, which are responsible for the development of ocular pathological disorders. Keratinocyte cells in the skin are also important contributors of the first line of defense and they are also exposed to UV-B radiation. It has been published that keratinocyte cells express inflammasome members and the UV-irradiated keratinocytes secrete IL-1β through a caspase-1-dependent mechanism, but at the same time there was no information about the role of NLRs in the UV-B-initiated immune responses in human corneal epithelial cells. Therefore, we aimed to investigate the effect of UV-B irradiation on the expression of numerous NLRs in human corneal epithelial cells. We found that after UV-B treatment the expression of NLR family members is downregulated, in line with the immunosuppressive role of UV-B. These phenomena may increase susceptibility toward infection by pathogens. We also found that corneal epithelial cells do not secrete IL-1β in contrast to primary keratinocytes, but UV-B induces the secretion of another proinflammatory cytokine, IL-6. It has also been shown that HaCaT keratinocyte cell line does not secrete IL-1β in contrast to primary keratinocytes. In line with these findings we also found differences in NLRP gene expression between the primary corneal epithelial cells and the cell line.

We demonstrated that the expression level of NOD1 and NOD2 in HCE-T cell line was similar to that of primary cells, therefore we aimed to study whether HCE-T could serve as good cell line to model for NOD1/2 function in corneal epithelial studies. We demonstrated the functional activity of the NOD1/NOD2 system by increasing IκB phosphorylation upon specific agonist PGN stimuli in the cell line. Consequently, we established a good model system of corneal epithelial cells for

NOD protein studies in the eye, which are becoming particularly important. In recent studies it has also been described that corneal epithelial cells express functional NOD1 and NOD2. NOD1 stimulation is an important inducer of corneal neovascularisation and it can be a good target in the development of corneal neovascularisation therapies. It has also been demonstrated that NOD1 and NOD2 in corneal epithelial cells modulate the gene expression of antimicrobial peptide human β-defensin 9.

The expression and function of NLR family in corneal epithelial cells remain poorly investigated to date. Only a few articles were published about the studies of NLRs in corneal epithelial cells since our paper was accepted. We demonstrated for the first time the mRNA expression pattern of various NLR molecules and the expression of key inflammasome and signalosome components in primary human corneal epithelial cells and in the HCE-T cell line.

6. Summary

In our work we described for the effect of RWE on the LPS-induced IL-1β secretion of macrophages. We demonstrated that RWE (in the presence of NADPH) has no significant effect on the secretion of IL-1β, neither in the primary macrophages, dendritic cells nor on the macrophage cell line THP-1 cells, but it has robust increasing effect on the LPS-induced IL-1β production in both of these cells. We have shown that whereas RWE alone (but not NADPH) induced some ROS production, their combined effect yielded a continuously increasing ROS level. Using ROS inhibitors, we have shown that the majority of the ROS involved in the RWE-dependent enhancement of LPS-induced IL-1β production is generated by pollen-derived NADPH oxidases. We have also shown that the increased production of IL-1β is NLRP3 inflammasome-mediated and caspase-1-dependent. We reported that RWE augments the LPS-induced gene expression of pro- and cleaved IL-1β, and also the key components of NLRP3 inflammasome. We have also shown the increased protein expression of pro- and cleaved caspase-1 and IL-1β. We have also reported that RWE induces the phosphorylation of p38 MAPK, JNK, furthermore, the phosphorylation appears to be NADPH-dependent. RWE in the presence of NADPH substantially enhances the LPS-induced p38 MAPK and JNK phosphorylation. We have also demonstrated that RWE induces the phosphorylation of AP-1 complex members c-Jun and c-Fos, which are also important participants of signaling pathways involved in IL-1β expression. The observed cooperation of RWE and LPS suggests that bacterial endotoxin contamination has an important role in the ragweed pollen-induced allergic reactions.

In our second study we investigated the transcription pattern of Nod-like receptors and inflammasome components in primary human corneal epithelial cells and in HCE-T cell line. The transcription of NOD1, NOD2, NLRX1 and NLRP1 is similar in the primary cells and in the cell line. We found that the expression of NLRP3 and NLRP10 is higher in HCE-T cells, while ASC and caspase-1 show higher transcription levels in the primary cells. NLRC5 and NLRP7 are hardly detectable in any of these cell types. In our study we reported that while relatively short (6 hours) UV-B irradiation leads to the downregulation of both investigated NLRP and NOD mRNAs and also those of inflammasome components in HCE-T cells, longer incubation (24 hours) of the cells after UV-B exposure results in the recovery or

upregulation of only the NLRP sensors. We have presented a short isoform of NLRP1 protein, whose expression changes in a similar way as that of RNA. However, the protein expression of NLRP3 was not detectable. Our group also detected – among all of the studied cytokines – only the presence of IL-6 from the supernatant of HCE-T cells. The secretion level of IL-6 is only increased in the sample 24 hours after UV-B irradiation, with the increase being twofold. UV-B is not able to induce the secretion of IL-1β. We have demonstrated that NOD1 and NOD2 are functionally active in HCE-T cells, enabling us to establish that HCE-T is a good corneal epithelial model system for NOD protein studies in the eye. As this model is highly suitable for further investigations, therefore it opens a new frontier in the development of new therapeutic approaches targeting eye-related infectious diseases.

7. Publications

UNIVERSITY OF DEBRECEN
UNIVERSITY AND NATIONAL LIBRARY
PUBLICATIONS

Register number: DEENKÉTK/111/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Alíz Varga

Neptun ID: LKUMIN

Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. **Varga, A.**, Budai, M., Milesz, S., Bácsi, A., Tőzsér, J., Benkő, S.: Ragweed pollen extract intensifies LPS-induced priming of NLRP3 inflammasome in human macrophages. *Immunology. 138* (4), 392-401, 2013.
   DOI: http://dx.doi.org/10.1111/imm.12052
   IF:3.705 (2012)

2. Benkő, S., Tőzsér, J., Miklóssy, G., **Varga, A.**, Kádas, J., Csutak, A., Berta, A., Rajnavölgyi, É.: Constitutive and UV-B modulated transcription of Nod-like receptors and their functional partners in human corneal epithelial cells.
   Mol. Vis. 14 (187-188), 1575-1583, 2008.
   IF:2.464

H- 4032 Debrecen, Egyetem tér 1. ¤ E-mail publikaciok@lib.unideb.hu

List of other publications

3. Budai, M.M., **Varga, A.**, Milesz, S., Tőzsér, J., Benkő, S.: Aloe vera downregulates LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages.
Mol. Immunol. 56 (4), 471-479, 2013.
DOI: http://dx.doi.org/10.1016/j.molimm.2013.05.005
IF:2.645 (2012)

Total IF of journals (all publications): 8.814

Total IF of journals (publications related to the dissertation): 6.169

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

16 May, 2014

Oral presentations

Aliz Varga: Nod-like receptors (NLRs) in epithelial cells. 4th Molecular Cell and Immune Biology Winter School, Galyatető, Hungary, January 2011.

Aliz Varga, Marietta Margit Budai, Réka Albert, Éva Rajnavölgyi, Goran Petrovski, József Tőzsér, Szilvia Benkő: Comparison of Nod-like receptors in limbal stem cell derived corneal epithelial cells and in immortalized corneal epithelial cell. „Fókuszban az őssejt-kutatás" - Az őssejt kutatás kardiovaszkuláris vonatkozásai, Debrecen, Hungary, March 2011.

Aliz Varga: Ragweed pollen extract generated ROS enhances LPS-induced IL-1βsecretion via NLRP3 inflammasome in human macrophages. 5th Molecular Cell and Immune Biology Winter School, Galyatető, Hungary, January 2012.

Poster presentations

Aliz Varga, Marietta Margit Budai, Attila Bácsi, József Tőzsér, Szilvia Benkő: Ragweed pollen extract generated ROS enhances LPS-induced IL-1β secretion via NLRP3 inflammasome in human macrophages. 16th International Summer School on Immunology, Hvar, Croatia September 3-11, 2011

Varga Aliz, Budai Marietta Margit, Bácsi Attila, Tőzsér József, Benkő Szilvia: A parlagfű pollenkivonat által termelt ROS az NLRP3-inflammoszómán keresztül fokozza az LPS-indukálta IL-1β szekréciót humán makrofág sejtekben. Conference of Hungarian Society for Immunology, Kecskemét, October 2011.

Varga Diána, **Varga Aliz**, Budai Marietta Margit, Bácsi Attila, Tőzsér József, Benkő Szilvia: A parlagfű pollen kivonat IL-1β expresszióra kifejtett hatása humán THP-1 makrofág sejtekben. A Magyar Élettani Társaság, a Magyar Anatómusok Társasága, a Magyar Biofizikai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa, Debrecen, Hungary, June 2012.

Aliz Varga, Marietta Margit Budai, Attila Bácsi, József Tőzsér, Szilvia Benkő: Ragweed pollen extract enhances LPS-induced IL-1β secretion via NLRP3 inflammasome in human macrophages. European Macrophage and Dendritic Cell Society 2012 Meeting, Debrecen, September 1-3, 2012

Aliz Varga, Marietta Margit Budai, László Csernoch, József Tőzsér, Szilvia Benkő: Effect of ragweed pollen extract on the IL-1β expression of macrophages and dendritic cells. Conference of Hungarian Society for Immunology, Pécs, Hungary, October 2013.

8. Keywords

Nod-like receptors, NLRP3 inflammasome, IL-1β production, reactive oxygen species, ragweed pollen, NADPH oxidase, macrophages, corneal epithelial cells, UV-B

9. Acknowledgements

I would like to render my special thanks to my supervisor, Dr. Szilvia Benkő for leading my journey through the world of research, supporting me in my efforts in mastering the science of molecular biology, being a role model in persistence and precision both inside and outside the laboratory. Thank you very much.

I would like to thank Prof. József Tőzsér for the professional advice he provided throughout many years. I would like to thank Prof. László Csernoch for making it possible for me to reach my professional goals at the Department of Physiology. I am also grateful to Prof. Éva Rajnavölgyi for enabling me to do my previous research at the Institute of Immunology.

I would like to thank my colleague and friend Marietta Margit Budai for the help she provided all the way through our laboratory duties.

I am also thankful to the whole staff of Department of Immunology and Department of Physiology for the supporting and friendly environment. I also would like to thank all of our collaborative partners for their help and excellent work.

And last, but not least I am sincerely grateful to my family and to my dear friends helping me get through the difficult times, and for their emotional support and encouragement.