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

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DEBRECEN, 2014

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1. Introduction

Part 1

Nucleotid-binding oligomerization domain-like receptors (Nod-like receptors or 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), such as bacterial, viral or fungal-derived motifs, ultraviolet (UV)-irradiation, uric acid, asbestos, silica, etc. It is unlikely that these various kinds of activator agents are directly detected by NLRP3 inflammasomes. There are several theories about the mechanism of NLRP3 inflammasome activation, such as disruption of lysosomal membranes and the release of cathepsin B, furthermore potassium efflux. It is also 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 interleukin-1β (IL-1β). This cytokine has an important role in various diseases, such as the endemic allergic rhinitis. The main producers of IL-1β that are implicated in this disease are macrophages. Allergic rhinitis is mainly caused by ragweed pollen. It has been described that ragweed pollen grains and their extracts have intrinsic NADPH oxidase activity, which has an important role in the protection against pathogens and regulates the expansion of cells in root hairs. When inhalating of pollen grains, pollen-NADPH oxidase activity induces oxidative stress in the airway epithelium by initiating ROS production, which is an important contributor to the manifestation of the allergic inflammation. It has been shown that concomitant exposure to pollen grains and endotoxin such as bacterial lipopolysaccharide (LPS) triggers substantially stronger allergic inflammation in patients as compared to that caused by allergen exposure alone. In our work we aimed to study whether ragweed pollen influences the function of NLRP3 inflammasome and the IL-1β production in 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. They protect the eye not only from microbial infection, but from environmental stresses such as UV-induced photodamage as well. UV-B irradiation may increase the susceptibility to pathogens responsible for the development of ocular pathological disorders. PRRs sense microbial structures; in addition they are activated by dangerous environmental stresses, like UV-B radiation. At the time of our studies on corneal epithelial cells, the expression of Toll-like receptors (TLRs) was described in human corneal epithelial cells and in human immortalized cell lines, but there was no information about the expression and the possible roles of NLR family in these cell types. Therefore we aimed to show the mRNA expression pattern of NLRs and key inflammasome components in 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, as the innate immune cells defend the host from invading microorganisms in a non-specific manner, like secreting soluble factors (e.g. cytokines, antimicrobial peptides, complement factors), and it also activates the adaptive immune system through a process known as antigen presentation. The innate immune system provides immediate defense against pathogens, the adaptive immune system ensures long-lasting and antigen-specific immunity to the host [1].

The innate immune system exists in all classes of plants and animals. It is the dominant immune system of plants, fungi, insects and primitive multicellular organisms. The first appearance of the adaptive immune system dates back to the first vertebrates. [2].

The rapid recognition of danger signals by the innate immune system is critical for the generation of proper adaptive immune responses. Danger signals from evolutionarily conserved pathogen- or danger-associated molecular patterns (PAMPs and DAMPs) are recognized by pattern recognition receptors (PRRs) situated on the surface or in the cytosolic compartments of host cells [3], furthermore PRRs may also be secreted by the producer cells.

2.1.1. Soluble pattern recognition receptors

Certain PRRs are secreted by the cell that produces them. These PRRs are referred to as soluble PRRs.

Mannose-binding lectin is a serum protein secreted by the liver. It is able to recognize certain sugar groups on the surface of microorganisms and activate the lectin pathway of complement activation [4, 5].

Pentraxins are another group of soluble PRRs. C-reactive protein (CRP) and serum amyloid P component are pentraxins in humans and mice [6]. CRP was the firstly identified innate immunity receptor. It is secreted mainly by the liver and found in the blood during inflammatory conditions [7]. The immunological role of CRP is to bind to phosphocholine expressed on the surface of dead or dying cells besides certain types of bacteria that subsequently leads to the activation of the complement system [8].

Antimicrobial peptides have an important role in the killing of bacteria, enveloped viruses, fungi, protozoans and other parasites. Recently it has also been described that antimicrobial peptides have many properties as potential antibiotics of therapeutic use for these microbial infections [9]. In mammals the three important classes of antimicrobial peptides are the defensins, the cathelicidins, and the histatins [10].

2.1.2. Membrane-bound pattern recognition receptors

A wide range of conserved PRR families have been described that remain associated with the producing cells. These are located either on the cellular surface or in endosomal compartments [11].

a, Toll-like receptors

Toll-like receptors (TLRs) are key molecular sensors of the innate immune system in recognizing various PAMPs such as glycolipids, proteins and nucleic acids, that have been taken into cells by phagocytosis, receptor-mediated endocytosis or macropinocytosis [12]. The role of Toll gene in innate immune responses was first described in *Drosophila melanogaster* in 1996 [13]. In 1998 TLR4 has been identified as the elusive mammalian receptor that detects lipopolysaccharide (LPS) [14] which is the cell wall component of Gram-negative bacteria. Following the characterization of TLR4, proteins that are structurally related to TLR4 were identified and named Toll-like receptors [15]. The importance of TLRs is highlighted by the Nobel Prize in Physiology or Medicine in 2011 being awarded to Dr Jules A. Hoffmann and Dr Bruce A. Beutler for their discoveries of the role of Toll-like receptors.

TLRs are type I integral membrane glycoproteins consisting of ligand-binding ectodomains, single transmembrane helix and cytoplasmatic signaling domains. The cytoplasmic part of TLRs, the Toll/IL-1 receptor domain (TIR) [16] shows a high degree of similarity to that of the IL-1 receptor family. The ligand-binding ectodomains contain approximately 19-25 leucin-reach repeats (LRR). The ligand sensing of TLRs triggers activation of signaling pathways [17-19] and the most important consequences of TLR activation are the production of proinflammatory milieu, and the cellular activation in response to microbes.

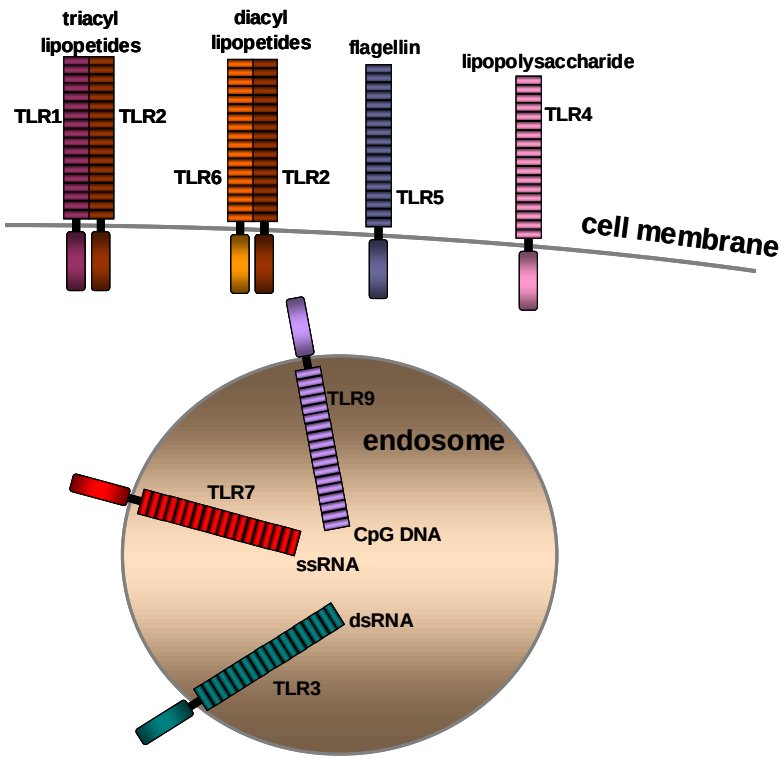

Figure 1. TLRs and their ligands

10 functional Toll-like receptors (TLR1-10) have been identified in humans [20, 21] and 13 in mice (TLR1-13). Most of the TLRs are expressed on immune cells, including macrophages, dendritic cells, B and T cells, but they have been detected on non-immune cells such as epithelial cells [22]. 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 [11] (Figure 1). Recently a new group of TLRs has been described including TLR11, TLR12 and TLR13, which are located within endosomal compartments. Although all of these TLRs are expressed in mice, human TLR11 is a non-functional pseudogene and humans completely lack TLR12 and TLR13 [23].

b, Dectins

Dectin-1 and Dectin-2 are type II transmembrane proteins belonging to the C-type lectin family. They are predominantly expressed in macrophages [24, 25] and dendritic cells [25]. Dectin-1 recognizes β-glucans, whereas Dectin-2 recognizes α-mannans. Both β-glucans and α-mannans are major cell wall components of fungi, therefore dectins have an important role in host defense against fungal infection [26]. Ligation of dectins induces intracellular signaling and mediates a variety of cellular responses such as cytokine production [27].

2.1.3. Intracellular pattern recognition receptors

While transmembrane TLRs and dectins have an important role in the detection of pathogens from the extracellular milieu or the lumen of membrane-enclosed intracellular compartments, intracellular pattern recognition receptors play substantial roles in the sensing of both the intracellular and the extracellular danger-signals. One group of the intracellular pattern recognition receptors is the Nod-like receptor family, which will be described in more details in the following chapters (2.2.). Another well studied group is the RIG-like helicases. Intracellular dsRNA is a common replication intermedier of many viruses. TLR3 is known as an endosomal membrane-bound receptor for dsRNA [28]. RIG-like helicase family has also been identified to be involved in the recognition of dsRNA. RIG-I belongs to the RIG-like helicases, because it has a helicase domain with intact ATPase activity responsible for the dsRNA-mediated signaling. RIG-1 also has caspase recruitment domain (CARD) [29] which transmits the dsRNA-induced downstream signals, leading to the activation of NF-κB and IRF-3 [30]. Another member of RIG-like helicases is melanoma differentiation-associated gene 5 (MDA5), which is also specialized in detecting viral RNAs in infected cells. While RIG-I is activated by 50-triphosphate containing RNAs as well as short (<2 kbp) dsRNAs, it is suggested that MDA5 recognizes long dsRNAs [31]. Upon ligand sensing, RIG-I and MDA5 interact with mitochondrial-antiviral signaling protein (MAVS), which leads to the expression of type-1 interferons (IFNs). Type-1 IFNs have an important role in immune responses against viral infection.

2.2. Nod-like receptors

In 1994 Whitham et al. reported new types of plant proteins which are responsible for the recognition of invading pathogens [32]. These proteins were named plant resistance proteins or R-proteins. R-proteins consist of an amino-terminal domain, a central nucleotide-binding domain (NBD) and an LRR domain at the carboxyl terminus. Almost 15 years ago a new PRR family was identified in vertebrates with similar tripartite domain structure than that of the R-proteins and TLRs. Proteins belonging to this PRR family are the nucleotide-binding and oligomerization domain (Nod)-like receptors. These were first referred to as CATERPILLER family, later in 2006 their naming changed to Nod-like receptors (NLRs) [33, 34].

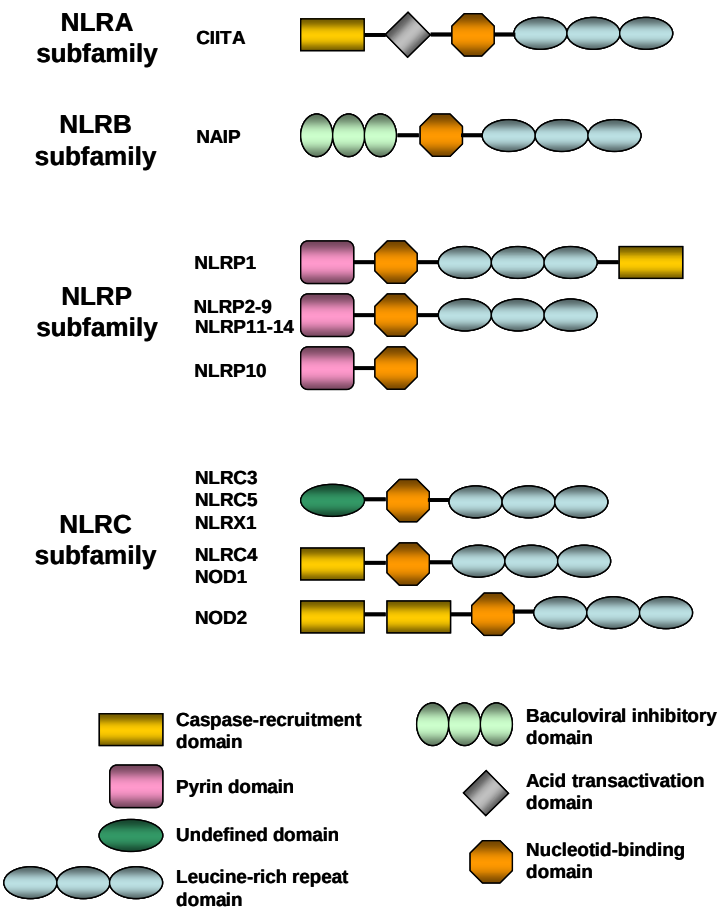

Figure 2. The Nod-like receptor family

In NLRs the C-terminal LRR domain senses danger signals and autoregulates the NLR activity. The centrally located 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: the acidic transactivation domain containing NLRA subfamily, the baculoviral inhibitory domain containing NLRB subfamily, the pyrin domain (PYD) containing NLRP (Nod-like receptor family pyrin domain containing protein) subfamily, and the CARD domain [29] or undefined domain containing NLRC subfamily (Figure 2).

It has been described that a range of NLRs evolved at a very early stage in animal evolution. It has been supposed that a common metazoan ancestor might have used nucleotide-binding domain proteins for cytoplasmic defense as well [35]. Recently 23 members of the Nod-like receptor family have been identified in humans, while more than 30 in mice [21].

2.2.1. NLRC subfamily

NLRC or NOD proteins are members of the NLRC subfamily of NLR family. These proteins consist of N-terminal typical or undefined domain, NBD and LRR domains (Figure 2).

a, NOD1, NOD2

NOD1 (also called NLRC1) contains a single CARD domain, while NOD2 (also called NLRC2) has two CARD domains. NOD1 senses the iE-DAP dipeptide and Tri-DAP, which are found in peptidoglycan (PGN), a cell wall component of all Gram-negative and certain Gram-positive bacteria. NOD2 recognizes MDP (MurNAc-L-Ala-D-isoGln, also known as muramyl dipeptide), which is the minimal bioactive PGN motif common to all bacteria and the essential structure required for adjuvant activity in vaccines (Figure 3) [36, 37].

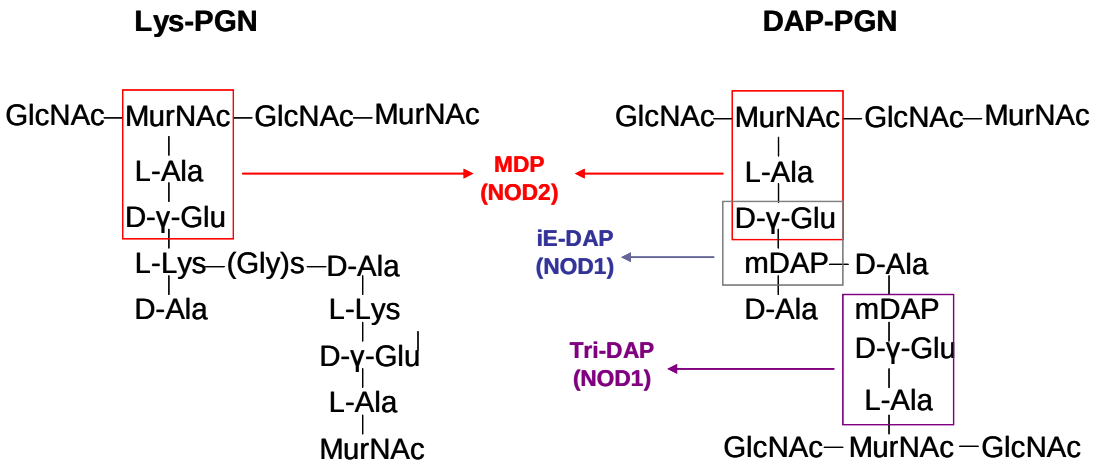

Figure 3. Schematic structure of Lys-PGN and DAP-PGN

Upon activation of these NLR proteins by their specific ligands, the oligomerization of the NBD domain initiates the binding of serine/threonine protein kinase RIP2 (receptor-interacting protein-kinase) adaptor protein via the CARD domain. This protein complex is referred to as NODosome in some articles (Figure 4).

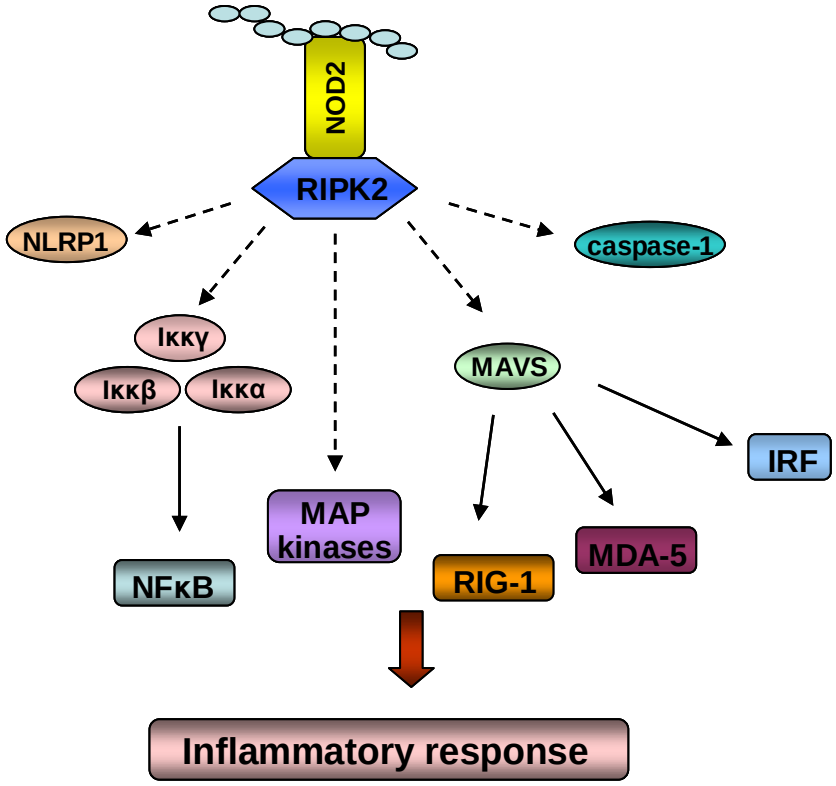

Figure 4. Signaling pathways via NODosome

After the assembly of NODosome, RIP2 has an important role in NFκB activation induced by NOD1 and NOD2. Moreover, the stimulation of NODosomes leads to MAPK activation and results in the activation of p38, ERK and JNK signaling pathways as well. These pathways together upregulate the expression of proinflammatory cytokines, therefore are able to stimulate both innate and adaptive immune responses

NOD1 and NOD2 are associated with severe diseases. NOD1 gene polymorphisms may be associated with several types of malignancies, including gastric, ovarian, prostate and lung cancer as well as lymphoma [38]. NOD2 polymorphisms have been associated with different kinds of human inflammatory disorders, including Crohn's disease, Blau syndrome, early-onset sarcoidosis, atopic diseases and asthma [39, 40].

b, NLRC5, NLRX1

NLRC5 is the largest member of the NLR family, consisting of 1866 amino acids with C-terminal 27 LRRs. This protein has an atypical CARD domain [41].

NLRC5 is a cytosolic protein, but after activation it was found to shuttle between the cytosol and the nucleus [42]. It has been described that NLRC5 is important in the regulation of MHC-I expression, accordingly acts as ”CITA” [43, 44]. It enhances MHC-1 expression,

by association and activation of the promoters of MHC class I genes [45]. NLRC5 expression is induced by TLR ligands, such as LPS and poly(I:C), interferons, or viral infection, but the most prominent effect is observed with IFN-γ. The role of NLRC5 in regulating innate immune signaling has been controversial. Studies reported that NLRC5 negatively regulates NF-κB and type I interferon signaling while other studies suggested a positive function in virus-induced type I interferon response. It has been suggested that the role of NLRC5 in regulating innate immune signaling seems to be highly cell type and context dependent.

Another protein of the NLR family belonging to the NLRC subfamily is NLRX1 (also known as NOD5). It is possible that the NBD domain of NLRX1 has uncommon ATP-binding motif.

NLRX1 is the first NLR protein that has been described to interact with MAVS. The literature is discrepant about the function of NLRC5 in the regulation of MAVS-dependent antiviral responses. It has been shown that silencing of NLRX1 with siRNA promotes virus-induced type I IFN production and decreases viral replication [46]. In contrast to these findings it has also been demonstrated that NLRX1 has an augmenting effect on virus-induced autophagy [47]. Nevertheless, another study reported that wild type and NLRX1-deficient mice exhibited unaltered antiviral and inflammatory gene expression [48]. It is also demonstrated that NLRX1 negatively regulates the TLR-mediated NFκB activation upon LPS treatment [49]. Furthermore, it is known that NLRX1 augments reactive oxygen species (ROS) production from the mitochondria following *Chlamydia trachomatis* infections [50], therefore it is possible that NLRX1 contributes to the link between mitochondrial ROS generation and innate immune responses against bacterial infections.

2.2.2. Inflammasomes

Upon activation by danger signals during infections, tissue damage and metabolic imbalances (Figure 5), a subgroup of NLRs and some non-NLR proteins can form multiprotein complexes. These complexes are called inflammasomes, which were described for the first time by the team of Prof. Jürg Tschopp in 2002 [51].

Inflammasome	Ligands or triggers
NLRP1	muramyldipeptide (MDP) anthrax lethal toxin
NLRP2	ATP, LPS, IFNγ, IFNβ
NLRP3	bacterial and viral compounds, ROS, crystals, alum adjuvant, etc.
NLRC4	flagellin
NLRP6	peroxisome proliferator-activated receptor (PPAR)γ agonist, bacterial compounds
NLRP7	microbial acetylated lipopeptides
NLRP12	*Yersinia pestis*, LPS
AIM2	dsDNA

Figure 5. The main activators of inflammasomes

The inflammasomes consist of a sensor molecule, an adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) [52] and an effector caspase-1 enzyme. After the sensor protein is activated, it connects to the PYD domain of ASC adaptor, subsequently ASC interacts with the pro-caspase-1 enzyme via CARD domain. This mechanism initiates caspase-1 self-cleavage and the formation of active caspase-1. The active caspase-1 enzyme of the inflammasome proteolytically cleaves pro-IL-1β and 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, with the latter being essential for its association with caspase-5 in humans. The NLRP1 inflammasome was the first member of the inflammasomes to be discovered. NLRP1 inflammasome in humans consisted of NLRP1 sensor, caspase-1 and caspase-5 (not present in mice) enzymes, and ASC adaptor protein (Figure 6).

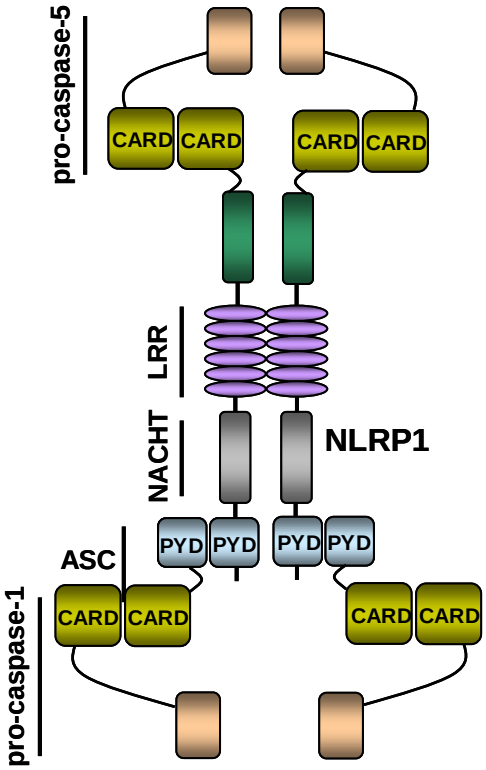

Figure 6. Schematic structure of NLRP1 inflammasome

One of the most known ligands of NLRP1 inflammasome is the anthrax lethal toxin, which is secreted by the bacterial pathogen *Bacillus anthracis*. The NLRP1 inflammasome activation is required for toxin-induced IL-1β release [53]. Another study shows that the assembly of the NLRP1 inflammasome is sufficient to initiate pyroptosis (caspase-1-dependent programmed cell death) [54]. Therefore it is suggested that NLRP1 has an important role in the regulation of survival-apoptosis balance. It has also been reported that NLRP1 is able to respond to NOD2 ligand MDP as well [55]. MDP-sensing induces the association of NOD2 with NLRP1 and the formation of a complex consisting of NOD2, NLRP1 and caspase-1 [56].

The gene polymorphisms of NLRP1 are implicated in the enhanced activity of the inflammasomes, leading to the risk of several autoimmune diseases, including vitiligo, type 1 diabetes, Addison disease [57], rheumatoid arthritis [58] and Alzheimer's disease [59]. It is also hypothesized that variants in NLRP1 gene might be involved in the susceptibility to autoimmune thyroid disease [60] and leprosy [61].

b, NLRP2 inflammasomes

The 1062 amino acid NLRP2 protein contains an N-terminal 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, including TNFα and IL-1β [62]. In addition, NLRP2 is also found to inhibit NFAT and AP-1 transcription factors [63]. It is also described, that NLRP2 enhances the activation of caspase-1 [64]. The mechanism how NLRP2 affects inflammasome activation is not entirely understood, but it is known, that besides LPS, IFNγ and IFNβ [64], ATP [65] also activates NLRP2. It is hypothetized that NLRP2 is able to regulate the expression of key NLRP3 inflammasome components as well [66].

Meyer et al. reported that a frameshift mutation in exon 6 of NLRP2 is associated with Beckwith-Wiedemann syndrome, which is a fetal overgrowth and human imprinting disorder [67].

c, NLRP6 inflammasomes

The 892 amino acid NLRP6 protein has an N-terminal PYD domain, a central NBD domain, and a C-terminal domain containing at least 5 LRR motifs. NLRP6 is a yet poorly characterized member of the NLR family, since little is known about the function of this protein. It is assumed that NLRP6 is an important player in the maintenance of intestinal homeostasis, because it restricts commensal bacteria through promoting IL-18 secretion. It is also demonstrated that NLRP6-deficient mice developed significantly larger and more tumors compared to wild-type mice [68]. The known agonists for NLRP6 inflammasomes are the nuclear receptor PPAR (peroxisome proliferator –activated receptor) γ agonist rosiglitazone [69] and LPS.

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 IL-1β secretion. There are several possible mechanisms on the inhibitory role of NLRP7, including direct interaction with pro-caspase-1 and pro-IL-1β which might prevent their activation or assembly of inflammasome, impairing transcription of pro-IL-1β and modulating the trafficking and release of IL-1β [70]. However, there is also evidence for a

pro-inflammatory role of NLRP7. It has been described that NLRP7 is activated by microbial acylated lipopeptides.

Mutations in NLRP7 gene (19q13.42) are associated with *Hydatidiform mole*. The *Hydatidiform mole* predisposes women towards molar pregnancy and may develop into a choriocarcinoma, an abnormal human pregnancy with no embryo and cystic degeneration of placental villi [71]. It has been described that NLRP7 alters DNA methylation and accelerates trophoblast lineage differentiation. Accordingly, NLRP7 may play a role in chromatin reprogramming and DNA methylation in the germline or early embryonic development [72].

e, NLRP12 inflammasomes

NLRP12 is a 1061 amino acid protein that has an N-terminal PYD domain, a central NBD domain, and a C-terminal domain with at least 12 LRR motifs. NLRP12 is mainly expressed in dendritic cells, neutrophils and monocytes/macrophages [73, 74]. The literature is discrepant about the role of NLRP12 in innate immunity. Both inflammatory and anti-inflammatory functions have been assumed. NLRP12 is an important player in the maintenance of intestinal homeostasis. It has been demonstrated that NLRP12-deficient mice are highly resistant to *Salmonella typhimurium* infection, which is a leading cause of food poisoning worldwide. The *Salmonella*-infected macrophages induce NLRP12-dependent inhibition of NFκB and ERK activation by suppressing phosphorylation of IκBα and ERK. Accordingly, NLRP12 can be a negative regulator of innate immune responses during *Salmonella* infection [75].

Mutations of NLRP12 are linked to hereditary inflammatory disease [76], and may lead to increased caspase-1 activity leading to increased inflammasome activation. [77]. In addition, a single nucleotide polymorphism in intron 9 of the protein is associated with atopic dermatitis [52].

f, NLRC4 inflammasomes

The full-length 1024 amino acid NLRC4 (also known as IPAF) contains CARD domain, NBD domain and 4 LRRs. NLRC4 is a member of the NLRC subfamily of NLRs. In contrast to NLRP proteins NLRC4 interacts directly with pro-caspase-1 through a CARD–CARD interaction. NLRC4 is mainly expressed in hematopoetic cells, macrophages, dendritic cells and neutrophils.

NLRC4 has been shown to be responsible for caspase-1 activation in response to bacterial flagellin. After macrophage infection with *Pseudomonas aeruginosa*, the activation of caspase-1 leads to the secretion of IL-1β. Macrophages deficient in NLRC4 or caspase-1 are markedly more resistant to *P. aeruginosa*-induced release of IL-1β [78].

g, AIM2 inflammasomes

The PYHIN (pyrin and HIN domain-containing protein) family member absent in melanoma 2 (AIM2*)* does not belong to the NLR family. 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 adaptor ASC leading to the activation of caspase-1 and consequently the cleavage of pro-IL-1β and pro-IL-18 to active cytokines (Figure 7).

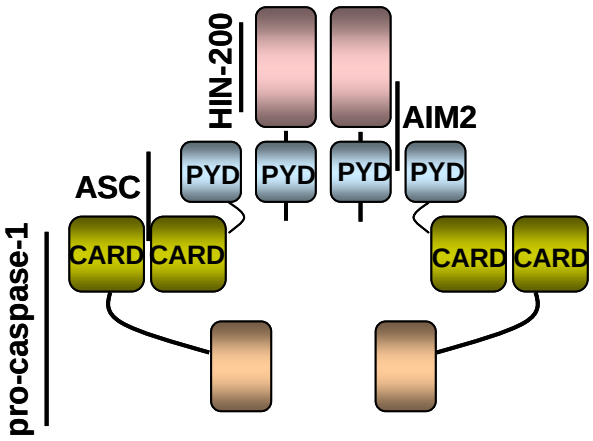

Figure 7. Schematic structure of AIM2 inflammasome

AIM2 inflammasomes are associated with severe diseases. Increased AIM2 expression has been demonstrated in keratinocytes in psoriatic lesions but not in healthy skin [79]. It has been shown that AIM2 facilitates the apoptotic DNA-induced systemic lupus erythematosus through macrophage functional maturation [80].

2.3. NLRP3 inflammasomes

One of the most widely studied member of the NLR family is the NLRP3, which was initially described as an activator of caspase-1 in 2002 [81]. NLRP3 is a 1036 amino acid protein contains a PYD domain, an NBD domain and 9 LRRs. The NLRP3 inflammasome complex contains NLRP3 sensor, ASC adaptor, and caspase-1 effector enzyme (Figure 8).

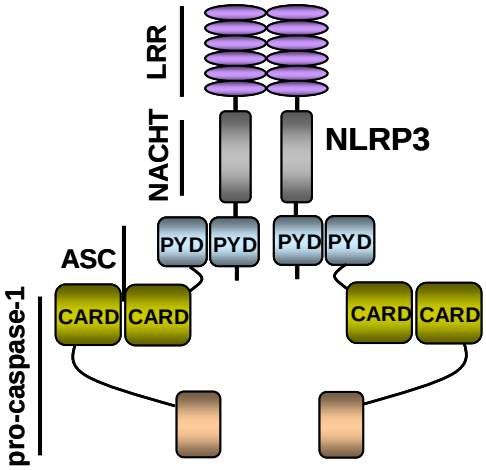

Figure 8. Schematic structure of NLRP3 inflammasome

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 maladiesF including chronic inflammatory diseases (such as Muckle-Wells syndrome, chronic infantile neurological cutaneous and articular syndrome), autoinflammatory diseases (such as familiar cold autoinflammatory syndrome, Familial Mediterranean Fever, pyoderma gangrenosum and acne), cancer predisposition and metabolic diseases (such as gout, type 2 diabetes, obesity-induced insulin resistence, atherosclerosis, intestinal inflammation) [82] (Figure 9). Therefore this is evident that the proper regulation of NLRP3 inflammasome activation is of high importance.

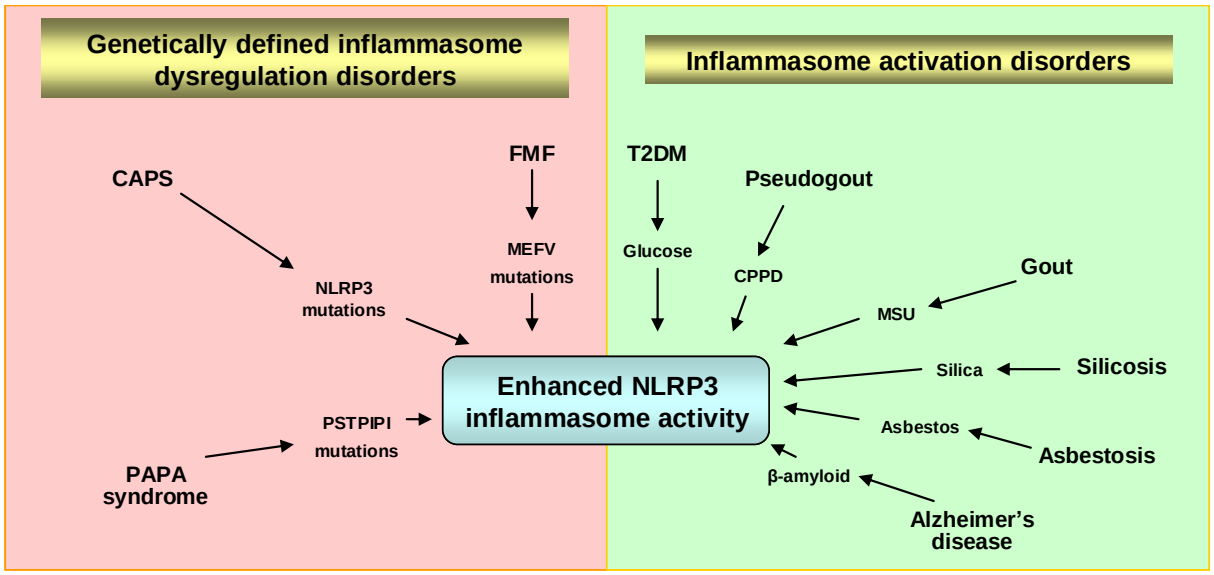

Figure 9. NLRP3-related diseases

The activation of NLRP3 inflammasomes is triggered by a wide range of danger signals derived from microorganisms, such as bacterial or viral RNA [83, 84], bacterial MDP, LPS [85], and non-microbial agenst such as ATP, monosodium-urate, calcium pyrophosphate dehydrate crystals and amyloid, UV-B radiation, etc. However, the mechanism of the activation of NLRP3 inflammasome remains unknown. It is unlikely that these different kinds of activator agents can be directly detected by NLRP3 inflammasomes. It has been suggested that secondary messengers are required in the signaling mechanisms of inflammasome activation. These messengers are commonly activated in response to the NLRP3 ligand sensing [86-88].

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

2.3.1.1. IL-1β as a 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. IL-1β is a potent proinflammatory cytokine, which is involved in many inflammatory conditions including autoinflammatory and allergic disorders. In these diseases, such as bronchial asthma, contact hypersensitivity and atopic dermatitis, dysfunctional inflammasome activity has been demonstrated to account for the IL-1β-induced inflammation [89]. Blood monocytes, tissue macrophages and dendritic cells are the primary sources of IL-1β. This cytokine is able to elicit fever after peripheral administration, therefore it is called ”endogenous pyrogen”. IL-1β regulates inflammatory

responses by supporting T-cell survival, upregulates the IL-2 receptor on lymphocytes, increases the antibody production of B-cells and promotes B-cell proliferation and T-helper 17 cell differentiation. It is also known that IL-1β secretion is a powerful stimulus for leukocyte recruitment. Moreover, IL-1β induces β-cell death, which cells have an important role in type 2 diabetes [90]. It has also been described that cancer cells directly produce IL-1β or induce cells within the tumor microenvironment to do so [91-93]. High IL-1β concentrations within the tumor microenvironment are associated with a more virulent tumor phenotype.

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

IL-1β is a potent multifunctional and pivotal proinflammatory cytokine, subsequently the production and the secretion of this cytokine have to be strictly regulated. Based on the current dogma the production of IL-1β requires two signals (Figure 10).

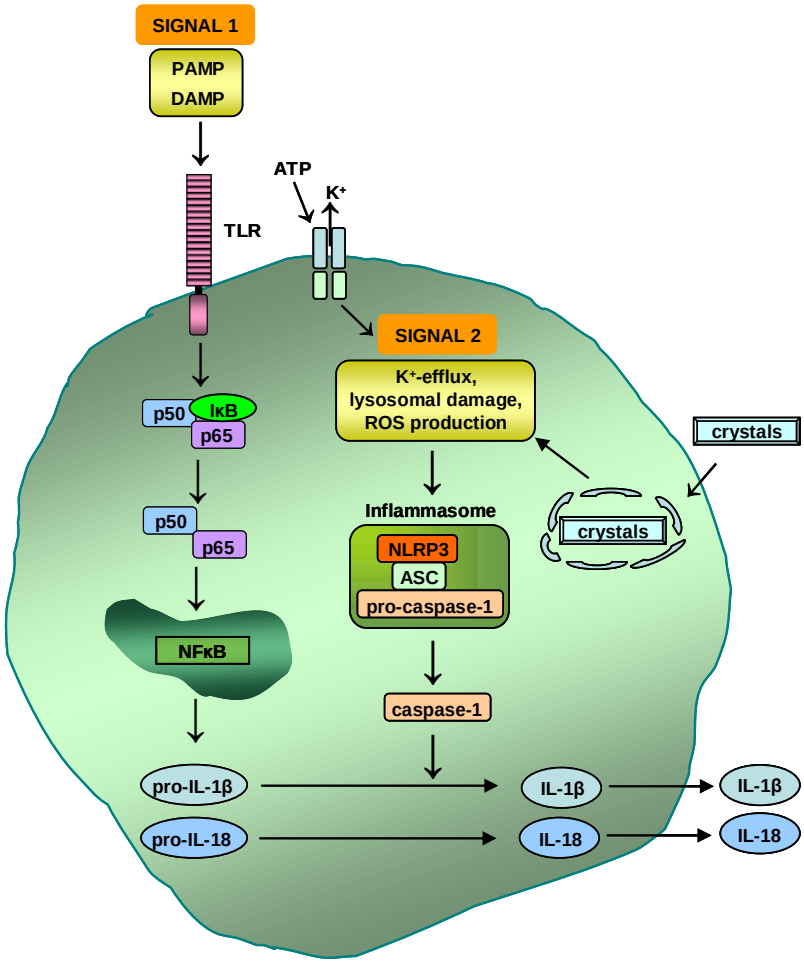

Figure 10. The activation model of NLRP3 inflammasome

The first signal, referred to as ”priming” is necessary for the induction of pro-IL-1β (and inflammasome members) mRNA expression in a NFκB-mediated process through TLRs [94]. It has been described that most PAMPs, which are known NLRP3 activators appear to be required only for the priming step [95]. In consequence of this step, IL-1β is synthesized as an inactive 35 kDa precursor (pro-IL-1β) [96]. The second signal is required for inflammasome assembly and a subsequent cleavage of pro-IL-1β into mature IL-1β by caspase-1. This second signal is frequently associated with the production of ROS or endosomal rupture [86, 87]. Changes in intracellular and extracellular calcium [97] and potassium efflux [98] also play an important role in the activation of NLRP3 [99, 100], but the regulatory mechanism of NLRP3 inflammasome by these DAMPs is still poorly understood. It is possible that the alteration in ion concentrations from normal conditions triggers the inflammasome activation. It is also a possibility that the changes in cellular redox state can regulate NLRP3 activation. Post-translational modifications of NLRP3 [101], ubiquitination and deubiquitination [102] also have been reported to regulate the NLRP3 inflammasome activation. Autophagy, which is required for recycling damaged proteins, has been shown to inhibit NLRP3 activation.

The activation of NLRP3 inflammasomes consists of three main steps: 1., the detection of the danger signal by sensor, 2., subsequent oligomerization of the inflammasome members, 3., activation of the effector enzyme [100]. The effector enzyme is the caspase-1 which cleaves pro-IL-1β protein to mature IL-1β. For this reason caspase-1 is also called as IL-1β-converting enzyme (ICE). Caspase-1 is a cysteinyl aspartate-specific protease synthesized as a zymogen with specificity for aspartic acid. The enzyme is composed of 10 (p10) and 20 kDa (p20) subunits. These subunits are derived from a 45 kDa precursor protein (p45) by an autocatalytic cleavage [103, 104]. The active site of caspase-1 is comprised of amino acid residues from both p10 and p20 polypeptides [105]. YVAD is the specific recognition sequence of caspase-1. A widely used caspase-1 specific inhibitor Z-YVAD-FMK is a synthetic peptide that irreversibly inhibits caspase-1 activity.

Similarly to IL-1β, the secretion of IL-18 is also mediated by the cleavage of caspase-1. However, the regulation of the transcription and translation of IL-18 appears to be distinct from that of IL-1β. While the secretion of IL-1β is regulated both at transcription and translation levels, IL-18 mRNA expression appears to be constitutive and the secretion of IL-18 is mainly regulated at protein level in stimulated conditions. Both IL-1β and IL-18 are secreted by an unconventional secretion pathway from the cells [106], because unlike other secreted proteins, pro-IL-1β and pro-IL-18 lack a hydrophobic leader sequence and are not found in organelles composing the classical secretory pathway. While the mechanism of

secretion of IL-1β and IL-18 is unknown, several non-conventional routes of secretion have been proposed including exosome shedding, shedding of plasma membrane microvesicles and lysosomal secretion.

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

Recent findings suggest that most identified NLRP3 activator agents induce the production of ROS, which are required as secondary messenger molecules in the signaling mechanisms of inflammasome activation. It has also been shown that antioxidants inhibit the activation of NLRP3 inflammasomes. These findings suggest that ROS play an important role in the NLRP3 inflammasome activation. ROS may directly trigger inflammasome activation or they may be indirectly sensed through cytoplasmic proteins that mediate the inflammasome assembly. Thioredoxin (TRX)-interacting protein (TXNIP) has been identified as an endogenous inhibitor of antioxidant TRX. 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 the LRR of NLRP3, leading to the NLRP3 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 [107].

ROS are a family of molecules that include highly reactive free oxygen radicals, such as superoxide anion ($O._2-$) and the stable non-radical oxidants, such as hydrogen peroxide (H_2O_2). ROS are mainly produced as byproducts during the mitochondrial electron transport of aerobic respiration, but ROS are also generated by the activity of cellular enzymes such as NADPH oxidases (nicotinamide adenine dinucleotide phosphate-oxidase). While ROS are produced as a product of normal cellular functioning, ROS-mediated oxidative stress plays an important role in pathological processes, such as hypertension, atherosclerosis, cancer, ischemia, neurodegenerative diseases and diabetes. During inflammatory conditions, stimulated phagocytic cells, such as neutrophil granulocytes, generate an ROS-dependent respiratory burst that kills the microorganisms, but this effect is nonspecific and host cells can also be damaged. Besides the antiseptic function of ROS, the release of H_2O_2 by damaged tissues forms a decreasing concentration gradient that directs the recruitment of leukocyte

cells at the site of tissue injury. These events demonstrate that ROS regulate inflammatory responses in tissues.

2.4.1. Antioxidants

The proper regulation of ROS generation is very important. The imbalance between ROS production and the ability to neutralize these oxidizing radicals generate oxidative stress. Physiologically cells protect themselves against ROS damage with enzymes displaying antioxidant activity such as TRX, alpha-1-microglobulin, superoxide dismutases, catalases, etc. Small cellular antioxidant molecules are also involved in neutralizing ROS, such as ascorbic acid (vitamin C), tocopherol (vitamin E), and gluthation. Antioxidants take their effect by removing free radical intermediates, inhibiting the oxidation of other molecules.

One of the most studied antioxidants is a general ROS scavenger N-acetyl cysteine [36] [36], which is a synthetic precursor in the formation of the intracellular antioxidant glutathione. It is commonly used as a dietary supplement due to its antioxidant and liver protecting effects.

Mitochondrial oxidative damage is a cause of various serious diseases. Therefore, the selective inhibition of mitochondrial ROS production can be a good therapeutic target. The mitochondria-targeted antioxidant, MitoTempo (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride monohydrate is one of the most studied molecules with superoxide scavenging capabilities [108].

Diphenyleneiodonium (DPI) is widely used as an inhibitor of NADPH oxidase, furthermore it also inhibits the production of ROS by mitochondria [109, 110] .

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

The incidence of allergic rhinitis and asthma is increasing to epidemic proportions (allergic rhinitis: 10-50%; and asthma: 5-15%), both in the developed and the developing world. Patients with allergic rhinitis are at three times the risk of developing asthma compared with those without allergic rhinitis. Allergic rhinitis is mainly caused by *Ambrosia Artemisiifolia* ragweed pollen. This plant is an annual, extremely allergenic weed that produces enormous amounts of pollen. After hydration in rainwater or in high humidity conditions pollen grains release respirable-sized subpollen particules that penetrate into lower airways and exacerbate the symptoms of allergic rhinitis and asthma as chronic inflammation,

airway hyperresponsiveness, bronchial constriction, increased fluid and mucus secretion. It has been demonstrated that granulocytes and peripheral blood monocytes collected from the blood of asthma patients produce a higher level of ROS than healthy controls [111] [112]. In allergic rhinitis and asthma patients the high level of ROS generates oxidative stress as a danger signal in the nearby airway epithelium, which is involved in the exacerbating of symptoms. It has been described that ragweed pollen grains and their extracts contain NADPH oxidases, similar to those found in mammalian phagocytes [113]. These enzymes generate ROS, playing an important role in the protection of plants against pathogens and regulating the expansion of cells in root hairs. After inhalation, ragweed pollen induces ROS production in airway epithelial cells [114]. It has been demonstrated that antioxidants inhibit ROS generation by ragweed pollen NADPH oxidases and intracellular ROS generation in airway epithelial cells [115]. Therefore, it is suggested that antioxidants in the airways may constitute a therapeutic strategy to prevent ragweed pollen induced allergic airway inflammation. Environmental pollutants such as ozone, diesel exhaust and cigarette smoke also enhance ROS production in lungs and exacerbate the symptoms of allergic asthma [116]. It has been described that ozone increases ragweed pollen allergenicity through stimulation of NADPH oxidase [117]. Ragweed pollen contains various antigenic proteins as well, that thus not possess NADPH oxidase activity, such as the pectate lyase Amb a 1, the major ragweed pollen protein. This antigen can be processed in antigen-presenting cells and presented to Th2 cells triggering an adaptive immune response. However, Amb a 1 induces a low-grade allergic airway inflammatory response.

Human airway epithelial cells contain functional NLRP3 inflammasomes that respond to urban particular matter exposure with the production of IL-1β [118]. The soluble IL-1β secretion is also increased after exposure to ozone and this elevated IL-1β production is dependent on the NLRP3 inflammasome [119]. The role of NRLP3 inflammasomes in the pathomechanism of allergic rhinitis and asthma 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 [120]. Another research group has shown that the expression of NLRP3 in nasal epithelial cells is down-regulated during pollen season among patients with allergic rhinitis [29]. In contrast to these findings it has also been described that allergic airway inflammation depends on NLRP3 inflammasome activation. Dendritic cell recruitment into lymph nodes, T-helper 2 lymphocyte activation in the lung, secretion of T-helper 2 cytokines and chemokines are reduced in the absence of NLRP3 [121]. It has also been shown that elevated levels of the acute-phase protein serum

amyloid A are present in patients with allergic asthma. The serum amyloid A activates the NLRP3 inflammasomes and this mechanism leads to the increased production of IL-1β in mice models of allergic asthma [122].

2.5. Macrophages

Monocytes differentiate *in vivo* into macrophages while entering into the tissues from the blood. Macrophages were characterized by Ilya Mechnikov in 1884 and he was awarded with a Nobel Prize in 1908 for discovering the major types and functions of phagocytes, such as macrophages. These cells can be identified by specific cell surface protein markers (CD14). The main roles of macrophages in innate immune responses are the recognition and the phagocytosis of pathogens. Macrophages also have an important role in the removal of cellular debris generated during tissue remodeling [123]. The binding of bacterial compounds to the cell surface receptors of macrophages triggers them to engulf and destroy the bacteria through the generation of a "respiratory burst", leading to the release of ROS. After the digestion of pathogens, macrophages will be able to present antigens of the digested pathogens to the corresponding helper T cells with their MHCII molecules. Therefore macrophages are also called antigen-presenting cells and act not only in the innate immunity, but they help to initiate the adaptive immunity as well.

Macrophages detect danger signals using PRRs located on the cell surface, in phagosomal vacuoles, like TLRs and in the cytosol, like NLRs. Since 2000, the number of articles published about the expression and function of Nod-like receptors in macrophages has been rising steadily [124, 125]. Tschopp et al. discovered the functional inflammasome in 2002, which was first identified in THP-1 macrophage cell line [51]. 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 [126], for example in allergic diseases [89].

2.6. Corneal epithelial cells

The ocular immune system protects the eyes from infection and dangerous environmental effects. The major cell types of the corneal immune system are corneal epithelial cells. They present a physical barrier to prevent pathogenic microbes from reaching the interior of the eye chamber, but they also actively secrete cytokines, chemokines, antimicrobial peptides to activate microbial defense [127]. Corneal epithelial cells express different kinds of PRRs for the recognition of PAMP or DAMP-derived danger signals. In 2008, at the time of our studies, the expression of several TLR family proteins was studied in primary corneal epithelial cells and cell lines [128-133], but there was no information about the expression of various NLR molecules and key inflammasome components. Our research group was the first to show the expression of Nod-like receptors in human corneal epithelial cells. Only three additional articles have been published ever since. The first article describes that NLRP3 is expressed in primary human corneal epithelial cells, the second article introduces that NOD2 ligand MDP modulates the human β-defensin 9 gene expression in corneal epithelial cells [134, 135]. Another group demonstrated the expression of NLRP1 in corneal epithelial cells [136].

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

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 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. Differences from these treatments are indicated in the legend of corresponding figures. 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 chloride (DPI, 10 μM, Sigma-Aldrich) or caspase-1 inhibitor (Z-YVAD-fmk, 20 μM, BioVison) were added to the cells 1 hour before treatments.

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 [137] 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.

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).

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 μg/ml RWE and 100 μM 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.

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.

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.

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.

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).

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 for NLRP1 (Hs00248187_m1), NLRP2 (Hs215284), NLRP3 (assay ID Hs00918082_m1), NLRP6 (Hs00373246_m1), NLRP7 (Hs00373683_m1), NLRP10 (Hs00738590_m1), NLRP12 (Hs00376282_m1), caspase-1 (Hs00354836_m1), ASC (HS00203118_m1), IL-1β (Hs00174097_m1), NOD1 (Hs00196075_m1), NOD2 (Hs00223394_m1), NLRC5 (Hs00260008_m1), NLRX1 (Hs00226360_m1) 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*.

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.

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 g/ml aprotinin, 0.5 g/ml pepstatin, 1.25 g/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).

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.

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

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 [138]. In our previous studies we detected that LPS is able to induce IL-1β secretion that could be saturated with 100 ng/ml LPS in THP-1 cells (Figure 11). Therefore we used this LPS concentration in our further studies.

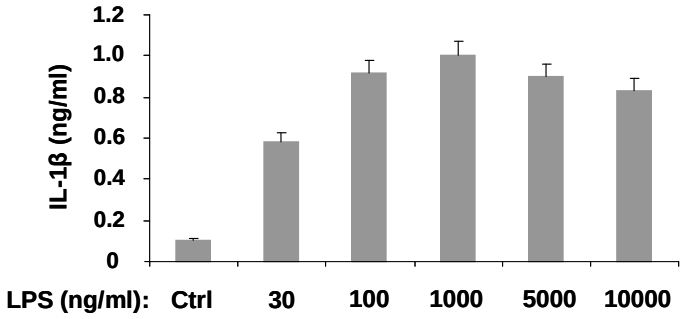

Figure 11. IL-1β secretion is saturated with 100 ng/ml LPS.
THP-1 macrophages were treated with an increasing amount of LPS and 24 hours after treatment the secreted IL-1β was measured from the collected supernatant by ELISA method.

First of all 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. 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 using ELISA method (Figure 12).

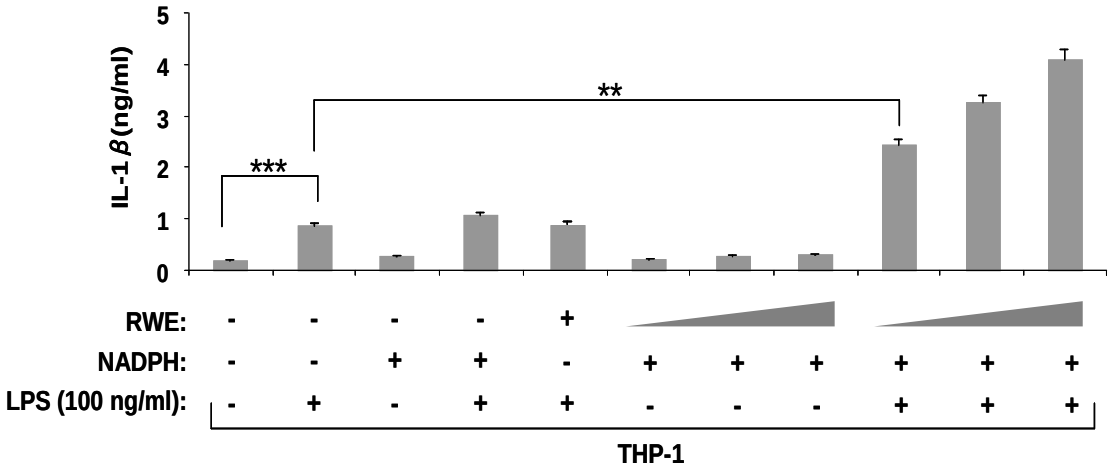

Figure 12. RWE enhances LPS-induced IL-1β production of THP-1 cells in the presence of NADPH. THP-1 macrophages were stimulated for 24 hours with an increasing amount (10, 30 and 100 μg/ml) of RWE in the absence or presence of LPS and 100 μM NADPH, as indicated. The supernatants were collected and their IL-1β content was measured by ELISA method in triplicates. The experiment was repeated five times and results of a representative set are provided. Mean ± SD values are provided. **P* < 0.05, ***P* < 0·005.

While in good agreement with previous findings [138] LPS treatment of THP-1 cells induced a substantial increase of the secreted IL-1β. The treatment of the cells with RWE in the absence or presence of NADPH or NADPH alone did not trigger the IL-1β secretion. However, RWE in the presence of NADPH significantly enhanced the LPS-induced IL-1β production, in a dose-dependent manner at the lowest saturating LPS concentration (100 ng/ml).

A similar induction was observed even at a 10-fold higher (1000 ng/ml) LPS concentration (Figure 13).

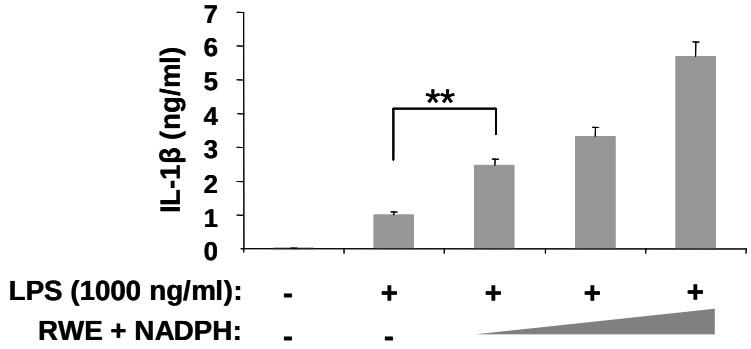

Figure 13. RWE further enhances the 1000 ng/μl LPS-induced saturated IL-1β secretion of THP-1 cells. THP-1 macrophages were treated with 1000 ng/ml LPS and increasing amounts (10, 30, 100 μg/ml) of RWE. Twenty-four hours after treatment supernatant was collected and IL-1β concentration was determined using ELISA method. The experiment was repeated five times and results of a representative set are provided.

We also investigated the effect of RWE on the LPS-induced IL-1β secretion in THP-1 cells in a time-dependent manner. We found that the IL-1β secretion was continuously

increased 6 hours after treatment and substantial further elevation was observed between 12-24 hours after treatment (Figure 14).

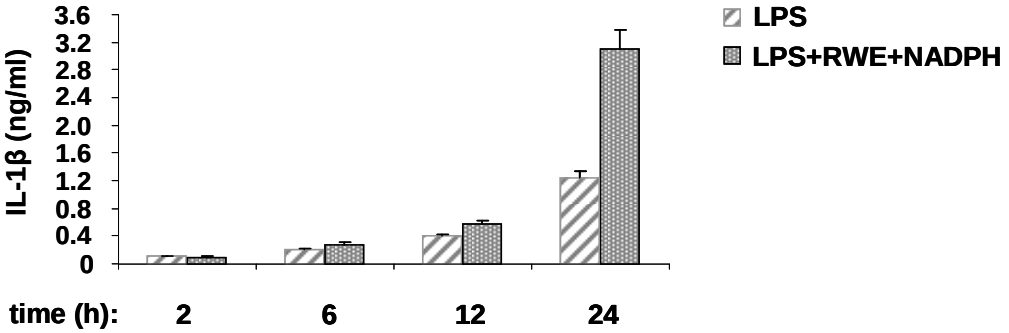

Figure 14. Time-dependency of LPS-induced and RWE-enhanced IL-1β secretion of THP-1 cells.
THP-1 macrophages were treated with 1000 ng/ml LPS in the presence or absence of 10 μg/ml RWE in a time-dependent manner. After the indicated times supernatants were collected and IL-1β concentration was determined using ELISA method. The experiment was repeated five times and results of a representative set are provided.

This effect required the concomitant administration of RWE and NADPH, as neither RWE nor NADPH alone has the intensifying effect on the LPS-induced IL-1β secretion, therefore in all of the following experiments RWE treatment was always performed with the concomitant administration of 100 μM NADPH.

To determine the effect of RWE on IL-1β secretion of primary human macrophages and dendritic cells, cells were differentiated from monocytes using GM-CSF for the former and GM-CSF and IL-4 for the latter, respectively, for five days.

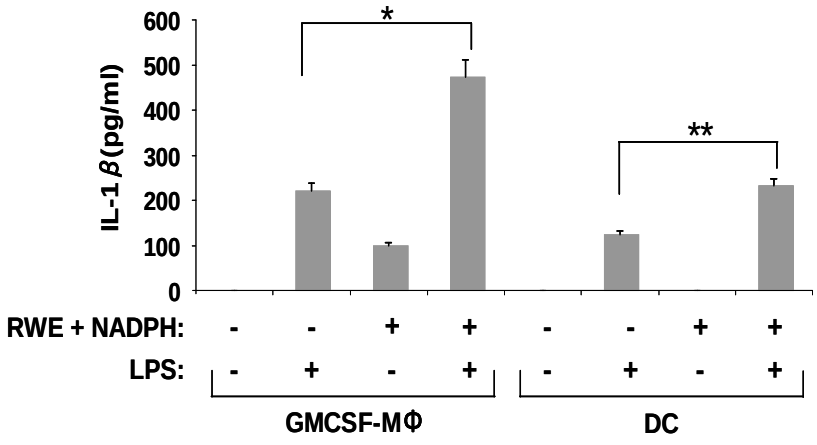

Figure 15. RWE enhances LPS-induced IL-1β production of human macrophages and dendritic cells.
Human monocyte-derived macrophages and dendritic cells were treated with 500 ng/ml LPS in the absence or presence of 100 ug/ml RWE and 100 μM NADPH. Twenty-four hours after treatment supernatants were collected and their IL-1β content was determined using ELISA method in triplicates. Experiments were repeated four times and results of a representative set are provided. Mean ± SD values are provided. *$P < 0{\cdot}05$, **$P < 0{\cdot}005$.

We found that LPS alone or in combination with RWE leads to results similar to those found with the THP-1 cell line (Figure 15). While LPS is capable of inducing IL-1β secretion by itself, RWE significantly enhanced the LPS-induced IL-1β secretion. These results show that while RWE (in the presence of NADPH) alone is not able to induce IL-1β secretion in dendritic cells, it activates a modest IL-1β secretion in human macrophages, however, it significantly intensifies the LPS-induced IL-1β secretion in both macrophages and dendritic cells.

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 [113], for this reason we aimed to study if pollen extract could induce ROS production in THP-1 macrophages. Therefore we measured ROS production of THP-1 cells after the treatments (Figure 16).

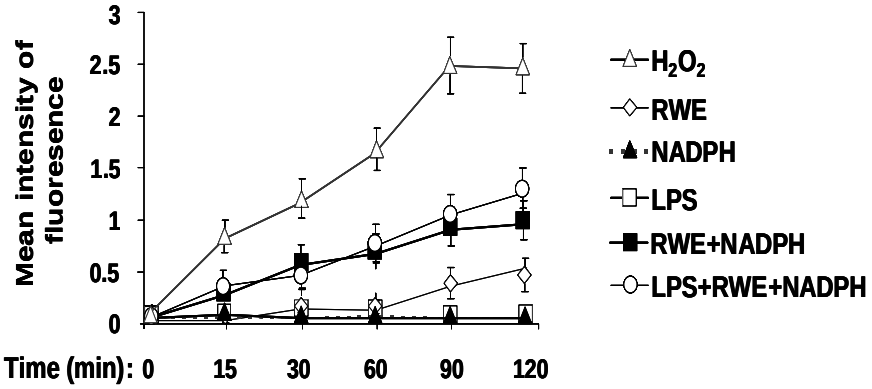

Figure 16. RWE increases ROS production in THP-1 cells.
THP-1 cells were loaded with H_2DCFDA, treated with various combinations of 100 μg/ml RWE, 100 μM NADPH and 1000 ng/ml lipopolysaccharide (LPS), and changes in the intracellular ROS level were measured using flow cytometry for the indicated time interval; 1 mM H_2O_2 was used as a positive control. Mean intensity of fluorescence was calculated from the positive area defined by the stained cells.

In this experiment H_2O_2 was used as a positive control. H_2O_2 induced a fast increase in intracellular ROS level. Our results show that whereas RWE alone (but not NADPH) induced some ROS production, 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 [139] 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 either the ROS-scavenger NAC or with the inhibitors of ROS production, MitoTEMPO and DPI (Figure 17).

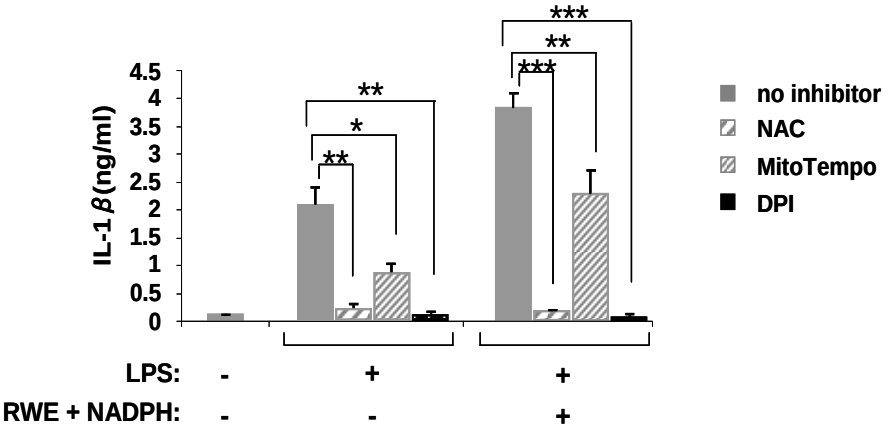

Figure 17. ROS inhibitors abolish IL-1β secretion.
THP-1 cells were pre-treated with 30 mM NAC, 300 μM MitoTempo or 10 μM DPI for 1 hour then treated with 100 ng/ml LPS in the presence or absence of 10 μg/ml RWE and 100 μMNADPH. Twenty-four hours after treatment the secreted IL-1β was measured from the collected supernatants in triplicates by an ELISA method. Results were obtained in three independent experiments, and a representative result set is shown. *$P < 0{\cdot}1$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

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 the 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.

To expel the possibility that the apparent inhibitory effect of ROS scavenger and ROS production inhibitors on the IL-1β production was due to cell death, viability assay was performed in the presence of the used agents (Figure 18). Methanol was used as positive cell death control.

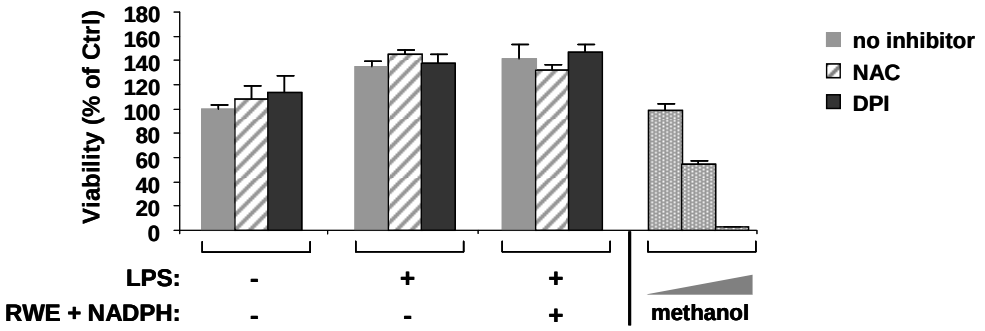

Figure 18. ROS inhibitors are non-toxic for THP-1 cells.
THP-1 cells were pre-treated with 30 mM NAC or 10 μM DPI for 1 hour then treated with 100 ng/ml LPS in the presence or absence of 10 μg/ml RWE and 100 μMNADPH. Methanol was used as positive control. Twenty-four hours after treatment the cell viability was measured by fluorescent viability/cytotoxicity kit.

Our results show that none of the utilized agents caused cell toxicity at the applied concentrations.

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

Maturation of IL-1β requires the cleavage of pro-IL-1β to active IL-1β via a process that is mediated by caspase-1. 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 with ELISA (Figure 19). We demonstrated that the specific caspase-1 inhibitor significantly reduced the LPS-induced RWE-enhanced IL-1β production. This phenomenon suggests that caspase-1 has a crucial role in RWE-enhanced IL-1β production.

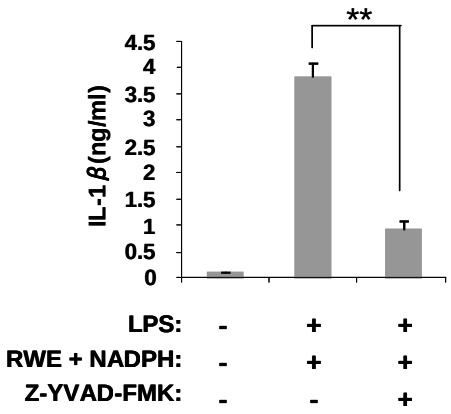

Figure 19. Caspase-1 inhibition abolishes LPS-induced and RWE-enhanced IL-1β secretion.
THP-1 macrophages were treated with 100 ng/ml LPS and 10 μg/ml RWE in the absence or presence of the caspase-1-specific inhibitor Z-YVAD-FMK (20 μM), for 24 hours. IL-1β content of the supernatants was determined using an ELISA method. Results were obtained in three independent experiments, and a representative result set is shown. * $P < 0{\cdot}05$, ** $P < 0{\cdot}005$.

We have also studied the effect of silencing of NLRP3 expression using siRNA on the IL-1β secretion of THP-1 cells. To verify the efficiency of the silencing we investigated the NLRP3 gene as well as protein expression by real-time quantitative PCR and Western blot (Figure 20).

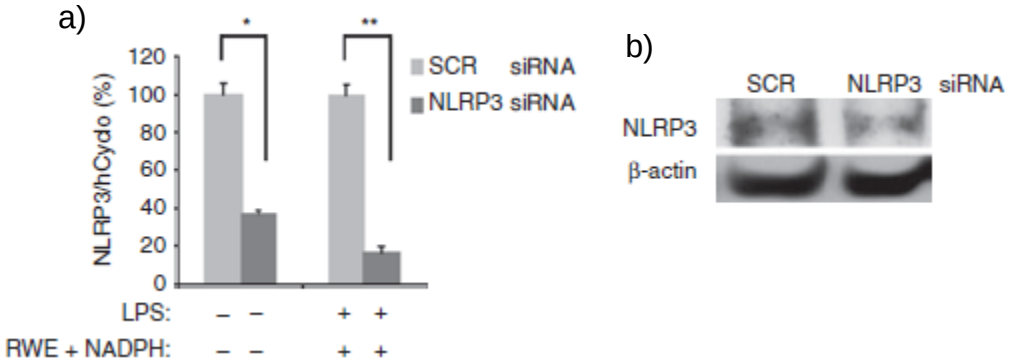

Figure 20. The silencing of NLRP3 gene expression is efficient.
THP-1 cells were electroporated with scrambled (SCR) small interfering (si) RNA, as negative control or siRNA specific for NLRP3. Two days after electroporation, cells were stimulated with LPS (100 ng/ml), RWE (10 μg/ml) and NADPH (100 μM) for 24 hours. After harvesting the cells, RNA was purified and NLRP3 transcription was measured by quantitative real-time PCR (**a**), while NLRP3 protein expression of NLRP3 was detected by Western blot technique (Alexis Biochemicals) (**b**). Gene expression is shown as the ratio of the studied transcripts relative to human cyclophilin expression (± SD) measured in triplicates. The gene expression levels of SCR siRNA-transfected samples correspond to 100%. Equal amount of protein sample loading was verified by detecting β-actin protein expression (rabbit polyclonal antibody, Sigma-Aldrich). Results were obtained in three independent experiments, and a representative result set is shown. * $P < 0{\cdot}05$, ** $P < 0{\cdot}005$.

We have shown that the NLRP3 silencing was efficient (Figure 20) and completely inhibited the IL-1β secretion of stimulated THP-1 cells (Figure 21).

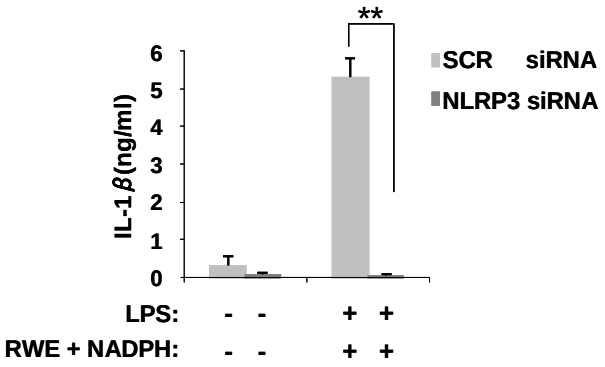

Figure 21. The NLRP3 silencing inhibits the IL-1β secretion of activated THP-1 cells.
THP-1 cells were electroporated with scrambled (SCR) small interfering (si) RNA, as negative control or siRNA specific for NLRP3. Two days after electroporation, cells were stimulated with LPS (100 ng/ml), RWE (10 μg/ml) and NADPH (100 μM) for 24 hours. The concentration of secreted IL-1β was measured from the supernatant of the cells in triplicates using ELISA method. Results were obtained in three independent experiments, and a representative result set is shown. * $P < 0{\cdot}05$, ** $P < 0{\cdot}005$.

We have also determined that not only the LPS-induced IL-1β production but also the LPS-induced RWE-enhanced IL-1β secretion is controlled by the NLRP3 inflammasome.

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

For NLRP3 inflammasome activation the elevated expression of inflammasome components and substrates are required, furthermore, assembly of inflammasome members is also collectively required. To investigate the effect of RWE on NLRP3 and caspase-1 expression, 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 by real-time quantitative PCR and Western blot techniques (Figure 22 a-e).

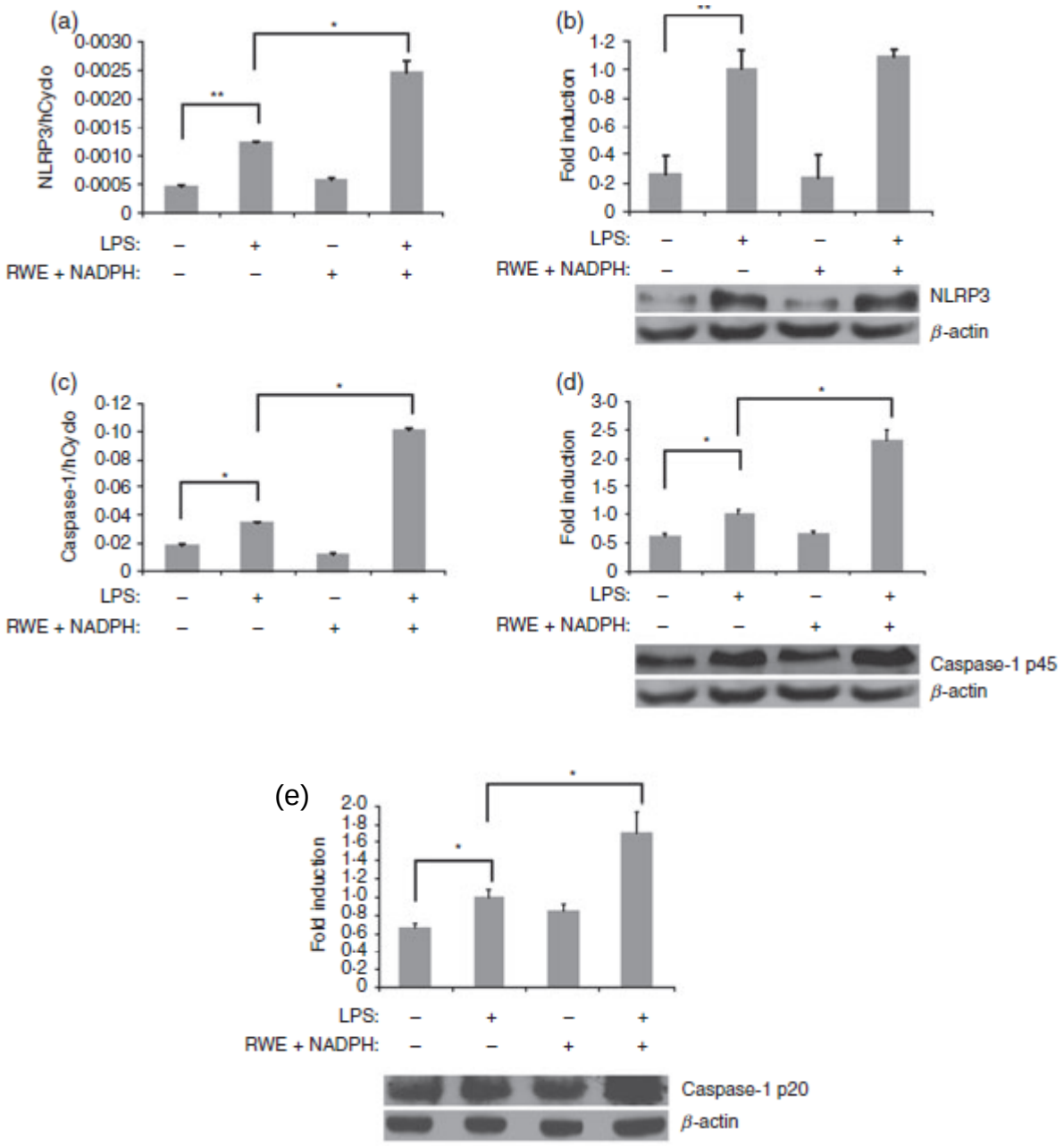

Figure 22. RWE increases NLRP3 (a, b), pro-caspase-1 (c, d) and cleaved caspase-1 (e) expression of LPS-treated cells.

THP-1 macrophages were treated with LPS (100 ng/ml), RWE (10 μg/ml) alone or together with NADPH (100 μM). Twenty-four hours following treatment cells were collected and gene expression of (a) NLRP3, (c) and caspase-1 was determined using quantitative RT-PCR. Protein expression was determined by Western blot technique using antibodies recognizing (b) NLRP3, (d) procaspase-1, and (e) caspase-1. Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit, Amersham). Gene expression is shown as the ratio of NLRP3 or caspase-1 transcripts relative to human cyclophilin expression (± SD) measured in triplicates. For Western blot an equal amount of protein sample loading was verified by detecting β-actin protein expression. Protein levels were estimated by densitometric analysis of the bands using KODAK 1D Image Analysis Software. A representative immunoblot is also shown for NLRP3, and caspase-1. Densitometry values provided are the average of Western blot runs of three independent experiments.

We demonstrated that LPS significantly induced the mRNA expression of NLRP3 (Figure 22 a) and pro-caspase-1 (Figure 22 c), furthermore the protein expression of pro-caspase-1/caspase-1 p45 (Figure 22 d) and cleaved caspase-1/caspase-1 p20 (Figure 22 e) of THP-1 macrophages. While RWE in the presence of NADPH did not influence the expression of these molecules, it further enhanced the LPS-induced mRNA and protein expression of pro-caspase-1 and the protein expression of cleaved caspase-1. (Figure 22 c, d, e). Although we found an enhanced transcription of NLRP3 (Figure 22 a), but the increase was not significant in the protein level (Figure 22 b). Similar results have been published that demonstrate the different expression of NLRP3 mRNA 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 cell lysates using a fluorescent substrate (Figure 23).

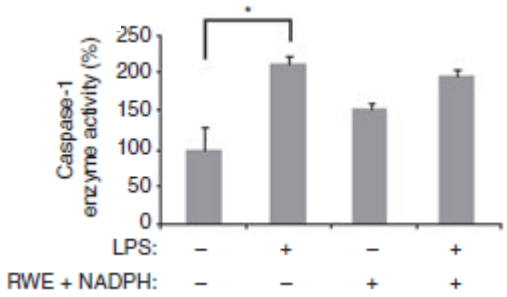

Figure 23. LPS induces caspase-1 activity, but RWE does not result in further enzyme activation.
THP-1 macrophages were treated with LPS (100 ng/ml), RWE and NADPH in the absence or presence of RWE (10 μg/ml) and NADPH (100 μM). Twenty-four hours following treatment cells were collected and from the lysate of THP-1 macrophages the activity of caspase-1 enzyme was determined using fluorometric peptide substrate in triplicates. Mean ± SD values of three independent experiments are shown. **P* < 0·1, ***P* < 0·01

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. We also investigated how RWE treatment (in the presence of NADPH) by itself or in combination with LPS affects the expression of caspase-1 substrate pro-IL-1β (Figure 24).

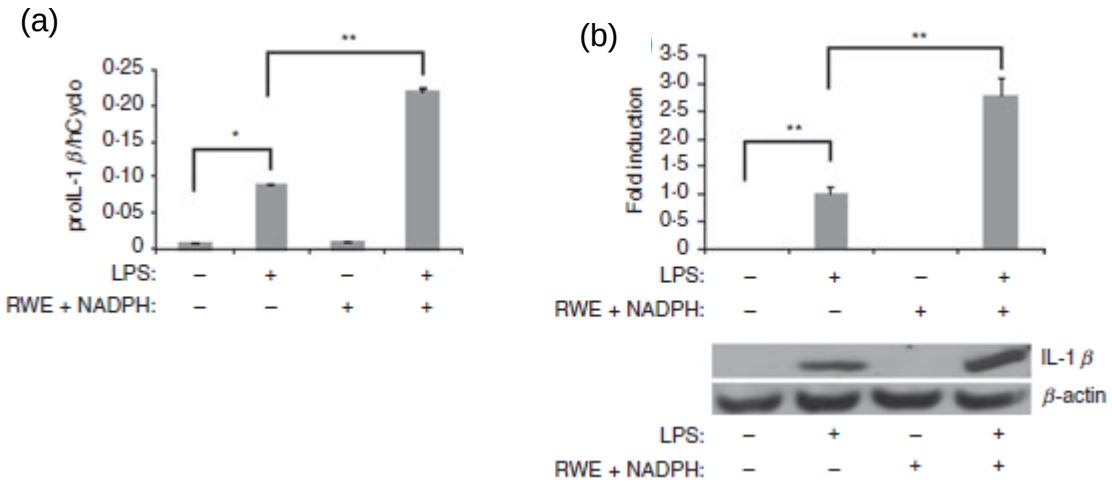

Figure 24. RWE enhaces LPS-induced pro-IL-1β and mature IL-1β expression in THP-1 cells.
THP-1 macrophages were treated with LPS (100 ng/ml), RWE and NADPH in the absence or presence of RWE (10 μg/ml) and NADPH (100 μM). Twenty-four hours following treatment cells were collected and gene expression of (a) pro-IL-1β was determined using quantitative RT-PCR. Protein expression was determined by Western blot technique using antibodies recognizing (b) IL-1β. Primary antibody was detected using horseradish peroxidase-conjugated secondary antibody (anti-rabbit, Amersham). Gene expression is shown as the ratio of pro-IL-1β transcripts relative to human cyclophilin expression (± SD) measured in triplicates. For Western blot an equal amount of protein sample loading was verified by detecting β-actin protein expression. Protein levels were estimated by densitometric analysis of the bands using KODAK 1D Image Analysis Software. A representative immunoblot is also shown for IL-1β. Densitometry values provided are the average of Western blot runs of three independent experiments.

Our results show that LPS treatment significantly induced the expression of pro-IL-1 β. This elevated expression of pro-IL-1β was further enhanced by RWE treatment. We found a substantially stronger production of processed IL-1β protein in the lysate of LPS and RWE (in the presence of NADPH) treated cells compared to the LPS treated ones.

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 (NLRP3 and caspase-1) is due to the presence of active NADPH oxidase in the pollen extract, we studied the RWE-induced transcription of these genes using a real-time quantitative PCR technique in the presence or absence of NADPH (Figure 25).

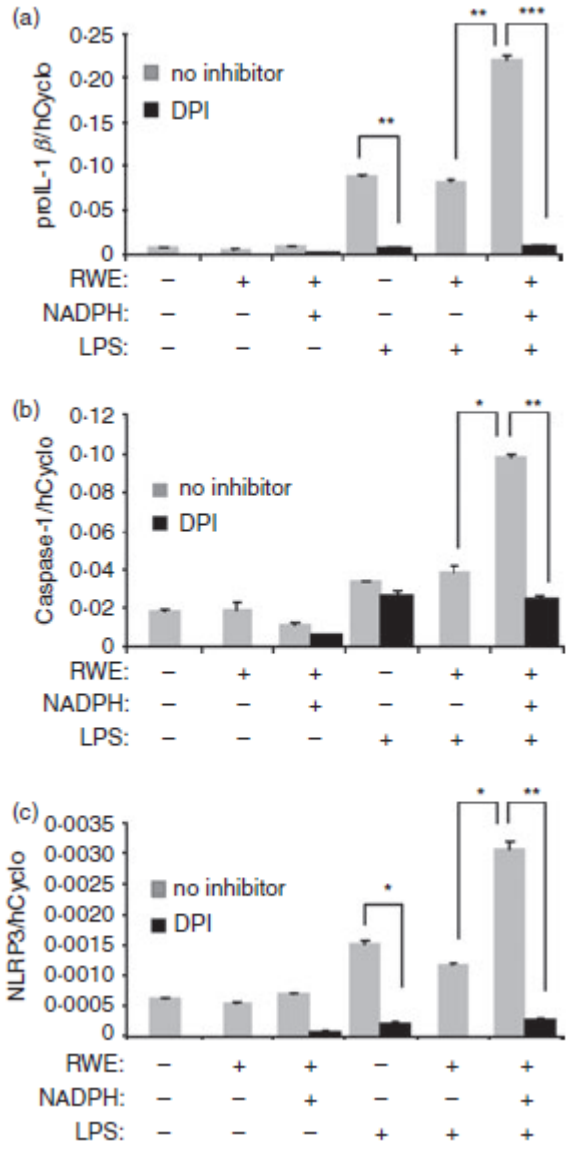

Figure 25. Ragweed pollen extract (RWE)-induced gene expression is NADPH-dependent and is suppressed by a reactive oxygen species (ROS)-inhibitor.
THP-1 macrophages were treated for 24 hours with the compounds as indicated, DPI was used in 10 μM. After harvesting the cells, the gene expression of (a) pro-interleukin-1β (pro-IL-1β), (b) caspase-1 and (c) NLRP3 was determined by quantitative real-time PCR. Gene expression is shown as the ratio of the studied transcripts relative to human cyclophilin expression (± SD) measured in triplicates. Results were obtained from four independent experiments, and a representative result set is shown.*$P < 0{\cdot}05$, **$P < 0{\cdot}005$, ***$P < 0{\cdot}0005$.

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. Interestingly, while the LPS-induced caspase-1 production was not affected by DPI, significant down-regulation was observed in the case of the RWE-treated

THP-1 macrophages, regardless of the LPS treatment. Our results suggest that the RWE-enhanced gene expression of inflammasome components and pro-IL-1β is NADPH and ROS-dependent.

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.

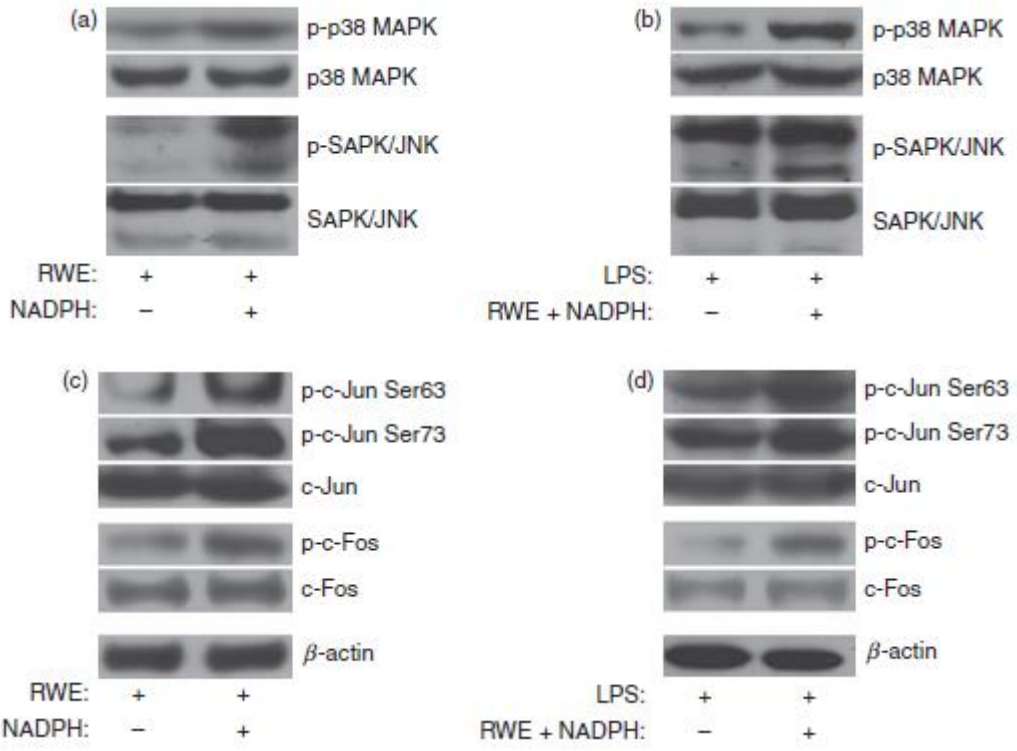

Figure 26. RWE induces (A) and further enhances the LPS-triggered (B) phosphorylation of SAPK/JNK and p38 MAPK.
THP-1 macrophages were treated with 30 μg/ml RWE in the absence or presence of 100 μM NADPH, or (b, d) with 100 ng/ml lipopolysaccharide (LPS) in the absence or presence of RWE plus NADPH. Five hours after treatment, cells were harvested, protein lysates were prepared and the phosphorylation of (a, b) p38 MAPK, SAPK/JNK, (c, d) c-Jun (phosphorylated at serine 73 or 63) and c-Fos; furthermore total c-Jun and c-Fos were analysed by Western blot method. To verify the loading of equal amounts of protein sample, the β-actin protein expression was detected (c, d) or total p38 and SAPK/JNK were used as loading controls (a, b). Results obtained from three independent experiments, and one representative Western blot is shown.

We found that unlike the phosphorylation of IκBα (data not shown), RWE induced the phosphorylation of p38 MAPK and JNK (54 and 46 kDa) compared to the non-treated cells (data not shown), furthermore the phosphorylation appeared to be NADPH-dependent (Figure 26 a). We also found that RWE in the presence of NADPH substantially enhanced the LPS-induced p38 MAPK and JNK phosphorylation (Figure 26 b). We further investigated the phosphorylation of AP-1 complex members c-Jun (phosphorilated at serine63 or 73) and c-Fos, which are also important participants of signaling pathways involved in IL-1β expression (Figure 26 c, d). The results are similar to those found in the case of the phosphorylation of p38 and JNK. 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. These results suggest that the ROS-dependent enhancement of LPS-induced IL-1β production by RWE may involve the p38 MAPK, JNK and AP-1 signaling pathways.

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 [128-133], but there was no information about the expression of various NLR molecules and key inflammasome components. Therefore we aimed to measure the transcription of NLRP proteins and inflammasome components in immortalized and primary human corneal epithelial cells by RT-PCR method. In the course of these experiments we studied the relative mRNA levels of NLRP1, NLRP2, NLRP3, NLRP4, NLRP6, NLRP7, NLRP10 and NLRP12, ASC and caspase-1 in HCE-T cell line as well as in primary corneal epithelial cells obtained from five healthy individuals undergoing photorefractive keratectomy (PRK) treatment. (Figure 27.)

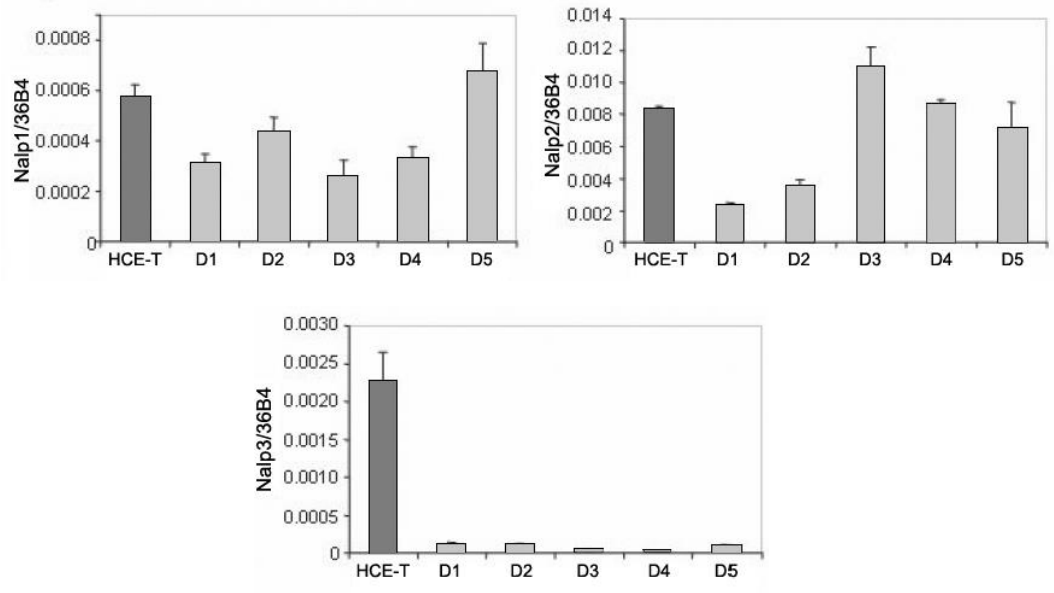

Figure 27. Relative mRNA expression of NLRP family proteins in corneal epithelial cells.
The relative expression of NLRP (Nalp) family member mRNA was measured by real time RT–PCR in HCE-T cells and in primary corneal epithelial cells derived from five individuals referred to as donors (D1–D5). Relative expression levels of NLRPs in HCE-T cells (mean values of three independent experiments) and in PRK samples are shown in the charts. Relative gene expression is shown as the ratio of the indicated transcripts relative to *36B4* expression±SD measured in triplicates.

We demonstrated that the mRNA expression of all the NLRP proteins was approximately uniform in the different donors, except for NLRP2 mRNA expression that showed up to a fivefold difference among the different PRK donors (D1-D5). The relative expression of NLRP1 mRNA was comparable in the cell line and primary cells. Interestingly, 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 cell line (data not shown), whereas NLRP10 mRNA expression was dramatically higher in HCE-T cells than in primary cells from PRK samples (data not shown). We could not detect the mRNA expression of NLRP4, NLRP6 or NLRP12, neither in the cell line nor in the primary cells, although the corresponding assays were proved themselves to be sensitive enough to detect these genes in other cell types.

We aimed not just to investigate the mRNA, but also the protein expression of NLRP1 and NLRP3 in HCE-T cells. We used LPS-treated THP-1 cells as positive control for these experiments (Figure 28).

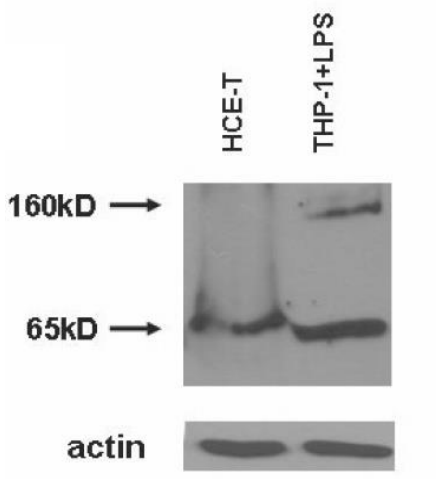

Figure 28. NLRP1 protein expression in HCE-T cells.
Detection of Nalp1 protein in HCE-T and LPS-treated THP-1 cell lysates by Western blotting is illustrated. The uniform loading of the sample amounts was verified by using β-actin antibody.

Our results show that while we detected a longer (160 kDa) and a shorter (65 kDa) isoform of NLRP1 protein in THP-1 cells, only the shorter isoform could be detected in HCE-T cells. We also found that in contrast to the NLRP3 mRNA expression, we could not detect the protein expression of NLRP3 (data not shown). Therefore HCE-T cells may not serve as a good model for NLRP3 or NLRP1 inflammasome studies.

To determine the relative expression levels of ASC and caspase-1 in the cell line and in primary corneal epithelial cells, we detected the mRNA expression of these proteins by real-time quantitative PCR method (Figure 29).

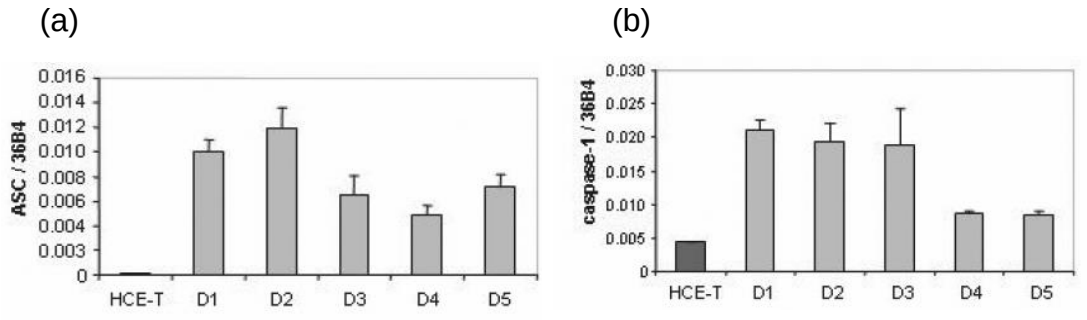

Figure 29. Relative mRNA expression of ASC and pro-caspase-1 in corneal epithelial cells.
The relative expression of ASC and caspase-1 mRNA was measured by real time RT–PCR in HCE-T cells and in primary corneal epithelial cells derived from five individuals referred to as donors (D1 – D5). Relative gene expression is shown as the ratio of the indicated transcripts relative to 3*6B4*expression±SD measured in triplicates.

We found that the mRNA expression of ASC (Figure 29 a) was dramatically lower in HCE-T cells compared to that of the primary cells. The pro-caspase-1 mRNA level (Figure 29 b) was significantly lower in HCE-T cells than in cells from PRK samples.

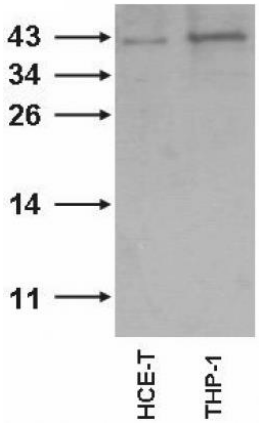

Figure 30. Pro-caspase-1 protein expression in HCE-T cells.
Detection of caspase-1 protein expression of HCE-T and THP-1 cell lysates by Western blotting is illustrated.

While we clearly detected the protein expression of pro-caspase-1 in HCE-T cells as well as in positive control THP-1 cells (Figure 30), 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 from PRK (Figure 31).

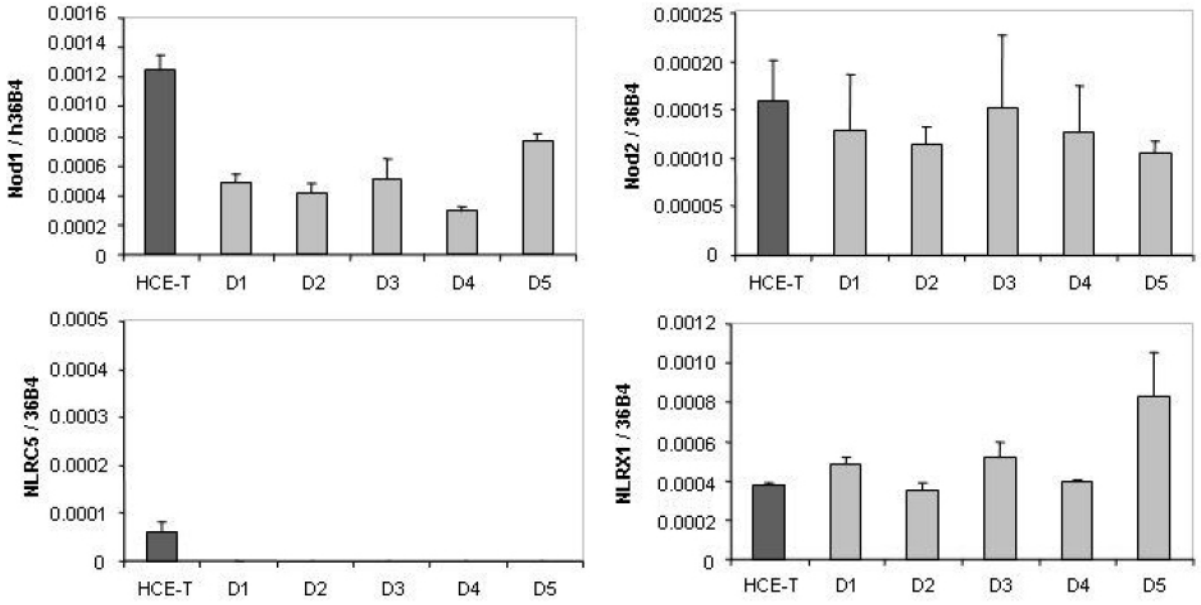

Figure 31. Relative mRNA expression of NLRC proteins in corneal epithelial cells.
The relative expression of NOD1, NOD2, NLRC5, and NLRX1 mRNA in HCE-T cells and in primary corneal epithelial cells derived from five individuals was measured and documented as described in the legend of Figure 27.

Our results show that 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.

We demonstrated that the expression level of NOD1 and NOD2 in the HCE-T cell line was similar to that of the primary cells. NOD1 and NOD2 is triggered by mainly bacteria-derived components. Therefore 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 (PGN-ECndss, a modified form of PGN from *E. coli*). This ultrapure PGN is a specific ligand of NOD1 and NOD2 that acts via NOD1 or NOD2 without the activation of TLR2. Triggering NOD1 or NOD2 activation by their specific ligands leads to the activation of NFκB (185), accordingly the phosphorylation of IκB is a marker of NFκB activation. Therefore we treated the HCE-T cells with 4 μg/ml ultrapure PGN-ECndss in a

time-dependent manner and detected the appearance of phosphorilated-IκB (pIκB) by Western blot method (Figure 32). LPS-treated (1 μg/ml) THP-1 cell lysate was used as positive control.

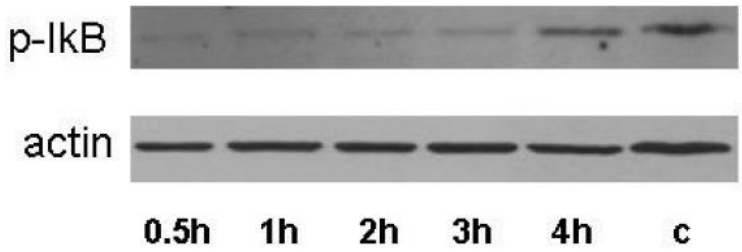

Figure 32. PGN triggers the phosphorylation of IκB.
Detection of phosphorylated IκB using Western blotting is illustrated. HCE-T cells were treated with 4 μg/ml ultrapure PGN-ECndss for the indicated time periods. The control THP-1 cells (c) were treated with 1 μg/ml LPS for 4 hours. Equal amount of sample loading was verified by detecting β-actin protein expression.

We detected the time-dependent appearance of the phosphorylated IκB after the PGN-ECndss treatments. We found that these proteins are able to recognize and respond to their specific ligand PGN and activate the NFκB signaling pathway via the phosphorylation of IκB. The phosphorylation peak was observed in the 4 h-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. We performed MTT assay 24 hours after exposure (with the dose we used in the experiments) to know the effect of UV-B irradiation on cell viability. We found that UV-B irradiation caused apoptosis in 10-13 % of all cells (data not shown).

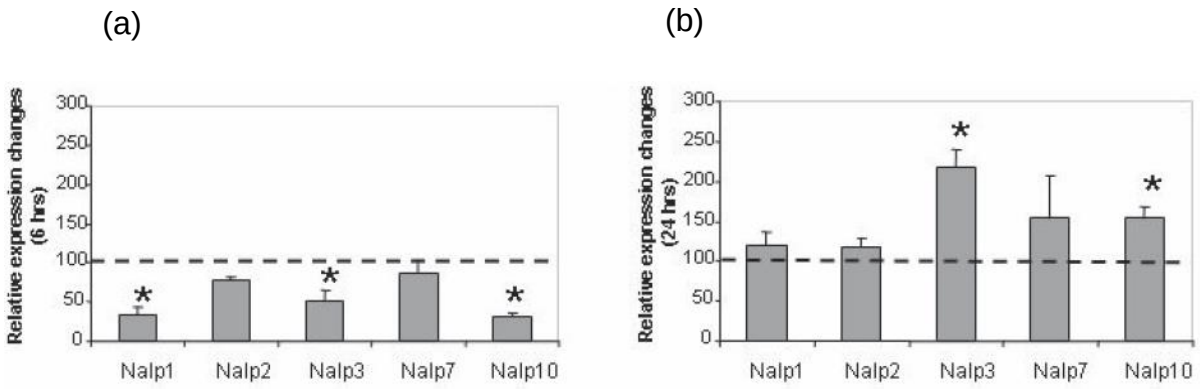

Figure 33. The effect of UV-B irradiation on the gene expression of NLRP proteins 6 hours (a) and 24 hours (b) after treatment in HCE-T cells.

The effect of UV-B treatment on the expression of NLRP subtypes in HCE-T cells. Changes of NLRP mRNA expression 6 hours (a) or 24 hours (b) after UV-B treatment are shown. The relative expressions of NLRP1, NLRP2,NLRP3, NLRP7, and NLRP10 were measured in 85%–90% confluent HCE-T cells irradiated with 30 mJ/cm^2 UV-B as described in the legend of Figure 27 and are shown compared to cells incubated in the same way but not irradiated. Mean values and ±SD were calculated from six independent measurements. The asterisk indicates a $p<0.005$.

Our results show that the mRNA expression of all of the studied NLRPs was downregulated 6 hours after UV-B exposure (Figure 33 a), with the exception of NLRP2 and NLRP7. Interestingly, 24 hours after UV-B irradiation the mRNA levels of NLRPs recovered (Figure 33 b). Moreover, the expression of NLRP3 and NLRP10 were significantly increased compared to the expression level of non-treated samples (Figure 33 b).

We also studied the effect of UV-B irradiation on the protein expression of NLRP1 (Figure 34) and NLRP3 by Western blot.

Figure 34. The effect of UV-B irradiation on the expression of NLRP1 protein 6 hours (a) and 24 hours (b) after treatment in HCE-T cells.

Changes of NLRP1 protein expression 6 hours (a) and 24 hours (b) after UV-B irradiation of HCE-T cells are illustrated. Equal amount of sample loading was verified by detecting β-actin protein expression.

We observed decreased protein expression of NLRP1 6 hours after UV-B exposure (Figure 34 a), but 24 hours after treatment protein level recovered nearly up to the protein

level of the non-treated control sample (Figure 34 b). Though the mRNA level of NLRP3 was significantly increased 24 hours after UV-B irradiation, we could not detect the protein expression of NLRP3 by the same antibody against NLRP3 that was successfully used in the case of other samples that express NLRP3 protein.

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

Besides the inflammasome sensors we also wanted to know the effect of UV-B irradiation on the expression of inflammasome adaptor ASC and caspase-1 enzyme (Figure 35).

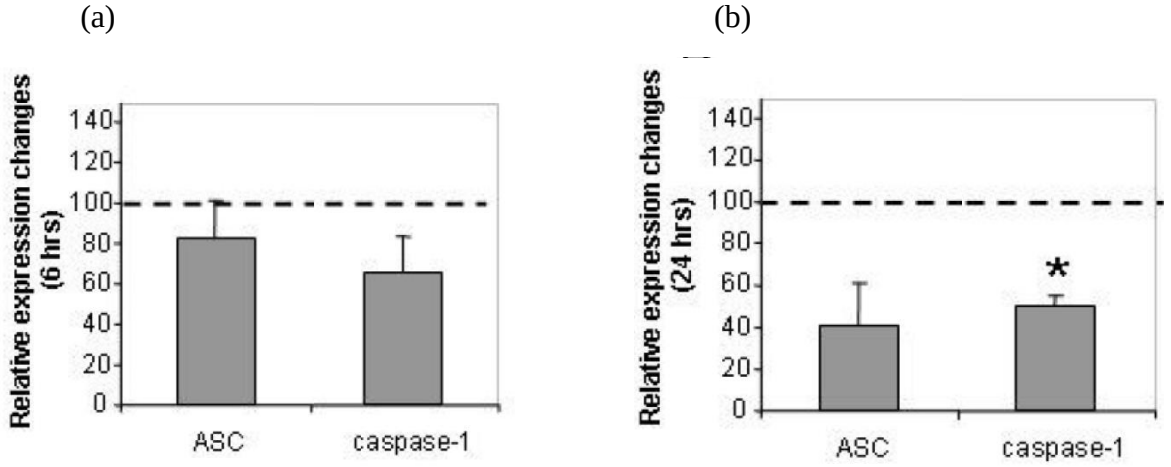

Figure 35. The effect of UV-B irradiation on the gene expression of inflammasome components 6 hours (a) and 24 hours (b) after treatments in HCE-T cells.
Changes of inflammasome component mRNA expression 6 hours (a) or 24 hours (b) after UV-B treatment are illustrated. The relative expression of ASC and caspase-1 was measured in 85%–90% confluent HCE-T cells irradiated with 30 mJ/cm^2 UV-B as described in the legend of Figure 27 and is shown as compared to cells incubated in the same way but not irradiated. Mean values and ±SD were calculated from six independent measurements. The asterisk indicates a $p<0.005$.

Our results show that the mRNA expression of all components was decreased 6 hours after UV-B exposure (Figure 35 a). We also found that 24 hours after irradiation the expression of these genes remained at lower levels, and the decrease of mRNA expression of caspase-1 also became significant as compared to the mRNA expression level of non-irradiated cells (Figure 35 b).

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

We also investigated the effect of UV-B irradiation on the transcription of NLRC proteins.

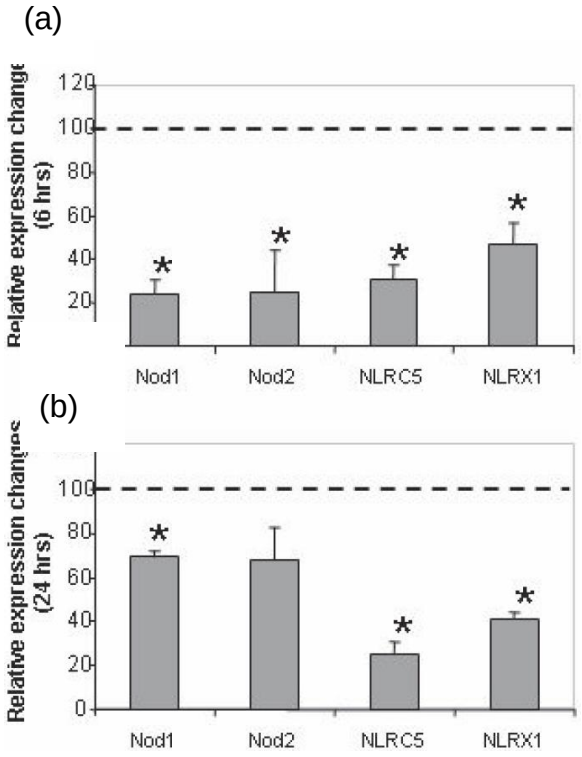

Figure 36. The effect of UV-B irradiation on the gene expression of NLRC proteins 6 hours (a) and 24 hours (b) after treatments in HCE-T cells.
Changes of NOD mRNA expression 6 hours (a) or 24 hours (b) after UV-B treatment are shown. The relative expression of NOD1, NOD2, NLRC5, and NLRX1 was measured in 85%–90% confluent HCE-T cells irradiated with 30 mJ/cm^2 UV-B as described in the legend of Figure 28 and is shown as compared to cells incubated in the same way but not irradiated. Mean values and ±SD were calculated from six independent measurements. The asterisk indicates a $p<0.005$.

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 (Figure 36 a). However, 24 hours after treatment the expression levels of NOD1 and NOD2 increased compared to the 6 hours treated cells (Figure 36 b), 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

It is well known that the activity of NLRP inflammasomes or NLRCs by different kinds of ligands is able to induce the secretion of certain cytokines. Therefore we wanted to know 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 by ELISA method.

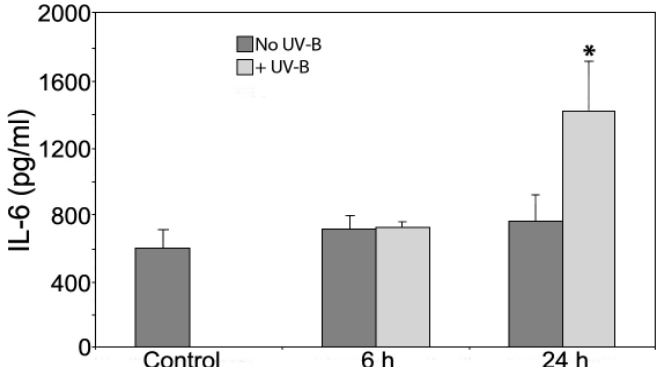

Figure 37. Secretion of IL-6 induced by UV-B irradiation in HCE-T cells
Cells were irradiated with 30 mJ/cm^2 UV-B at 85%–90% confluency, and culture supernatants were harvested at the indicated time points. Control cells were treated in the same way but were not irradiated. The concentration of IL-6 cytokine was determined by ELISA.

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 (Figure 37). 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 innate immune system is able to recognize a large variety of PAMPs or DAMPs by different kinds of germline-encoded PRRs, such as intracellular NLRs [140] and transmembrane TLRs [141]. In our studies we focused on NLRs, which are known to be able to cooperate with TLRs [142]. NLR proteins are expressed in professional immune cells, such as macrophages and dendritic cells and also in ”non-immune cells”, such as epithelial cells. So far 23 members of NLR family have been identified in humans and more than 30 in mice. Through protein-protein interactions, certain members of NLR family assemble to large multiprotein complexes, which are called inflammasomes and signalosomes. While the activation of signalosomes predominantly leads to activation of NFκB and MAPK, the inflammasomes are involved in the activation of caspase-1 leading to the production of IL-1β. The most studied inflammasomes are the NLRP3 inflammasomes. The NLRP3 inflammasome contains NLR family member NLRP3 protein as the sensor of the protein complex, ASC adaptor and caspase-1 effector enzyme. In the course of inflammasome activation the active caspase-1 is responsible for the cleavage of pro-IL-1β to mature cytokine.

IL-1β is a potent multifunctional proinflammatory cytokine, which is involved in inflammatory diseases, including autoinflammatory and allergic disorders. In these diseases the improper inflammasome processing is responsible for the elevated IL-1β secretion and the subsequent inflammatory conditions [89]. Monocytes, macrophages and dendritic cells are the main producers of IL-1β. Physiologically these cells do not constitutively express IL-1β, but in response to activating agents the secretion of IL-1β can be substantially induced. Accordingly, IL-1β is a pivotal inflammatory cytokine, therefore the regulation of the IL-1β production has to be strictly controlled.

Secretion of IL-1β requires two signals. The first (”priming”) step is necessary for the induction of pro-IL-1β (and inflammasome members) expression in a NFκB-mediated process via TLRs [94, 143], such as TLR4 which is triggered by bacterial LPS [144]. The second signal is activated by a large variety of microorganisms, different kinds of endogenous danger signals and environmental irritants [99, 100]. This step is necessary for the activation of the NLRP3, the assembly of the inflammasome and the subsequent cleavage of the precursor pro-

IL-1β into mature IL-1β by the caspase-1 enzyme of the inflammasome. Since the activation of NLRP3 inflammasomes is triggered by an incredibly broad range of activators, it is unlikely that these agents is sensed directly by NLRP3 inflammasomes. Recently it has been hypothesised that agents that activate NLRP3 inflammasome are also able to induce ROS production, and that the produced ROS are required in the signaling mechanisms of inflammasome activation.

ROS play an important role in physiologic conditions [145], but also have a crucial role in the pathogenesis of allergic rhinitis and asthma [146]. The main cause of seasonal allergic rhinitis is the ragweed pollen. It has been demonstrated that ragweed pollen grains and their extracts have intrinsic NADPH oxidase activity, which are similar to those found in phagocytes [113]. The generated ROS induces oxidative stress in the airway epithelium, which is an important contributor to the manifestation of the allergic inflammatory symptoms.

It has been shown that 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 inflammasomes The main sources of IL-1β are considered to be inflammatory cells, such as macrophages. It has also been described that macrophages collected from asthma patients generate higher levels of ROS than in healthy controls [116]. 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, which was differentiated into macrophage cells with PMA. 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 pollens [147, 148]. 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 has 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 [113].

Several studies describe the possibility of synergistic effects of allergens and microbes [149, 150]. It is known that pollen grains are generally contaminated with microbial products, such as LPS. It has been shown that the exposure to LPS-contaminated pollen grains triggers substantially stronger allergic inflammation in patients as compared to that caused by allergen exposure only [151, 152]. However, the distinct mechanisms responsible for allergic airway reactions are only partially understood. One possible mechanism of its development could be

the joint effect of the bacterial LPS and allergic pollens. After inhalation of LPS-contaminated pollens, LPS is able to activate the host's alveolar macrophages and the activated macrophages produce and secrete proinflammatory cytokines and chemokines. The endotoxin content of pollen extract we used is negligible compared to the LPS concentration that we used for the treatments. Since 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 [138], 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 [138]. 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 [138], 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 in THP-1 cells and in human primary macrophages and dendritic cells. However, we found that RWE (in the presence of NADPH) strongly induces the LPS-induced IL-1β secretion of these cells. It is in line with the observations that in the presence of an endotoxin trigger pollen grains substantially exacerbate allergic symptoms [151, 152]. Although various inflammasome complexes have been associated with IL-1β production, such as AIM2, NLRC4, NLRP1 or NLRP3 inflammasomes [153], only NLRP3 inflammasome-mediated IL-1β production has been previously demonstrated to be mediated by intracellular ROS [154, 155]. 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 NADPH-oxidase activity induces ROS production in various epithelial cells [115, 156] and dendritic cells [157]. 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 [156]. Still, in the case of LPS-treatment – in line with previous studies [139] – 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 [36] 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 in 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. We also established that the decreased IL-1β secretion is not due to apoptosis/necrosis because none of the utilized agents showed cell toxicity at the applied concentrations.

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 [154]. 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 [158]. 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 [127].

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 lines [133], 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 obtained from healthy individuals undergoing photorefractive keratectomy (PRK) treatment. 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 PRK-derived primary cells and SV-40 immortalized corneal epithelial cell line (HCE-T).

We found that the gene expression pattern of NLR in the different PRK donors (D1-D5) is approximately uniform. The only exception is the NLRP2 gene expression that shows up to a fivefold difference among the individual PRK samples. 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 [136]. 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 [134]. It has been shown that certain fungi induce the mRNA expression of 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.

Furthermore, we aimed to detect the expression of NLRP1 and NLRP3 proteins in the HCE-T cells using Western blot technique. In our experiments we used the LPS-stimulated THP-1 cells as a positive control to verify the antibodies and to show the protein weights of the NLRP1 isoforms. Two major isoforms are identified for NLRP1, which are produced by alternative splicing. The long (160 kDa) and the short (65 kDa) isoform differ in a 44 amino acid region within the LRR. Little is known about the function of the short isoform. It has been demonstrated that the short isoform is capable of inducing apoptosis but at much lower levels than the long isoform. Our results demonstrate that there are both the longer and the shorter isoforms of the NLRP1 protein in the LPS-treated THP-1 positive control, while only the shorter NLRP1 isoform was detected in HCE-T cell line. Furthermore, we could not detect the NLRP3 protein in HCE-T cells. In our previous studies we also found that LPS did not induce the expression of neither the NLRP1 nor the NLRP3. 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 cornea against pathogens and environmental stress factors. In our previous studies we examined the role of bacterial LPS on the expression of NLRs and their co-acting partners in corneal epithelial cells, as LPS is one of the most abundant components of Gram-negative bacteria, which cause keratitis. We found that LPS effects neither the expression of these proteins, nor the secretion of inflammatory cytokines. It is in line with recent studies that have shown corneal epithelial

cells intracellularly express the LPS receptor TLR4, and that the LPS treatment does not lead to the activation of the cells. Therefore, we decided to study the effect of another stress signal for corneal epithelial cells on the expression of NLRs. One of the major environmental stresses for cornea is the UV-B radiation. Cornea absorbs a large amount of UV-B radiation. UV-B causes 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 [159]. 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 interleukin-1β through a caspase-1-dependent mechanism [160, 161], 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 [162]. These phenomena may increase susceptibility toward infection by pathogens. We also found that corneal epithelial cells do not secrete IL-1β in contrast to 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 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 [163] 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 [135].

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 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.

Összefoglalás

Munkánk során kimutattuk a parlagfű pollen kivonat hatását az LPS-indukált makrofágok IL-1β termelésére. Eredményeink szerint a parlagfű pollen kivonat (NADPH jelenlétében) nem befolyásolja szignifikánsan sem a primer humán makrofágok és dendritikus sejtek, sem a THP-1 makrofágok IL-1β termelését, azonban jelentősen fokozza ezen sejtek LPS-indukált IL-1β szekrécióját. Kimutattuk továbbá, hogy míg a parlagfű pollen kivonat – ellentétben a NADPH-val – önmagában is ROS termelést generál, együttesen fokozatosan növekvő mennyiségű ROS termelést váltanak ki a THP-1 sejtekben. Megállapítottuk, hogy a ROS, mely az LPS-indukált, pollen kivonat által tovább növelt IL-1β termelés során keletkezik, nagymértékben a parlagfű pollen-eredetű NADPH oxidáz enzim működésének következménye. Azt is kimutattuk, hogy a növekedett IL-1β szekréció NLRP3 és kaszpáz-1 enzim-dependens. A pollen kivonat tovább fokozza az LPS által indukált kaszpáz-1, valamint az IL-1β protein expresszióját is. Az IL-1β expressziójában fontos szereppel bíró jelátviteli útvonalak foszforilációs vizsgálatai során azt az eredményt kaptuk, hogy a parlagfű pollen kivonat NADPH-dependens módon indukálja a p38 MAPK és a JNK foszforilációját, valamint jelentősen növeli ezen fehérjék LPS által indukált foszforilációjának mértékét. A parlagfű pollen kivonat az AP-1 komplex c-Jun és c-Fos LPS-indukált foszforilációját is tovább növeli. A parlagfű pollen kivonat valamint az LPS kooperációja arra enged következtetni, hogy a pollen szemek bakteriális endotoxin „szennyezettsége” fontos szerepet játszhat a parlagfű pollen által kiváltott allergiás folyamatok kialakulásában.

A disszertáció alapjául szolgáló másik munkánk során a Nod-like receptorok és az inflammoszómatagok expresszióját vizsgáltuk primer humán korneális epitél sejtekben és a HCE-T humán korneális epitél sejtvonalban. Megállapítottuk, hogy a NOD1, NOD2, NLRX1 és az NLRP1 mRNS hasonló mértékben expresszálódik mind a primer sejtekben, mind a sejtvonalban, azonban míg az NLRP2 és az NLRP10 a sejvonalban, az ASC és a kaszpáz- 1 a primer sejtekben fejeződik ki nagyobb mértékben. Az NLRC5 és az NLRP7 mRNS a detektálhatósági szint közelében fejeződik ki. Munkánk során kimutattuk, hogy UV-B sugárzás hatására a kezelés után 6 órával az általunk vizsgált NLRP és NLRC proteinek, valamint az inflammoszómatagok mRNS expressziója csökkent, míg az NLRP fehérjék mRNS kifejeződése az UV-B sugárzás után 24 órával újra elérte a nem-kezelt sejtekben mért szintet. Az NLRP3 és az NLRP10 esetében pedig magasabb expressziót mutattunk ki a kontrollhoz képest. Az NLRP1 protein kisebb molekulasúlyú izoformáját mutattuk ki a HCE-

T sejtekben, melynek expressziója UV-B hatására az mRNS-éhez hasonlóan változik. Az NLRP3 fehérje jelenlétét nem tudtuk detektálni a HCE-T sejtvonalban. Az általunk vizsgált proinflammatórikus citokinek közül az IL-6 szekréciója mutatható ki a sejtek felülúszójából, melynek mennyisége az UV-B sugárzás hatására a kezelést követő 24 óra múlva nőtt. Megállapítottuk, hogy a korneális epitél sejtvonalban expresszálódó NOD1 és NOD2 funkcionálisan is aktív, így jó modell rendszert nyújthat a korneában található NOD proteinek vizsgálataihoz, melyek új teret nyithatnak a szemet érintő „danger szignál”-eredetű betegségek elleni új terápiás lehetőségek kidolgozásához.

7. Keywords

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

Tárgyszavak

Nod-like receptorok, NLRP3 inflammoszóma, IL-1β termelés, reaktív oxigén gyökök, parlagfű pollen, NADPH oxidáz, makrofágok, korneális epitél sejtek, UV-B

8. LIST OF ABBREVIATIONS

AIM2	absent in melanoma 2
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
CARD	caspase activation and recruitment domain
CITA	MHC class I transactivator
CRP	C-reactive protein
DAMP	danger-associated molecular pattern
DC	dendritic cell
DCFA-DA	2′7′-dichlorofluorescein diacetate
DPI	diphenylene iodonium
dsRNA	double-stranded RNA
ELISA	enzyme-linked immunosorbent assay
GM-CSF	granulocyte-macrophage colony stimulating factor
HCE-T	human immortalized corneal epithelial cell line
IFN	interferon
IL	interleukin
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling protein
MDA5	melanoma differentiation-associated protein 5
MF	macrophage
MitoTEMPO	(2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride . monohydrate
NAC	N-acetyl cysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NBD	nucleotide-binding oligomerization domain
NLR	Nod-like receptor
NLRC	NOD-like receptor family caspase-associated recruitment domain-containing

NLRP	NOD-like receptor family pyrin domain-containing
NOD	nucleotide-binding and oligomerization domain
RT-PCR	real-time polymerase chain reaction
PMA	phorbol myristate acetate
PAMP	pathogen-associated molecular pattern
PGN	peptidoglycan
PRK	photorefractive keratectomy
PRR	pattern recognition receptor
PYD	pyrin domain
RIG-1	retinoic acid-inducible gene 1
RIP2	receptor interacting protein 2
ROS	reactive oxygen species
RWE	ragweed pollen extract
THP-1	human acute monocytic leukemia cell line
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TRX	thioredoxin
TXNIP	thioredoxin (TRX)-interacting protein
Z-YVAD-FMK	caspase-1 Inhibitor (fluoromethylketone), Z-Tyr-Val-Ala-Asp[164]-FMK

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 lost, 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.

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10.2. Publication list prepared by the Kenézy Life Sciences Library

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Register number: DEENKÉTK/111/2014.
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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.
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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

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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.
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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