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Interaction of Human Primary Keratinocytes with Toll-Like Receptor Ligands and Resulting Pro-inflammatory Signals

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2. List of abbreviations

APC	antigen presenting cell
HSP	Heat shock protein
HBD	Human beta defensin
IFN	Interferon
IG	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
LP	Lipoprotein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	mitogen activated protein kinase
MDC	Monocyte-derived dendritic cells
MKK	Mitogen activated kinase kinase
MPLA	Monophosphoryl-lipid-A
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa B
PDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
PI3K	Phosphatidyl-Inositol-3 kinase
PKR	Protein kinase R
PMBC	Poeripheral mononuclear blood cells
Poly (I:C)	Polyriboinosinic-polyribocytidylic acid (equivalent of viral ds RNA)
RIG-1	Retinoid inducible gene-1
SARM	Sterile alpha and HEAT/Armadillo motif
SIT	Specific immunotherapy
SNP	Single nucleotide polymorphism
TBK-1	Tank binding kinase 1
TGF	Transforming growth factor
TH	T-helper
TIRAP	Toll-IL-1 receptor domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor associated factor
TRIF	TIR-containing adaptor inducing IRF-β

3. Introduction

3.1. Skin

The skin is positioned at the interface between an organism's internal milieu and an external environment characterized by constant assault with potential microbial pathogens. While the skin was formerly considered an inactive physical protective barrier that participates in host immune defense merely by blocking entry of microbial pathogens, it is now apparent that a major role of the skin is to defend the body by rapidly mounting an innate immune response to injury and microbial insult. In the skin, both resident and infiltrating cells synthesize and secrete small peptides that demonstrate broad-spectrum antimicrobial activity against bacteria, fungi, and enveloped viruses. Antimicrobial peptides also act as multifunctional immune effectors by stimulating cytokine and chemokine production, angiogenesis, and wound healing.

The epidermis is divided into four or five layers (depending on the type of skin) based on keratinocyte morphology:

Stratum germinativum (also stratum basale or basal cell layer) is the layer of keratinocytes that lies at the base of the epidermis immediately above the dermis. It consists of a single layer of tall, simple columnar epithelial cells lying on a basement membrane. These cells undergo rapid cell division, mitosis to replenish the regular loss of skin by shedding from the surface. About 25% of the cells are melanocytes, which produce melanin which provides pigmentation for skin and hair (Figure 1).

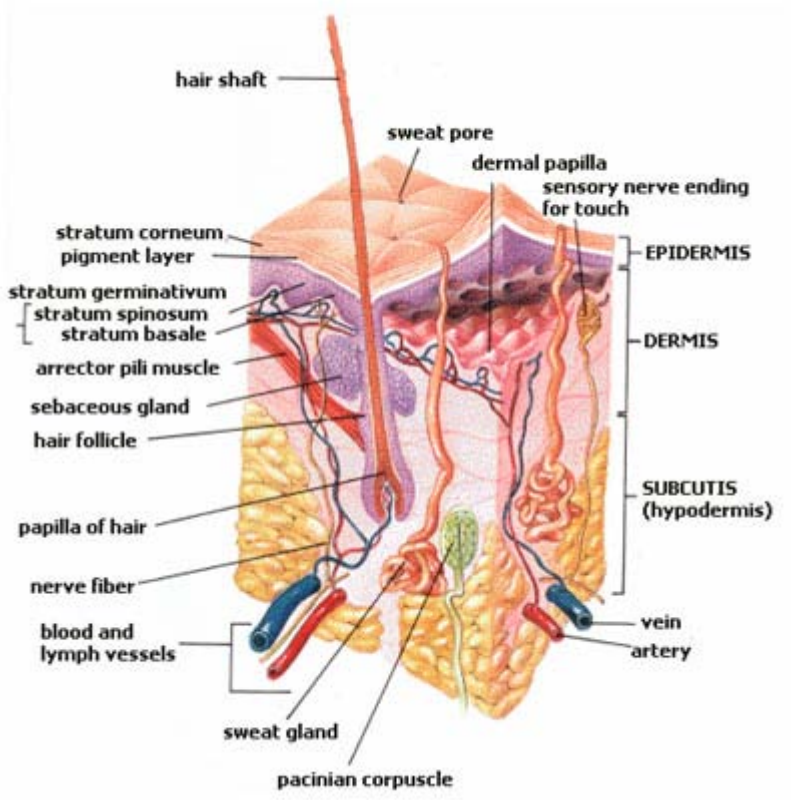


Figure 1. *Anatomic structure of human skin (www.web-books.com)*

The stratum spinosum is a multi-layered arrangement of cuboidal cells that sits beneath the stratum granulosum. Adjacent cells are joined by desmosomes giving them the spiny appearance from which their name is derived. Their nuclei are often darkened (a condition called pyknosis), which is an early sign of cell death. Their fate is sealed because the nutrients and oxygen in interstitial fluid have become exhausted before the fluid is able to reach them by diffusion. Cells of the stratum spinosum actively synthesize intermediate filaments called cytokeratins which are composed of keratin. These intermediatefilaments are anchored to the desmosomes joining adjacent cells to provide structural support, helping the skin resist abrasion (Figure 1).

In microscopic views of skin, the stratum granulosum layer of the epidermis lies between the stratum spinosum below and the stratum lucidum above. This layer typically contains 1 to 3 rows of squamous cells with many small basophilic granules

in their cytoplasm. These keratohyalin granules are a step in the synthesis of the waterproofing protein keratin, and contain large amounts of filaggrin. This is the highest layer in the epidermis where livingcells are found, the stratum lucidum above appears clear due to auto-digestion of cellular organelles.

The stratum lucidum (Latin for "clear layer") is a thin, clear layer of dead skin cells in the epidermis, and is named for its translucent appearance under a microscope. It is found beneath the stratum corneum of thick skin, such as that on the palms of the hands and the soles of the feet. The keratinocytes of the stratum lucidum do not feature distinct boundaries and are filled with eleidin, an intermediate form of keratin. The cells of the stratum lucidum are flattened and contain an oily substance that is thought to be the result of lysosome disintegration. It is this substance that gives the st ratum lucidum its waterproof properties and thus, it is also called the *barrier* layer of the skin (Figure 1).

The stratum corneum ("the horny layer") is the outermost layer of the epidermis (the outermost layer of the skin). It is composed mainly of dead cells that lack nuclei. As these dead cells slough off, they are continuously replaced by new cells from the stratum germinativum (basale). In the human forearm, for example, about 1300 cells/cm^2/hr are shed and commonly accumulate as house dust. Cells of the stratum corneum contain keratin, a protein that helps keep the skin hydrated by preventing water evaporation. In addition, these cells can also absorb water, further aiding in hydration and explaining why humans and other animals experience wrinkling of the skin on the fingers and toes (colloquially called "pruning") when immersed in water for prolonged periods. The thickness of the stratum corneum varies according to the amount of protection and/or grip required by a region of the body. For example, the hands are typically used to grasp objects, requiring the palms to be covered with a thick stratum corneum. Similarly, the sole of the foot is prone to

injury, and so it is protected with a thick stratum corneum layer. In general, the stratum corneum contains 15 to 20 layers of dead cells. In reptiles, the stratum corneum is permanent, and is only replaced during times of rapid growth, in a process called ecdysis or moulting. The stratum corneum in reptiles contains beta-keratin which provides much more rigid skin layer (Figure 1).

3.1.1. Skin a Physical Barrier

Physical structures prevent most pathogens and environmental toxins from harming the host. The skin and the epithelial lining of the respiratory, gastrointestinal, and the genitourinary tract provide physical barriers between the host and the external world. Skin, once thought to be an inert structure, plays a vital role in protecting the individual from the external environment. The epidermis impedes penetration of microbial organisms, chemical irritation, and toxins, absorbs and blocks solar and ionized radiation, and inhibits water loss.

3.1.2. Skin Immune System

The immune system of vertebrates has two components: Innate immunity and adaptive immunity. These two systems utilize two very different mechanisms for host defense. While adaptive immunity occurs only in vertebrates, the innate immune system exists in all multicellular organisms. Defense mechanisms that are used by the host immediately after encountering a forigne ligand are reffered to as innate immunity.The innate immune system relies on a set of germline-encoded receptors that are expressed on a wide variety of cells, like macrophages and neutrophils as well as on epithelial cells situated at host-environment boundaries. The skin represents the largest organ of the human body. In addition to its structural functions, a specific immunological environment has developed in the skin.

3.2. Langerhans cells

Several different dendritic cells (DCs) populations have been identified, each of which is differentiated via a unique pathway. Two DC subsets of myeloid origin have been described: Langerhans cells (LCs), present in the epidermis, which take up antigen and subsequently migrate to local lymph nodes to differentiate into DCs; and myeloid-lineage-derived DCs, located in the dermis, blood, and B-cell follicles, which lack LC markers (Vissers et al., 2001).

Cutaneous DCs (LCs as well as dermal DCs) function as sentinels that survey invading agents and transmit the information into immune responses by taking up exogenous antigens, migrating to local draining lymph nodes, and presenting the processed antigens to T cells resulting in T-cell differentiation and activation which means initiatation of protective immunity (Johnston et al., 2000).

LC not only act as professional antigen presenting cells to induce antigen-specific T cells for adaptive immune responses, but they also initiate a cascade of innate immune responses by sensing these danger signals. However, recent studies challenge the classical paradigm to position LC in the center of cutaneous immunity. Although LC express Toll-like receptors (TLRs) that recognize bacterial and viral products, exposure to pathogen-associated TLR ligands triggers neither sufficient LC maturation nor good production of cytokines and chemokines. LC also lack the ability to produce IFN-gamma by any stimuli, moreover, recent data suggest that LCs may not directly present viral antigens to T cells for their activation in mouse models of cutaneous viral infection. The alternative player in protective immune responses may be surrounding keratinocytes, which may modulate LC functions indirectly (Asahina and Tamaki, 2006) .

3.3. Keratinocytes

The keratinocytes are the major cell type of the epidermis, making up about 90% of epidermal cells. Once thought to be inert, keratinocytes can mount an immune response through secretion of light levels of several chemokines and antimicrobial peptides IL-1, IL-6, IL-8, IL-10, TNF-α, IFNs and β-defensins. More over, in response to different stimuli, keratinocytes are able to produce a broad panel of interleukins, chemokines, cytokines and cytokines receptors.

3.4. Toll-like receptors (TLRs)

The discovery of the TLR as sensors of microbial molecules transformed the views of discrimination between self and non-self, a key requirement of any immune system. It turns out that much of microbial recognition is served by only a handful of TLR. (TLRs) function as key regulators of both innate and adaptive immunity. TLRs are germline encoded type I transmembrane receptors which are expressed on numerous cell types including macrophages and dendritic cells (Carpenter and O'Neill, 2007). They function as pathogen recognition receptors (PRRs), recognizing pathogen-associated molecular patterns (PAMPs) which are unique to microbes and essential for their survival. Through the recognition of PAMPs the innate immune system is capable of discriminating between self and non-self. The term Toll was originally coined for a cell surface receptor governing dorsoventral orientation in the *Drosophila* embryo (Stein and Stevens, 1991). Toll was then found to be involved in immune defence in *Drosophila* specifically against the invading fungus *Aspergillus fumigatus* (Lemaitre et al., 1996). A human homologue to *Drosophila* Toll was identified 1 year later (Medzhitov et al., 1997) . A total of 10 TLRs are expressed in humans. Each TLR responds to distinct PAMPs, leading to the activation of specific signalling pathways. TLRs are characterized by the presence of an extracellular

leucine-rich repeat domain (LRR) and an intracellular Toll/IL-1 receptor (TIR) domain (Rock et al., 1998; Akira and Takeda, 2004b; Takeda and Akira, 2004; Takeda and Akira, 2003) (Figure 2).

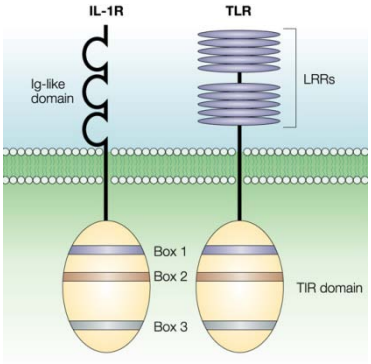


Figure 2. *TLR structure and signalling. Toll-like receptors (TLR) and interleukin-1 receptors (IL-1Rs) have a conserved cytoplasmic domain, known as the Toll/IL-1R (TIR) domain. The TIR domain is characterized by the presence of three highly homologous regions known as boxes 1, 2 and 3. Despite the similarity of the cytoplasmic domains of these molecules, their extracellular regions differ markedly: TLR have tandem repeats of leucine-rich regions (known as leucine rich repeats, LRR) whereas IL-1Rs have three immunoglobulin (Ig)-like domains (Akira and Takeda, 2004).*

LRRs are found on a diverse number of proteins and are involved in ligand recognition and signal transduction (Kobe and Deisenhofer, 1994; Kobe and Deisenhofer, 1995). The LRR domain is separated from the transmembrane region by a LRR carboxy-terminal domain. The TIR domain portion of the TLR is required for intracellular signalling. This domain spans about 200 amino acids, with varying degrees of sequence similarity among family members. Three particular boxes can be identified which are highly conserved among family members. Box 1 is considered the signature sequence of the family whereas boxes 2 and 3 contain amino acids critical for signalling. The crystal structure of the TIR domains of TLR1 and TLR2 has revealed a core structural element centred around box 2 (Xu et al., 2000). This region, termed the BB loop, forms an exposed surface patch and contains a critical proline or arginine residue. These amino acids are located at the tip of the loop and

are thought to form a point of contact with downstream signalling components. Toll-like receptors are just one class of PRRs. The Nod-like receptors (NLR) family are intracellular microbial sensors. They recognize microbial components present in the cytosol (Strober et al., 2006). The structural domains of the NLRs include a Pyrin domain or a caspase recruitment domain (CARD), which is believed to be involved in receptor binding. They also contain a nucleotide binding domain and the C-terminus is made up of leucine-rich repeats which are involved in ligand binding (Creagh and O'Neill, 2006). Although NLRs have specific functions in the innate immune system these roles can involve TLRs. TLRs can induce pro-IL-1b production and prime NLR multiprotein complexes (also known as 'inflammasomes') to respond to bacterial ligands and produce IL-1b and IL-18. The retinoic acidinducible gene (RIG)-like receptor family (RLR) include RIG-I, which is also a cytosolic receptor-recognizing double-stranded RNA (dsRNA) from viruses. Melanoma differentiation-associated gene (MDA5) is another member of the RLRs (Creagh and O'Neill, 2006).

3.4.1. TLR1/2/6

TLR2 seems to be capable of recognizing a large spectrum of microbes. This is partly due to its ability to form heterodimers with TLR1 and TLR6 (Carpenter and O'Neill, 2007). TLR2 responds to lipoproteins and lipopeptides from a wide range of pathogens including *Borrelia burgdorferi*, *Treponema pallidun* and *Mycoplasma fermentans* (Khor et al., 2007). Lipoproteins present in bacteria that are triacylated are recognized by TLR1/TLR2. This was demonstrated when TLR1-deficient macrophages showed impaired cytokine production when challenged with triacylated lipopeptides (Takeuchi et al., 2002). TLR2/TLR6 heterodimer responds to diacylated lipopeptides and can also respond to Zymosan, a component of the cell membrane of fungi (Underhill et al., 1999; Kataoka et al., 2002). TLR2- deficient cells confirmed

that TLR2 can act as a receptor for components of Gram-positive bacteria as they were highly susceptible to infection with *Staphylococcus aureus* and *Streptococcus pneumonia* (Echchannaoui et al., 2002; Takeuchi et al., 2002; Mempel et al., 2003). TLR2 and TLR6 knockout mice infected with *Mycobacterium tuberculosis* showed decreased TNF-a, IL-1b and transforming growth factor (TGF)-b mRNA levels. However, they showed increased levels of IL-4 and IL-6 (Sugawara et al., 2003). Other TLR2 ligands include lipotechoic acid from Grampositive bacteria, lipoarabinomannan from mycobacterial cell walls, porins present in *Nesseria* and GPI anchors and glycoinositolphopholipids from *Trypanosoma cruzi* (West et al., 2006).

3.4.2. TLR3

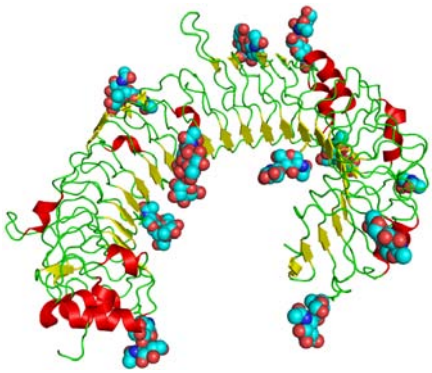


Figure 3. *Schematic 3D structure of TLR3 (http://en.wikipedia.org/wiki/image)*

Double-stranded RNA (ds RNA) is naturally a viral product which is produced by viruses during their life cycle and it is recognized by TLR3. Recently the crystal structure of the human TLR3 ectodomain has been obtained (Choe et al., 2005). This structure reveals that TLR3 is mostly masked by carbohydrate but there is one face which is glycosylation free and it is predicted that this is the region to which dsRNA binds. TLR3-deficient cells show impaired responses to the synthetic dsRNA ligand polyinosine-polycytidylic acid (Poly (I:C)) (Alexopoulou et al., 2001). Upon activation, TLR3 signals, in a MyD88- independent manner, through Toll-IL-1R domain-

containing adaptor-inducing IFN- (TRIF) to up-regulate type I IFN (Kirk and Bazan, 2005). TLR3 signals also lead to the activation of NF-κB and type I interferons (IFNs) (Carpenter and O'Neill, 2007) (Figure 4).

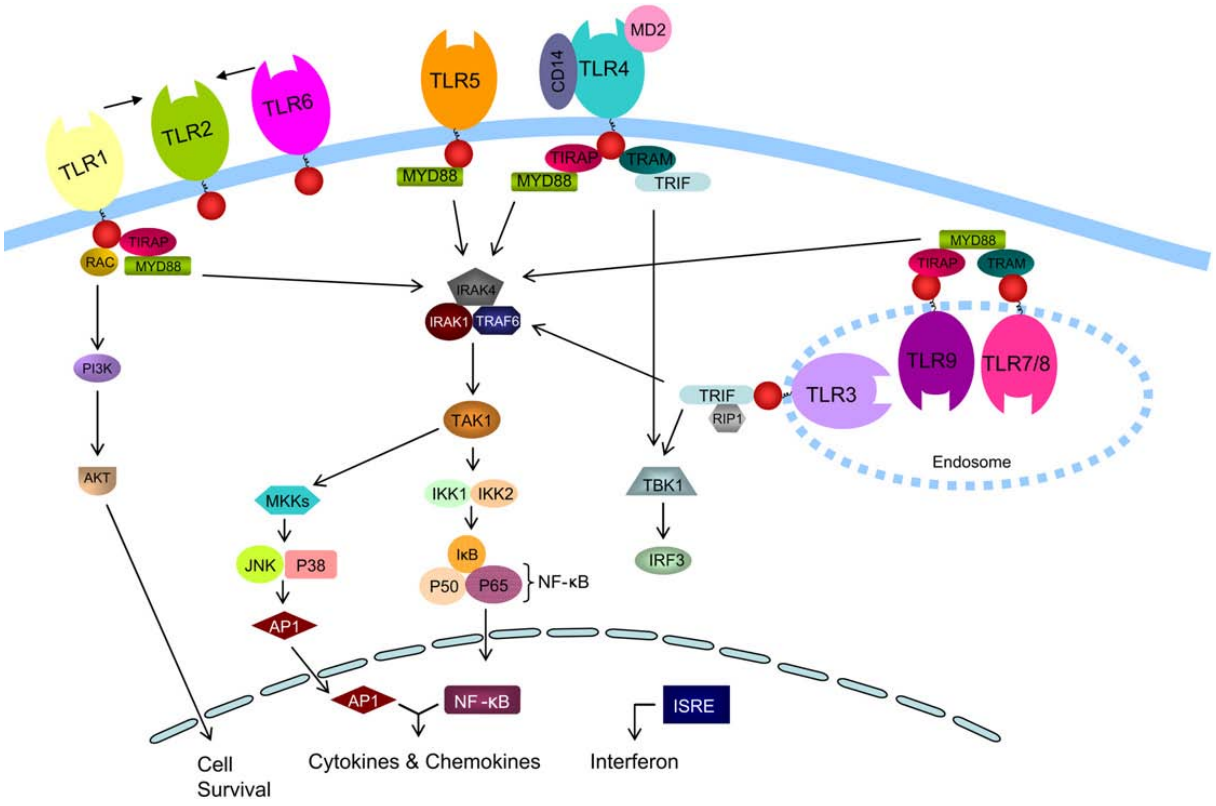


Figure 4. *Schematic illustration of TLRs signalling pathways (Mempel et al., 2007).*

Although viral dsRNA is known to be sensed by TLR3, DCs or fibroblasts that lack TLR3 still produce type I IFNs after intracellular introduction of dsRNA molecules. This TLR3-independent induction does not require TRIF, but depends on the kinase TBK1 (TANK-binding kinase-1) and the transcription factor IRF-3 (IFN regulatory factor-3) (Hemmi, Takeuchi et al. 2004; Wagner and Bauer 2006). Therefore, TLR3 is the only TLR which does not use the crucial adaptor molecule myeloid differentiation primary response gene-88 (MyD88) for intracellular signal transmission (Hemmi et al., 2004). In addition to direct NF-κB activation, TLR3 can also use an alternative signalling pathway leading to the activation of TANK-binding kinase-1 (TBK-1), which results in the phosphorylation and nuclear translocation of

the transcription factors interferon regulatory factor-3 (IRF3) and IRF7 (Sankar et al., 2006) followed by the production of type I interferons, namely IFN-β (Jiang et al., 2002).

Although viral dsRNA is known to be sensed by TLR3, dendritic cells or fibroblasts that lack TLR3 still produce type I interferons after intracellular introduction of dsRNA molecules. This TLR3-independent induction does not require TIR domain-containing adapter including IFN-β (TRIF), but depends on the kinase TBK-1 and the transcription factor IRF3 (Hemmi et al., 2004). In this pathway, the dsRNA is recognized by the cytoplasmic helicase domain of the RNA helicase protein retinoic acid–inducible gene-I (RIG-I). The downstream signaling events that result from this recognition require the NH2-terminal caspase recruitment domain (CARD) of RIG-I, which binds to the adaptor molecule Cardif (also known as IPS-1) (Yoneyama et al., 2004; Meylan et al., 2005). Another candidate for the sensing of cytoplasmic dsRNA is MDA5 (melanoma differentiation-associated gene 5 or Helicard) which is also interferon-inducible and belongs, like RIG-I, to the DExD/H-box-containing RNA helicases (Yoneyama et al., 2005). The third TLR3-independent mechanism by which mammalian cells recognise dsRNA is the dsRNA-dependent protein kinase R (PKR). PKR is activated upon binding of dsRNA and then undergoes dimerization and autophosphorylation. This 68-kDa, cytoplasmic serine/threonine kinase phosphorylates its physiological substrate eukaryotic initiation factor 2-α (eIF2-α) and inhibits translation and perhaps other substrates which results in activation of a panel of genes that ultimately leads to cessation of virus replication in infected cells (Parker et al., 1995; Clemens and Elia, 1997; Lemaire et al., 2005). It has also been shown that PKR regulates other pathways, including p53, p38, IRF1, and NF-κB (Kumar et al., 1997; Goh et al., 2000; Lemaire et al., 2005). Induction of NF-κB has a relevant role in mediating PKR functions, and NF-κB activation by PKR is involved in IFN-β

induction in response to dsRNA (Kline et al., 1999). Little, if anything, however, is known on the relative contribution of the various dsRNA sensing and signalling pathways in human keratinocytes. As viral infections such as human herpes virus or papilloma virus infections are common in human skin with keratinocytes as the targets for viral attack, the ability to induce a sufficient antiviral response appears to be crucial during the earliest phases of the response when innate defense mechanisms dominate (Kalali et al., 2008).

3.4.3. TLR4

TLR4 is the first described human homologue of *Drosophila* Toll (Medzhitov et al., 1997). Lipopolysaccharide (LPS) is the major component of the cell wall of Gram-negative bacteria. It is a potent immunostimulant and can cause the endotoxic shock. LPS consists of a lipid a portion which is the endotoxic component, a core oliogosaccharide and an O-antigen. To respond to LPS, TLR4 requires other coreceptors include CD14 which is a glycosylphosphatidyl inositol (GPI)-anchored glycoprotein (Wright et al., 1990) and MD2. LPS binds to LPS binding protein in serum which transfers LPS monomers to CD14 which in turn concentrates the LPS to allow binding to TLR4/MD2 complex (Takeda and Akira, 2003). This, triggers a pathway which leads to the activation of the transcription factor NF-κB which regulates the transcription of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, IL-1 and IL-8. It can also activate other transcription factors include members of the mitogen-activated proteins kinase family (MAPK) notably p38 and JUN N-terminal kinase (JNK) (Carpenter and O'Neill, 2007). TLR4 is also capable of responding to components of fungal pathogens such as mannans from *Saccharomyces cerevisiae* and *Candica albicans* and glucuronoxylomannan from *Cryptococcus neoformans* (Shoham et al., 2001; Netea et al., 2004; Netea et al.,

2006;; Netea et al., 2004b). Other TLR4 ligands include taxol (Perera et al., 2001; Toshchakov et al., 2003) and the fusion protein from respiratory syncytial virus (Kurt-Jones et al., 2000).

3.4.4. TLR5

TLR5 is predominantly expressed on epithelial cells, monocytes and immature cells. It is localized to the basolateral surface of intestinal epithelial cells where it is capable of recognizing flagellin from bacteria which have invaded the epithelia (Gewirtz et al., 2001). Flagellin is a 55 kDa monomeric component of bacterial flagella (Hayashi et al., 2001). A study on TLR5 knockout mice highlighted redundancy within the TLRs. It appears that TLR4 can function to induce antimicrobial responses in TLR5-deficient mice challenged by *S. typhimurium* and *Pseudomonas aeruginosa* (Feuillet et al., 2006). A recent study has shown that some bacteria are capable of evading recognition by TLR5 by possessing flagellin which has no immunostimulatory properties; these include *Helicobacter pylori* and *Campylobacter jejuni*. TLR5 knockout mice have been shown to be susceptible to *Escherichia coli*-induced urinary tract infection (ndersen-Nissen et al., 2007). This is the first evidence to implicate TLR5 in host protection in the urinary tract.

3.4.5. TLR7/8

TLR7 and TLR8 are structurally quite similar and are both localized to endosomal compartments. TLR8 is non-functional in mice. Murine TLR7 and human TLR8 both respond to a range of synthetic antiviral compounds including resiquimod (R848) and loxoribine, imiquimod (Hemmi et al., 2002; Tyring et al., 2002; Lee et al., 2003; Schon et al., 2003; Heil et al., 2004). It has recently been shown that TLR7 and TLR8 respond to guanosine (G)- and uridine (U)-rich singlestranded RNA (ssRNA) from the human immunodeficiency virus-1 (HIV-1) (Heil et al., 2004). TLR7 and TLR8

respond to ssRNA viruses such as *Dengue* and *Influenza.* TLR7-deficient dendritic cells showed impaired responses to ssRNA and failed to release IFN-a or TNF-α (Lund et al., 2004).

3.4.6. TLR9

Studies carried out on TLR9-deficient mice have shown that unmethylated CpG is the natural ligand for TLR9 (Hemmi et al., 2000). TLR9-deficient mice did not produce any inflammatory cytokines in response to CpG, their splenocytes failed to proliferate and there was no maturation of dendritic cells (Carpenter and O'Neill, 2007). TLR9, like TLR7 and TLR8, is localized to endosomal compartments which may allow them to discriminate between self and non-self, as host DNA and RNA tend not to enter into endosomal compartments (Barton et al., 2006). TLR9 has been shown to be able to respond to MCMV and herpes simplex virus (HSV), presumably through the presence of unmethylated CpG within these viral genomes (Krug et al., 2004).

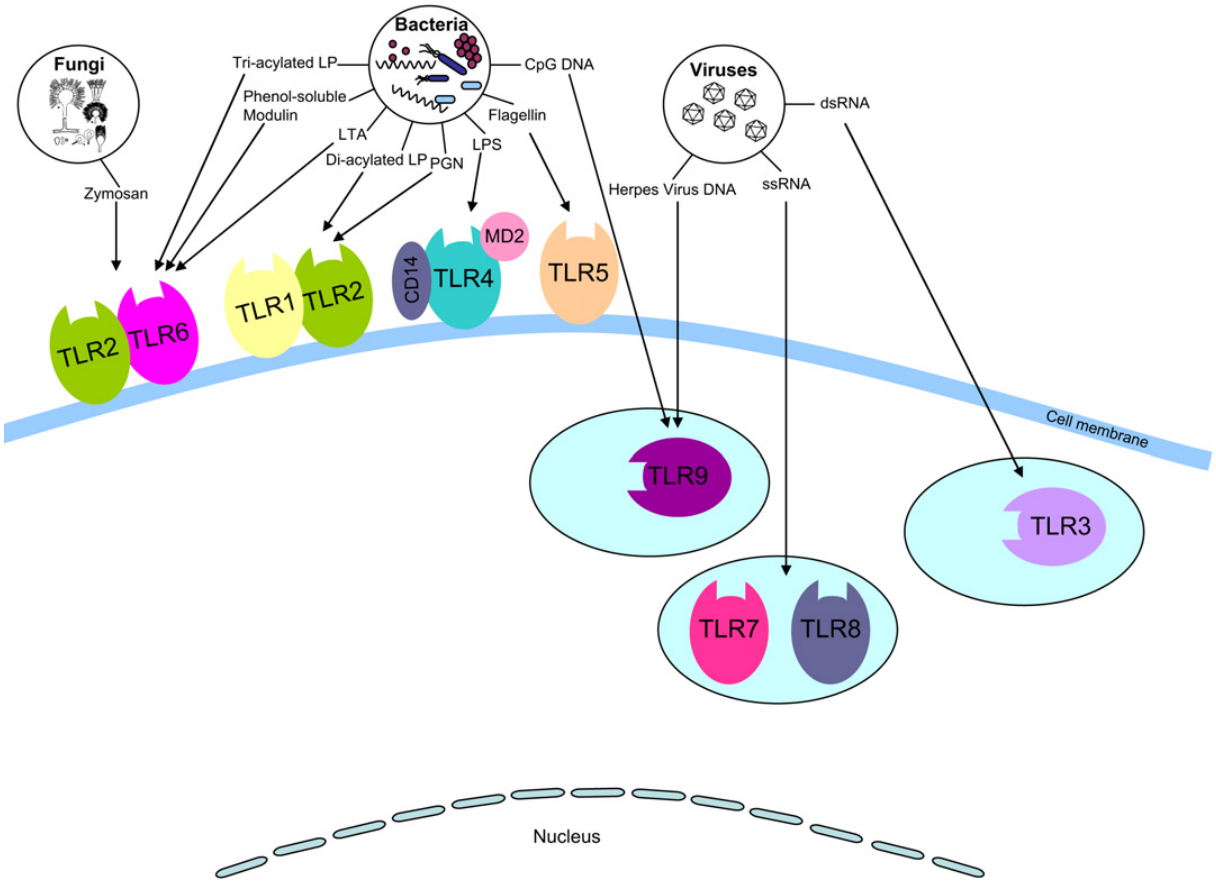


Figure 5. *Cellular localization and ligands for the family of TLRs. Although the surface-expressed TLRs recognize bacterial compounds, the intracellular receptors are active against virus-associated molecules (Mempel et al., 2007).*

3.4.7. TLR10

No ligand has yet been reported for TLR10, therefore it remains the only orphan member of TLR family. Activation of TLRs is believed to involve receptor oligomerization, and the current view is that the receptors are active as either homo- or heterodimers, depending on the receptor type. The cytoplasmic TIR domains dimerize to form a platform for the recruitment of adaptor proteins and additional signaling molecules. TLR10 has been shown to form homodimers and to interact with TLRs 1 and 2 and signal through the common TIR adaptor MyD88 (Hasan et al., 2005; Nyman et al., 2008).

3.5. TLRs signalling pathway

3.5.1. MYD88

MyD88 was the first adaptor protein identified and it is used by all TLRs except TLR3. The main evidence for the role of MyD88 in TLR signalling came from the study of MyD88-deficient mice. These mice were unresponsive to PAMPs detected by TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 (Takeuchi et al., 2000a; Takeuchi et al., 2000b; Carpenter and O'Neill, 2007). They were hyporesponsive to LPS and were susceptible to infection by a number of bacteria including *S. aureus* (Takeuchi et al., 2000a), *T. gondii* (Scanga et al., 2002), *Listeria monocytogenes* (Edelson and Unanue, 2002) and *M. tuberculosis* (Quesniaux et al., 2004; Ryffel et al., 2005). There were no reported developmental defects in the MyD88 knockout mice. Despite the fact that MyD88 knockout mice are susceptible to a number of bacterial infections, they have been shown to still be *Toll-like receptors as pathogen sensors* capable of mounting an adaptive immune response (Ryffel et al., 2005). Therefore, the role of MyD88 in initiating an adaptive immune response is dependent on the infection. MyD88 has a TIR domain like TLRs, and it seems that it interacts with TLRs through a TIR–TIR interaction. MyD88 recruits interleukin 1 receptorassociated kinase 4 (IRAK-4) which triggers phosphorylation of IRAK1 (Suzuki et al., 2002; Khor et al., 2007). IRAK1 can activate TRAF6. Both proteins leave the receptor complex and interact with TGF-b-activated kinase 1 (TAK1) and two TAK1 binding proteins TAB1, TAB2 (Jiang et al., 2002). TAK1 becomes phosphorylated and activates the IkB kinase (IKK) complex (Wang et al., 2001a; Wang et al., 2001b). IkB is then phosphorylated allowing NF-κB to translocate to the nucleus and induce expression of proinflammatory cytokines (Figure 4). TAK1 is also capable of phosphorylating MKK6 and 7 which leads to the activation of p38 and JNK.

The family of TLRs has shows great similarities with the IL1 receptor especially in its intracytoplasmatic domain with a highly conserved Toll-interleukin 1 receptor (TIR) motif (Bowie et al., 2000; Bowie and O'Neill, 2000). For the TIR domain five different adaptor molecules have been described, namely MyD88, TIRAP, TRIF, TRAM, and SARM of which the first four have activating functions and SARM inhibitory capacities (Carty et al., 2006b). The extracellular part is composed of repetitive leucine residues (leucine rich repeat). The intracytoplasmatic signaling structure is grossly divided in a MyD88-dependent and independent pathway both of which show recruitment of crucial adaptor molecules to the Toll-IL-1 receptor motif (Hashimoto et al., 1988). The majority of TLRs (namely TLR1,2,4,5,6,7,8, and 9) use the myeloid differentiation factor 88 (MyD88) to initiate a signaling cascade which leads to the downstream activation of kinases and which results in the translocation of the central transcription factors NF-κB and IRF-3 (Kawai and Akira, 2006). To this end, MyD88 associates with TIRAP to a complex which then recruits IRAK and subsequently TRAF-6 (O'Neill et al., 2003) finally resulting in an activation of the IKK complex (Figure 4). In MyD88-independent signaling which is used by TLR3 and to a certain degree also by TLR4, the adaptor molecule TRIF is recruited to the intracellular part of TLR3 directly (Yamamoto et al., 2003) or to tTLR4 via TRAM (Fitzgerald et al., 2003) which in consequence leads to activation of both, TBK-1 and TRAF-6, a crucial checkpoint for the induction of a NF-κB-dominated immune response or a IRF-3-dominated immune response with an type I interferon activation pattern (Fitzgerald et al., 2003). Interestingly, it has become clear in the last years that the recruitment of TRAF-6 and TBK-1 is mutually exclusive, most probably due to steric hindrances which leads to the exclusive activation of the NF-κB or IRF-3 pathway depending on the accessibility of the binding sites (Kang and Chae, 2001).

The divergent activation of these two transcription factors then leads to a preferential state of pro-inflammatory immune responses (as for NF-κB) or antiviral immune responses (as for IRF-3). This activation has been shown to be under the negative regulatory control of SARM, an alternative adaptor molecule for the TIR domain (Carty et al., 2006a) (Figure 4).

3.6. Consequences of TLR activation

As mentioned above, activation of NF-κB is one of the central activation pathways after recognition of TLR-ligands. This activation not only provides the production of various chemo- and cytokines but also enhances the capacity of phagocytotic cells to ingest microbial compounds. As some of the TLRs, namely TLR2, and 4 are able to co-localize to phagosomes (Underhill et al., 1999; Blander and Medzhitov, 2004), a very early contact of the immune system with potentially hazardous microbial antigens is provided. Within the phagosome, two types of innate receptor co-operate to determine the type and magnitude of the immune response. These are phagocytic receptors such as the mannose-receptors and PAMP-receptors such as the TLRs (Blander and Medzhitov, 2006). This combination not only provides a maturation signal for the phagossomes, it seems also a crucial help to distinguish self-antigens (which should not be loaded on MHC-II molecules) and foreign antigens (which should be efficiently loaded on MHC-II molecules (Blander and Medzhitov, 2006). In addition to trigger phagocytotic and maturation signals, the engagement of TLRs also enhances co-stimulatory molecule expression such as CD80 and CD86 providing a second signal for the full immune response (Tsuji et al., 2000; Michelsen et al., 2001). Thus, the engagement of TLRs in the phagosome enhances killing of captured microorganisms and the efficient degradation of the ingested proteins for presentation on the cell surface.

The situation is different for the group of intracellular TLRs (TLR 3, 7, 8, and 9) which are located on the endosomal membrane where they sense virus derived pattern molecules and respond with the induction of antiviral genes such as type I interferons (Wagner, 2004). Besides the induction of soluble factors, the engagement of intracellular TLRs also enhances the presentation of antigens by the MHC-I pathway leading to the activation of CD8 cells which are the central weapon against virally infected cells (Perera et al., 2001; Heit et al., 2003). Besides their central role in triggering and shaping the cellular immune response, TLR activation also results in the production of antimicrobial defensins. Thus, several epithelial cell types including airway epithelial cells and keratinocytes react to the stimulation through TLR2 or TLR4 by the up-regulation of human β-defensin 2 (Becker et al., 2000; Hertz et al., 2001). Interestingly, after induction of HBD-2, this antimicrobial peptide itself can act as a ligand for the TLR4 and thereby enhance the immune response. In addition to defensin production (Biragyn et al., 2002), TLR-engagement also induces reactive oxygen and nitrogen species which are crucial for the killing of intracellular pathogens such as Mycobacterium tuberculosis. Of note, in contrast to murine cells the induction of mycobacterial killing in human macrophages and dendritic cells requires the parallel engagement of the vitamin D receptor (Liu et al., 2006).

The most important feature of TLR activation however, is the production of pro-inflammatory milieu which is provided by certain cyto- and chemokines. These are predominately TNFα and IL-12 for NF-κB signaling TLR-ligands and IFNα/β for IRF-3 signaling TLR-ligands. In fact, the combination of the induction of a robust cellular immune response together with a rapid skewing of the immune response towards a TH-1 dominated profile have rendered TLR-agonists interesting adjuvants for allergy treatment and the design of tumor vaccines (Dabbagh and Lewis, 2003).

Finally, the engagement of TLRs in some tissues leads to the induction of programmed cell death, using caspase 8 and the FAS-associated death domain protein. This pro-apoptotic effect has been shown for mycobacterial, bacterial, and mycoplasmal lipoproteins and to signal through TLR2 and TLR4 (Lopez et al., 2003; Into et al., 2004).

In summary, the sensing of microbial patterns through TLRs leads to the induction of a robust immune response of the TH-1 type including the induction of potent self-defense molecules, and the efficient conversion of immature antigen presenting cells in fully equipped amplifier of a cellular immune response. In tissues not capable of specific antigen presentation, TLR signaling might also induce apoptosis to minimize the spreading of infection.

3.7. Aim of the study

The presented work aims at identifying the expression patterns of Toll-like receptors in human keratinocytes, at evaluating their functional relevance, and identifying the central down-stream events. A special focus was attributed to the response of human keratinocytes to double-stranded RNA and to the alternative recognition patterns for this particular danger signal such as protein kinase R, retinoid inducible gene I, and melanoma differentiation antigen-5.

4. Methods and Materials

4.1. Cell culture and reagents

Primary human keratinocytes were obtained from neonatal foreskins and cultured in keratinocyte serum-free medium (Gibco/Life Technologies, Eggenstein, Germany) In brief, surgical specimens of human foreskin were cut into pieces of 0.5 cm2 and exposed to dispase 2.4 U per mL (Roche, Mannheim, Germany) for 12 h at 41C. The epidermis was then mechanically removed from the dermal layer, homogenized by repeated aspirations in Pasteur pipettes, and incubated in ethylenediamine tetraacetic acid (EDTA) trypsin 0.25% (Roche) for 60 min. Cells were washed three times in SFM (Gibco/Life Technologies, Eggenstein, Germany), stained for viability with 0.5% trypan blue (Sigma), and seeded into 75 cm2 culture flasks (Becton Dickinson, Heidelberg, Germany) with keratinocyte SFM. Cells were kept at 371C and 5% CO2.

Whole human epidermis was obtained according to the same procedure. Condyloma specimens were obtained during routine surgery and were snap-frozen in liquid nitrogen. For *in vitro* stimulation assays of human keratinocytes the following substances were used: Poly (I:C) (Invivogen, CA, USA), flagellin purified from *Salmonella typhimurium* (Invivogen), and gardiquimod (GDQ) (Invivogen). For inhibition experiments of Poly (I:C)-induced keratinocyte responses, the following inhibitors were selected: 2-aminopurine (2-AP; PKR inhibitor) (Sigma, Deisenhofen, Germany), bafilomycine A1 (BFA; TLR3 inhibitor) (Sigma) and SU6668 (TBK-1 inhibitor) (kindly provided by GPC Biotech, Munich, Germany).

For our study in human mDCs, highly atopic individuals were classified by their total IgE levels of at least 1000 IU/ml and by at least one specific sensitization

within a panel of commonly screened environmental allergens (DPC Biermann, Germany). All atopic individuals suffered from at least one of the typical atopic diseases such as eczema (AE), rhinoconjunctivitis (RCA), and bronchial asthma (AB). Healthy control individuals lacked all of these characteristics and showed total IgE values of < 70 IU/ml. A detailed summary of the included individuals is given in table 1. All AE-patients were treated with topical corticosteroids at the time of study recruitment and systemic treatment (corticosteroid, cyclosporine A) 3 months prior to blood withdrawal disqualified from participation in the study. All individuals gave informed consent for a venous puncture and withdrawal of 50 ml heparinized blood.

4.2. Cytokine and chemokine immunoassays

To analyse IL12 and TNF production in mDCs, 5 x 105 mDCs were stimulated for 24 hours with the respective ligands before culture supernatants were recovered and diluted 1:10 in PBS. ELISA kit for hIL-12 and hTNF-α (eBioscience, San Diego, USA) were used according to the manufacturer's instructions.

IL-8 in keratinocytes culture supernatants was quantified using a standard sandwich ELISA. Microtiter plates (Costar, Cambridge, MA, USA) were coated with an unconjugated anti-IL-8 capture antibody (R&D Systems, MN, USA) overnight (15 h), followed by incubation with blocking buffer for 1 h. Wells were then incubated for 2 to 3 h with culture supernatant samples or varying concentrations of recombinant human IL-8 (0-2,500 pg/ml). Detection of IL-8 was achieved using a biotinylated anti-IL-8 antibody (BD Biosciences, Heidelberg, Germany). The plate was developed using Immunopure HRP-conjugated streptavidin (Pierce, IL, USA) and ABTS Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories, MD, USA). The absorbance at 405 nm was read using a microtiter plate reader (Cambridge Technology, MA, USA), and concentrations of IL-8 were calculated from

a standard curve of recombinant human IL-8 (R&D Systems) and normalized according to the total protein levels of related samples measured by using a standard Bradford protein assay (Pierce). IFN-β was measured in culture supernatant using an ELISA kit according to the manufacture's protocol (PBL Biochemical Laboratories, NJ, USA). Data were normalized based on total protein levels of related samples.

4.3. Microarray analysis

Third passage human primary keratinocytes were cultured on 10 cm^2 Petri dishes to reach 80% of confluency. Cells were stimulated with medium containing 20 µg/ml Poly (I:C) for 2, 8 and 24 h. Cells coming from three independent individual experiments were pooled and total RNA was isolated from stimulated and unstimulated cells via TRIzol, subjected to DNase I digestion, and purified by using a commercially available kit form Qiagen (Hilden, Germany. Following quality control of the RNA on a formaldehyde agarose gel, double-stranded cDNA was prepared and subjected to T7-based in vitro transcription following the standard Affymetrix protocol. 15 µg of biotinylated cRNA was hybridized overnight to HG U133A GeneChips, followed by washing, staining and scanning procedures according to Affymetrix protocols. The present call rate for the different samples ranged from 38.3% to 45.9%. The raw expression data (CEL files) were normalized using the robust multi-array average algorithm (Bolstad et al., 2003). For identification of differentially expressed genes, data were analyzed using the Bioconductor package LIMMA (Smyth et al., 2005) that combines the pairwise comparison between different conditions into one *F*-test, yielding a p-value similar to one-way ANOVA. P-values were subjected to correction for multiple testing. For further data analysis the Spotfire Decision Site 9.0 software was used. To increase the stringency of the analysis, additional filters for relative and absolute changes across all arrays were applied

(max/min ratio > 3, max-min >50). The resulting set of 685 regulated probe sets was subjected to clustering by Self-Organizing Maps (SOM function of Spotfire Decision Site), followed by testing for over-represention of Gene Ontology Biology Process terms (using Genomatix Bibliosphere software). The microarray expression data set will be submitted to NCBI's Gene Expression Omnibus database after acceptance of this manuscript.

4.4. Separation of keratinocytes into basal and suprabasal layer cells

Cell suspensions were obtained from normal human skin specimens. A third portion of this suspension was kept as whole epidermis sample. The rest of the cell suspension was added to tissue culture flasks coated with rat tail collagen (Macklis et al., 1985). In this assay basal layer cells maintain their capacity to rapidly adhere to the flasks whereas suprabasal cells keep floating in the medium. Accordingly, after 1 h non-attached cells were collected in the suprabasal fraction and adherent cells were scratched off using a rubber policeman and collected as basal cell fraction.

4.5. FACS analysis of cell surface markers and purification for mDCs

Flow cytometry using three color stainings were carried out using the FACS star (BD Dickinson, Heidelberg, Germany) and the CellQuest software. Surface labelling was carried out using the following antibodies: CD1a, CD11c, CD14, CD40, CD80, CD83, CD86, CXCR4, CCR3, HLA-DR, Mannose receptor (all antibodies from BD Biosciences, Heidelberg, Germany), anti-HLA-ABC (Sigma, Deisenhofen, Germany), and anti-Fc□RI from Cosmo Bio (Tokyo, Japan). Anti-TLR3 was from Imgenex (San Diego, CA, U.S.A), anti-TLR4 from Serotec (Düsseldorf, Germany), and anti-TLR5 from Santa Cruz Biotech (Santa-Cruz, Ca, U.S.A.). For staining with anti-TLR3 (intracytoplasmatic distribution), cells were permeabilized using the Cell Perm kit from BD Biosciences.

To highly purify mDCs within the cultures, cells were incubated with PE-anti CD1a followed by MACS-anti-PE beads (Miltenyi, Bergisch Gladbach, Germany). To obtain purities >98% cells were passed twice through the corresponding columns.

4.6. Preparation of mDCs

Human mDCs were generated as previously described (Bellinghausen et al., 2000). In brief, ~ 60 Mio PBMCs were seeded in 75 cm2 flasks and incubated for 2 hours at 37 °C in RPMI medium containing 10% FCS (Invitrogen, Eggenfelden, Germany) and penicillin/streptomycin (Gibco). After that time, adherent cells were recovered and incubated in 20 ml RPMI /10 % FCS medium containing 100 IU of hIL-4 and 100 IU of hGM-CSF (both from AL-ImmunoTools, Friesoythe, Germany) for seven days by changing the cell culture media every two days. This protocol was shown to induce immature, CD1a+, CD11c+ mDCs. For stimulation experiments, cells were seeded in 24 well plates and were incubated with the indicated ligands for 24 hours. Due to limited amounts of cells, not all donors were analyzed for the entire set of markers. Detailed numbers are given with the corresponding figures.

4.7. RNA inhibition

Double-stranded siRNA to knock down endogenous TLR3, RIG-I and MDA5 and a scrambled-sequence (control) siRNA were chemically synthesized (Ambion, Inc., TX, USA). Three different siRNA were applied for each individual target gene. For TLR3, RIG-I and MDA5 knockdown experiments, subconfluent proliferating keratinocytes cultured in 6-well dishes were treated with TLR3-siRNA, RIG-I-siRNA, MDA5-siRNA or control-siRNA (100 nM) using siPORT Amine transfection agent (Ambion) according to the manufacturer's instructions. Cells were stimulated with 20 µg/ml Poly (I:C) 12 h after transfection. Then, IL-8 and IFN-ß production were investigated at mRNA level after 36 h.

4.8. RNA isolation

Keratinocytes were grown in six-well dishes to 70-80% confluency and were stimulated using the indicated conditions. mDCs were purified to >98 % CD1a positive cells. Thereafter, cells were washed with PBS and treated with 1 ml TriZol per well. Cell lysates were centrifuged for 10 min at 13000 RPM, 4°C. Then, chloroform was added and mixed gently to extract RNA from cell lysates. After centrifugation (13000 RPM, 10 min, 4°C) the aqueous phase was transferred to a new tube. RNA was then precipitated by adding isopropanol (500 µl/1ml TriZol). After 10 min incubation at room temperature, tubes were centrifuged (13000 RPM, 15 min, 4°C) to get RNA pellet. Supernatant was discarded and RNA pellet was washed with ethanol (1 ml, 70% ethanol). After centrifugation, ethanol was discarded. For the further cleanup of the RNA, pellet was dissolved in 100 ml RNase free water, then 350 µl RLT working buffer (1ml buffer+10µl 2ME, usable for 1month) was added and mixed. After adding 250 µl absolute ethanol, mixture was applied to an RNeasy mini column (Qiagen). After columns were centrifuged (13000 RPM, 15 sec, 4 °C), flow through was discarded. RNA was washed by adding 700µl of RW1 buffer (Qiagen) and centrifuged (13000 RPM, 15 sec, 4 °C). Flow through was discarded, then 80 µl of the work solution of RNase free DNase (Qiagen) was applied to the middle of the silica-gel membrane in the column and was incubated for 15 min. Reaction was stopped and filter was washed by adding 500 µl of RW1 buffer. Flow through was discarded, then 500 of RPE buffer was applied to the column, tubes closed gently and centrifuged (13000 RPM, 15 sec, 4 °C) and flow through was discarded. Another 500 µl of RPE was added and centrifuged. Columns were transferred to the new 2 ml collection tubes, and centrifuged (13000 RPM, 2 min, 4 °C). RNeasy columns were transferred to a new 1.5 ml tube, then 25-40 ml of RNase free water was pipette to

the center of the silica-gel membrane to elute RNA. RNA was collected through centrifugation of the columns for 1 min.

4.9. RNA quantification

Isolated RNA was diluted 1:50 with RNase free water. Then, RNA was quantified using a biophotometer (Eppendorf, Germany). The spectrophotometer calculates the RNA concentration based on the following equation:

1 A_{260} unit of RNA= 40 µg/ml H_2O

4.10. RT-PCR

RNA was then transcribed to cDNA using Superscript II reverse transcriptase enzyme based on the manufacture's protocol (Invitrogen). Briefly, a 20-µl reaction was used for 1-2 µg of total RNA. Mixes were prepared based on the following protocol:

1. The following components were added to a nuclease-free microcentrifuge tube:

1 µl Oligo(dT)12-18 (500 µg/ml)

1 - 2 µg total RNA *or* x µl

1 µl dNTP Mix (10 mM each)

Distilled water to 12 µl

2. Mixtures were heated to 65°C for 5 min and quick chilled on ice. The contents of the tube were collected by brief centrifugation and following components were added:

5X First-Strand Buffer: 4 µl

0.1 M DTT: 2 µl

RNaseOUT™ (40 units/µl: 1 µl (Cat. No. 10777-019)

3. Contents of the tube were mixed gently and incubated at 42°C for 2 min.

4. 1 µl (200 units) of SuperScript™ II were added and mixed by pipetting gently up and down.

5. Tubes were incubated at 42°C for 50 min.

6. Reaction was stopped by heating at 70°C for 15 min.

The cDNA was used as a template for amplification in PCR

4.11. Real-time PCR

As a read-out system for RNA and cDNA yields, real-time PCR was applied by using ABI Prism 7000 Sequence Detector System (Applied Biosystems, CA, USA). Measurements in each sample were performed in duplicate. For semi-quantitative real-time PCR of TLR expression, primers and TaqMan probes (Table 1) were synthesized according to Zarember and Godowski (Zarember and Godowski, 2002), real-time PCR for IFN-ß was performed using assay on demands (Applied Biosystems, Foster City, CA, USA) and for IL-8 gene expression assay primers and TaqMan probes were synthesized according to Mempel *et al.* (Mempel et al., 2003). Keratinocyte differentiation markers (transglutaminase-1, involucrin and β1-integrin), PKR, RIG-I and MDA5 were evaluated with SYBR Green and primers as described (Kang and Chae, 2001; Shoham et al., 2001; Toshchakov et al., 2003; Westergaard et al., 2003; Terhorst et al., 2007). A summary of all primers used in the study is shown in Table 1. All primers and probes were purchased from MWG Biotech (Ebersberg, Germany). Input cDNA was normalized according to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control gene. The PCR reactions were cycled during the real-time detection through the following thermal program: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

Gene name	Sequences (5'→3')	Size of amplicon (bp)
GAPDH	Forward GAAGGTGAAGGTCGGAGTC Probe 5'FAM-CAAGCTTCCCGTTCTCAGCC-3'TAM Reverse GAAGATGGTGATGGGATTTC	225
TLR1	Forward CAGTGTCTGGTACACGCATGGT Probe 5'5'FAM-TGCCCATCCAAAATTAGCCCGTTCC-3'TAM Reverse TTTCAAAAACCGTGTCTGTTAAGAGA	56
TLR2	Forward GGCCAGCAAATTACCTGTGTG Probe 5'5'FAM-TCCATCCCATGTGCGTGGCC-3'TAM Reverse AGGCGGACATCCTGAACCT	48
TLR3	Forward CCTGGTTTGTTAATTGGATTAACGA Probe 5'FAM-ACCCATACCAACATCCCTGAGCTGTCAA-3'TAM Reverse TGAGGTGGAGTGTTGCAAAGG	61
TLR4	Forward CCAGTGAGGATGATGCCAGAAT Probe 5'5'FAM-TGTCTGCCTCGCGCCTGGC-3'TAM Reverse GCCATGGCTGGGATCAGAGT	46
TLR5	Forward TGCCTTGAAGCCTTCAGTTATG Probe 5'5'FAM-CCAGGGCAGGTGCTTATCTGACCTTAACA-3'TAM Reverse CCAACCACCACCATGATGAG	76
TLR6	Forward GAAGAAGAACAACCCTTTAGGATAGC Probe 5'5'FAM-TGCAACATCATGACCAAAGACAAAGAACCT-3'TAM Reverse AGGCAAACAAAATGGAAGCTT	87
TLR7	Forward TTAACCTGGATGGAAACCAGCTA Probe 5'5'FAM-AGAGATACCGCAGGGCCTCCCG-3'TAM Reverse TCAAGGCTGAGAAGCTGTAAGCTA	69

TLR8	Forward TTATGTGTTCCAGGAACTCAGAGAA Probe 5'5'FAM-TGATTTCCAGCCCCTGATGCAGC-3'TAM Reverse TAATACCCAAGTTGATAGTCGATAAGTTTG	82
TLR9	Forward GGACCTCTGGTACTGCTTCCA Probe 5'5'FAM-ACGATGCCTTCGTGGTCTTCGACAAA-3'TAM Reverse AAGCTCGTTGTACACCCAGTCT	150
TLR10	Forward TGTTATGACAGCAGAGGGTGATG Probe 5'5'FAM-TGACCCCAGCCACAACGACACT-3'TAM Reverse GAGTTGAAAAAGGAGGTTATAGGATAAATC	150
IL-8	Forward GCCAACACAGAAATTATTGTAAAGCTT Probe 5'FAM-AGAGCTCTGTCTGGACCCCAAGGAAAAC-TAM Reverse AATTCTCAGCCCTCTTCAAAAACTT	87
TLR7	Forward TTAACCTGGATGGAAACCAGCTA Probe 5'FAM-AGAGATACCGCAGGGCCTCCCG-3'TAM Reverse TCAAGGCTGAGAAGCTGTAAGCTA	69
MDA5	Forward TGTATTCATTATGCTACAGAACTG Reverse ACTGAGACTGGTACTTTGGATTCT	219
IRF5	Forward GCCTTGTTATTGCATGCCAGC Reverse AGACCAAGCTTTTCAGCCTGG	406
PKR	Forward TCTGACTACCTGTCCTCTGGTTCTT Reverse GCGAGTGTGCTGGTCACTAAAG	75
RIG-I	Forward CAGTATATTCAGGCTGAG Reverse GGCCAGTTTTCCTTGTC	389
Involucrin	Forward CTCCTCAAGACTGTTCCTCC Reverse GCAGTCATGTGCTTTTCCTCTTGC	143
Transglutaminase-1	Forward GCGGCAGGAGTATGTTCTTA Reverse GATGTGTCTGTGTCGTG	444
Beta 1-integrin	Forward AGTTGCAGTTTGTGGATCACTGAT Reverse AAAGTGAAACCCGGCATCTG	81

***Table 1**. Oligonucleotide sequences used for PCR*

4.12. Immunofluorescence staining

TLR7 was identified on frozen cryostat sections of skin biopsy specimens using specific antibodies. Briefly, cryostat sections (4 µm) were fixed with cold acetone for 10 min and dried for at least 1 h at room temperature. Sections were incubated with a primary polyclonal rabbit IgG antibody against TLR7 (IMG-581, Imgenex, CA, USA) as well as with the corresponding isotype control antibody for 60 min. Sections were then washed and incubated with secondary antibody conjugated with the fluorescence dye Alexa 488 (Invitrogen) for 1 h. Detection of TLR3, PKR, RIG-I and MDA5 in cultured keratinocytes was performed with the same protocol after fixation with 3.7% paraformaldehyde using immunofluorescence staining and confocal microscopy with primary antibodies against TLR3 (SC-12509, Santa Cruz, CA, USA), PKR (SC-707, Santa Cruz, CA, USA), RIG-I (SC-48929, Santa Cruz, CA, USA) and MDA5 (SC-48031, Santa Cruz, CA, USA). For the staining of the endosomal compartments, keratinocytes were incubated for 1 h in the presence of 50 µg/ml Alexa594-labeled dextrane prior to fixation.

4.13. RelA and IRF3 nuclear translocation analysis.

Primary keratinocytes were grown on chamber slides to approximately 70% confluency. Subsequently, cells were pre-incubated with 2-aminopurine (2-AP), bafilomycine A1 (BFA) or SU6668 for 45 min. Incubation was followed by adding Poly (I:C) or flagellin for 3 h. Cells were then washed twice with PBS, and fixed in PBS containing 3.7% paraformaldehyde for 20 min at room temperature. Cells were washed again with PBS and permeabilized by incubation with PBS containing 0.2% Triton X-100 and 10% FCS for 20 min at room temperature. Cells were then incubated for 1 h at room temperature with 1:100 diluted mouse anti-human IRF3 monoclonal antibody (BD Bioscience) or 1:500 diluted rabbit anti-human NF-κB p65

antibody (Rockland, Gilbertsville, PA, USA) followed by a 1 h incubation with 2 µg/ml Alexa-488 conjugated chicken anti-rabbit or anti-mouse antibodies (Molecular Probes, Germany) diluted in PBS at room temperature in the dark. Cells were washed three times with PBS followed by nuclear staining using TO-PRO-3 iodide (Molecular Probes) (10 mM) for 20 min. Slides were then washed with PBS, observed, and photographed with a confocal microscope (LSM510, Carl Zeiss Jena, Göttingen, Germany).

4.14. Cell lysis, gel electrophoresis and immunoblotting.

Keratinocytes were solubilized for 20 min at 4°C in RIPA lysing buffer containing 1 x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, 50 kIU aprotinin, 100 mM sodium orthovanadate and 10 µl/ml protease inhibitor cocktail (Sigma). Cell lysates were then sedimented in a microfuge for 15 min at 15,000 x g. Soluble supernatant was collected and utilized for SDS-PAGE. After cell lysis, the supernatant was titrated in reducing SDS-PAGE loading buffer (Invitrogen), treated at 70°C for 10 min, separated in a 10% Bis-Tris gel (Invitrogen) with MOPS or MES Buffer, according to the manufacturer's instructions, and transferred to a polyvinylidene fluoride membrane (PVDF; Immobilon P, Millipore, MA, USA) for 60 min using transfer buffer (Invitrogen). Membranes were blocked for 30 min at room temperature (Blocking buffer: 20 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.05% Tween20, 0.5% BSA), incubated at 4°C overnight with the following primary antibodies: anti-β-actin (Sigma) (0.25 µg/ml), anti-JNK1/JNK2 (BD Biosciences) (1 µg/ml), anti-phospho-JNK1/JNK2 (Cell Signaling, MA, USA) (1 µg/ml), anti-P38 and anti-phospho-P38 (Cell Signaling) (diluted 1:200 in Tris-buffered saline containing 0.5% BSA and 0.05% Tween20). Blots were washed repeatedly in washing buffer (15 mM NaCl, 50 mM Tris-HCl, 0.05% Tween20; pH 7.6) and

incubated for 1 h at room temperature with 0.1 µg/ml peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK) in blocking buffer. Peroxidase activity was detected using chemiluminescence substrate (Pierce) and recorded with a chemiluminescence detector (Vilber Lourmat, Eberhardzell, Germany).

4.15. Gardiquimod treatment and cell viability

The viability of keratinocytes treated with Poly (I:C) and gardiquimod was assessed by the CellTiter-Blue cell viability assay (Promega, Madison, WI). Keratinocytes were stimulated with 20 µg/ml Poly (I:C) for 24 h prior to 24 h incubation with medium, Poly (I:C) or 1 mg/ml gardiquimod. The medium was then removed and replaced with 360 µl of keratinocytes serum free medium, and 40 µl of CellTiter-Blue reagent was added to each well. The plate was incubated at 37°C for 2 h in 5% CO_2, and the fluorescent intensity of each well was measured with a microplate reader. Briefly, the assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal. The homogeneous assay procedure involves adding the single reagent directly to cells cultured in serum-supplemented medium.

5. Results

5.1. Expression of Toll-like receptors in human epidermis and cultured keratinocytes

When analysing the mechanisms of TLR–mediated interaction of the important human skin pathogen S. aureus with cultured human foreskin keratinocytes, it was recently found TLR2- but not TLR4-mediated signal transduction pathways to be elicited by S. aureus mainly through PGN and LTA. In addition to TLR2, other members of the TLR family were also expressed in cultured keratinocytes as shown by RT–PCR. To show a relevance of the TLR expression pattern described for cultured human keratinocytes, the mRNA expression of TLR1-TLR10 was analysed in freshly prepared human epidermis and compared to cultured primary unstimulated keratinocytes from human foreskins (Table 2).

RNA source	TLR									
	1	2	3	4	5	6	7	8	9	10
Human epidermis	+	+	+	–	+	+	–	–	–	+
Primary keratinocytes	+	++	+	–	+	++	–	–	–	+

***Table 2**: Comparison of mRNA expression of TLR 1–10 between human primary keratinocytes and human epidermis. The mRNA expression of TLR1–10 in freshly prepared human epidermis and cultured human primary keratinocytes was analysed by real-time PCR normalized to GAPDH. The amount of expressed RNA is indicated by: (–) no detectable expression (+) normal expression (++) at least 10-fold > normal expression. The PCR reaction was run in duplicates either with epidermis from three unrelated donors or with cultured keratinocytes from three different foreskins.*

This analysis revealed a very similar expression pattern on the mRNA level for TLRs in whole human epidermis and cultured primary keratinocytes. Expression was observed for members of the TLR2 subfamily (TLR1, -2 and -6), for TLR3 and TLR5. In addition, expression was found for TLR10, whose ligand still needs to be defined. Based on the demonstrated close similarities in the TLR expression profile between

whole epidermis and cultured keratinocytes, further quantitative and functional analyses were carried out with cultured primary keratinocytes. Likewise, the spontaneously immortalized human keratinocyte cell line HaCaT, which shows almost normal differentiation and keratinization in skin models was used throughout all experiments in comparison to primary keratinocytes. The expression of TLR mRNA was investigated by quantitative real-time PCR in primary keratinocytes and HaCaT cells (Figure 6). The most pronounced expression on the mRNA level was found for TLR2 and TLR3 both in primary keratinocytes and HaCaT cells. TLR4 was not expressed in primary keratinocytes but in HaCaT cells. The expression of TLR4 in HaCaT cells was a stable and constant finding that was seen in two independent cell line stocks. TLR1 mRNA was detectable in primary keratinocytes at a 62.5-fold higher level than in HaCaT cells. No detectable signals were observed for the TLR9 subfamily members TLR7, TLR8, and TLR9 in both cell types. TLR5 and TLR10 mRNA were expressed at 8.8-fold and 22.5-fold higher levels in primary keratinocytes as compared to HaCaT cells. TLR6 mRNA was present only in primary keratinocytes but not in HaCaT cells. Furthermore, the constitutive expression of the TLR4 cofactors CD14 and MD-2 was analyzed (Figure 5). A 39.8-fold higher expression of CD14 mRNA was found in HaCaT cells compared to primary keratinocytes. In contrast, MD-2 expression in primary keratinocytes exceeded MD-2 expression in HaCaT cells by 157-fold.

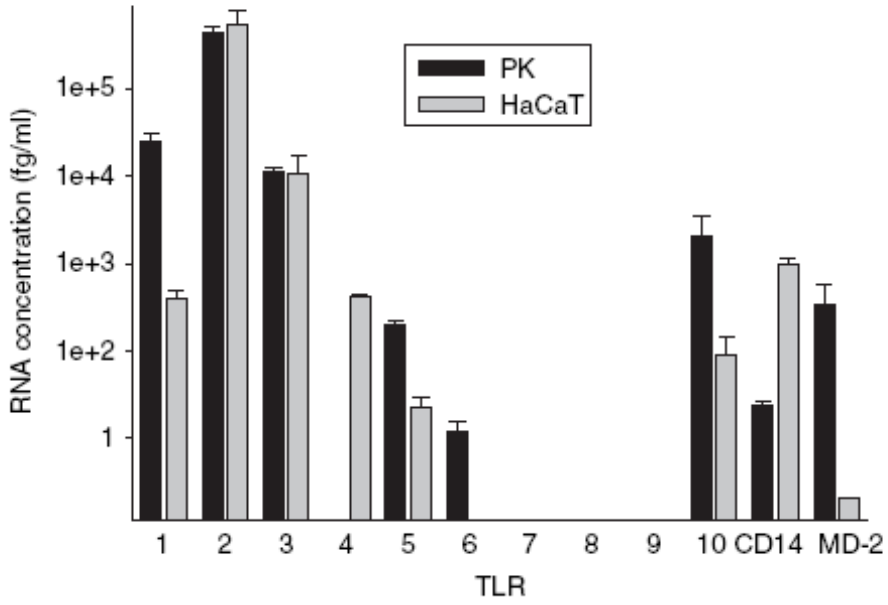


***Figure 6**. Real-time PCR for TLRs, CD14 and MD-2 in primary human keratinocytes (PK) and HaCaT cells. Cells were cultured in six-well-plates. Constitutive TLR expression of TLR1-10, CD14, and MD-2 mRNA was analysed by quantitative real-time PCR. Three different mRNA preparations of primary keratinocytes, each pooled from three independent donors, as well as three different cell preparations of HaCaT cells were used. Columns show the mean ± standard deviation of the mRNA amount. Real-time PCR was performed in duplicates.*

5.2. TLR-expression on dendritic cells from atopic versus non-atopic donors

Monocyte-derived dendritic cells were cultivated using standard conditions and immature mDCs from representative atopic and non-atopic individuals were purified with anti-CD1a to a purity of > 98% in order to avoid the bias of culture conditions when analyzing TLR-specific transcripts. From these pure DC-cultures cDNA was obtained and processed for Real-Time PCR targeting TLR1-10 expression. As previously described, mDCs from healthy donors express all TLRs with the exception of TLR7, TLR9, and TLR 10. Highest levels were seen for TLR2, 4, 5, 6 and 8, intermediate levels for TLR1 and 3 (Figure 7). Interestingly, the pattern of TLR-expression was very similar in mDCs from highly atopic patients with the exception of

a slightly higher expression of TLR 5. This experiment confirmed that mDCs derived from atopic individuals show a comparable expression pattern of TLRs as compared to healthy individuals.

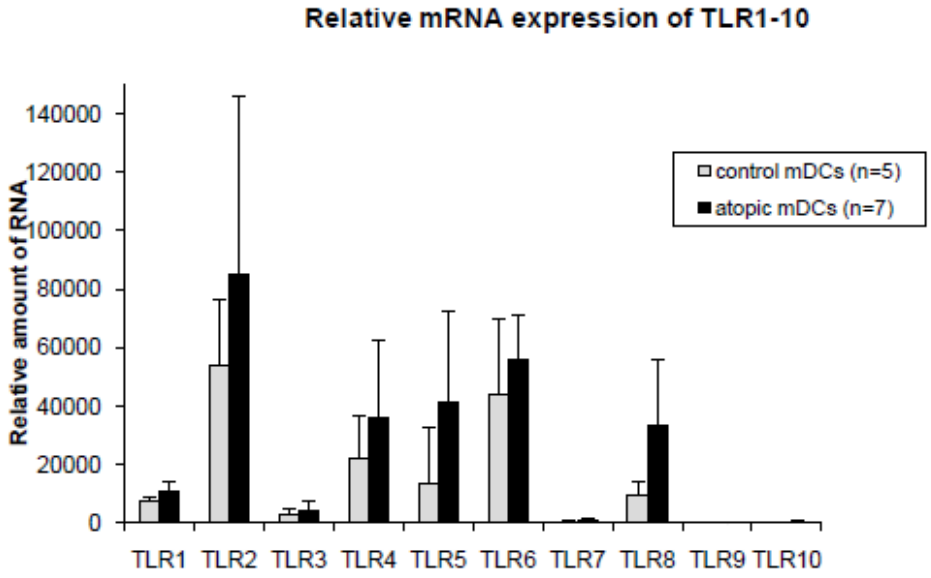


***Figure 7**. The relative expression of TLR1-10 in mDCs is depicted as copies of the gene of interest per 106 copies of the house-keeping gene GAPDH. In both groups TLRs 2, 4, 5, 6, and 8, showed the strongest expression, whereas we found very low or no expression for TLR7, TLR9, and TLR10. TLRs 1, and 3 were expressed at intermediate levels. The strongest difference was obtained for TLR5 which was higher expressed in atopic individuals. In general, mDCs from atopic individuals tended to express slightly higher RNA levels for the TLR genes.*

5.3. mDC's surface receptor expression profile and response to TLR-ligands

MDCs from atopic and non-atopic individuals were generated and cultivated with identical protocols for 7 days. When screening for surface markers without stimulation we found no significant differences in the baseline expression of several surface markers although some markers showed the tendency for higher expression in atopics (data not shown).

When stimulated with various ligands, we observed maturation of mDCs in both groups with an increased expression of HLA-DR, CD80, CD83, and CD86. However,

in atopic individuals the co-stimulatory molecules CD80, CD83, and CD86 are consistently up-regulated to a higher degree than control individuals. This was significant for CD83 and CD86 after stimulation with Poly (I:C) but showed a clear tendency for all stimuli even if not reaching significance levels. In contrast, non-atopic control persons tended to increase surface expression of MHC class I molecules (HLA-DR) with significant results after LPS stimulation (Figure 8). Of interest, there was a clear tendency for up-regulation of co-stimulatory molecules in atopic individuals combined with only moderate changes in MHC-expression which was found inverse in control subjects. As a control for the observed changes, both groups of DCs were incubated with CpG-ODNs and non-CpG-ODNs (as a ligand for TLR9) which showed no up-regulation for either surface marker in atopic donors and a rather non-specific induction of MHC molecules in control individuals (as seen for CpG and non-CpG) arguing against functional expression of TLR9 in mDCs.

For TLRs 3, 4, and 5, we applied pre- and post stimulation (24 hrs) staining in selected patients which showed TLR-down-regulation for all three receptors by Poly (I:C), LPS, and PGN consistent with previous reports (Visintin, Mazzoni et al. 2001). However, stimulation with flaggelin showed no changes for TLR3 and a slight up-regulation of TLRs 4 and 5 (data not shown).

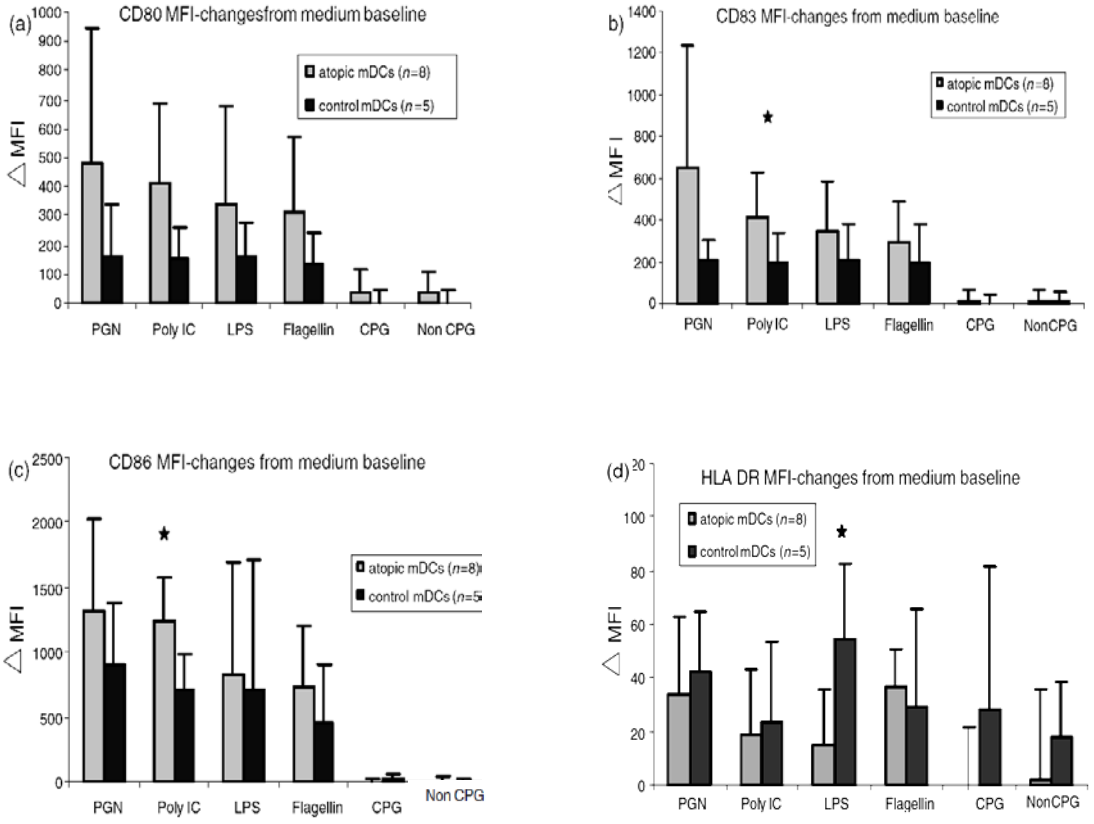


Figure 8. *Induction of surface molecules on mDCs after stimulation with various ligands. The graph shows changes in mean fluorescence intensity (MFI) from baseline values (medium control) after incubation with the respective ligand. Among the various analyzed surface molecules, CD80 (A), CD83 (B), CD86 (C) and HLA-DR (D) are shown. Significant changes between the atopic and control mDCs (p>0.05) as calculated by the Friedmann-test are indicated (*). In general, mDCs from atopic donors tended to up-regulate the co-stimulatory molecules CD80, CD83, and CD86 to a higher extent than controls, whereas cells derived from healthy individuals showed stronger induction of MHC molecules.*

Next we looked for IL-12- and TNF-α-expression by stimulated DCs. For this purpose, cells were incubated with the appropriate ligands and supernatants were analyzed after 48 hours (a time point which had been evaluated in preliminary experiments as adequate for highest cytokine production). Interestingly, there was a similar pattern of TH1-cytokine responses between atopic and not atopic individuals showing highest values for IL-12 and TNF-α after Poly (I:C) stimulation. For PGN, LPS and flagellin as ligands this picture changed slightly. Whereas LPS and flagellin

induced substantial amounts of IL-12 (higher for LPS in atopics and for flagellin in controls) (Figure 9), PGN was about 10 fold more potent than the medium control for induction of this cytokine in controls and about 100 fold more potent in atopics (Figure 8). As for TNF-α production, LPS and PGN were equally capable of inducing TNF-α with reduced potency for flagellin. CpG and non-CpG did not induce substantial amounts of either cytokine thus rendering unlikely a role for mDC activation through TLR9 (Jarrossay et al., 2001; Kadowaki et al., 2001).

In summary, atopic mDCs seem to mount TH1 responses of highly comparable magnitude as control subjects when challenged with various TLR ligands.

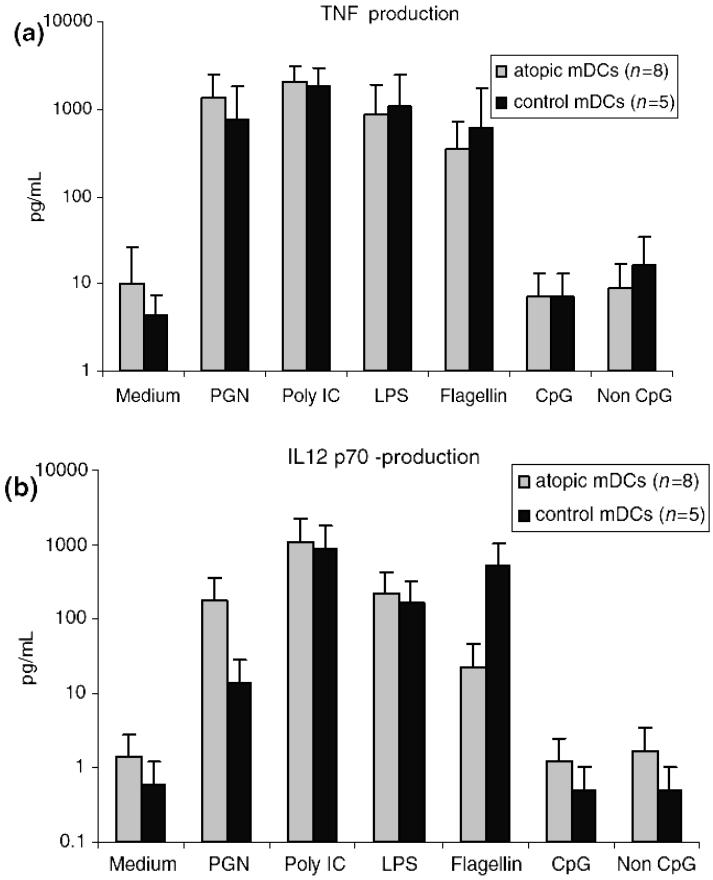


Figure 9. *Production of TNF-α and IL-12 p70 in mDCs stimulated with TLR ligands. Culture supernatants of stimulated mDCs were analyzed after 24 hours for the induction of TNF-α (a) and IL-12p70 (b). Whereas CpG and non-CpG DNA induced cytokine levels similar to medium control, PGN, Poly (I:C), LPS, and flagellin induced in both groups both types of pro-inflammatory cytokine. For TNF-α as for IL-12 highest values were seen after stimulation with Poly (I:C). Statistical evaluation was done with the Friedmann test.*

5.4. Expression of the dsRNA recognizing receptors PKR and RIG-I in mDCs

Besides TLR3, further molecules participate in the recognition of the dsRNA equivalent Poly (I:C), namely RIG-I and PKR which lead to the translocation of IRF3 and NF-κB, respectively (Sen and Sarkar, 2005). Having observed a strong pro-inflammatory response of mDCs to Poly (I:C) with only moderate expression of TLR3, we screened for the presence of further Poly (I:C) receptors. Indeed, RIG-I and PKR were expressed at high levels in mDCs of both groups showing no significant differences mDCs. Thus, Poly (I:C) most probably signals through all three receptors thereby providing a stronger pro-inflammatory signal in human mDCs than expected from the relatively moderate expression of the TLR3.

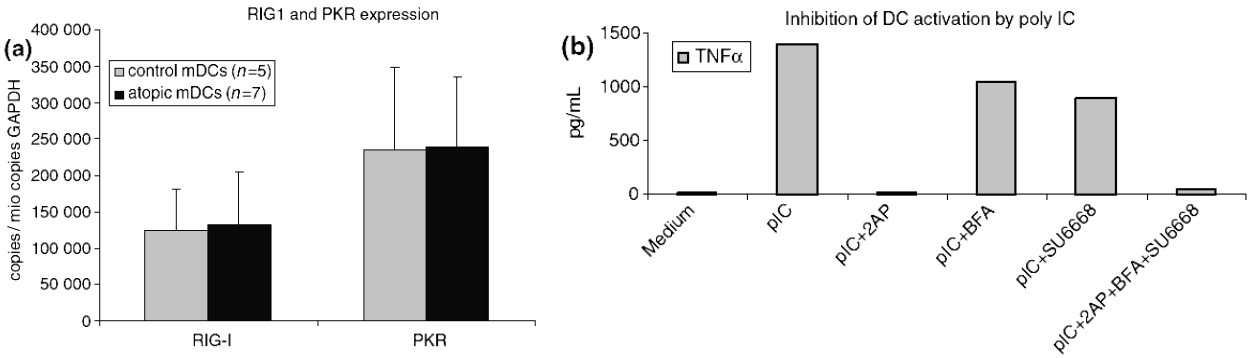


Figure 10. *Gene expression analysis of proteinkinase R (PKR) and retinoid-inducible gene-I (RIG-I), two alternative dsRNA-binding proteins in mDCs. Both genes are expressed at very similar levels in both groups of mDCs (a) and were shown to be functional by specific inhibitors (b). Shown is the the inhibition of TNFα production in the DCs of patient FPD with 2-aminopurine (inhibitor of PKR), bafilomycine A (inhibitor of endosome acidification/ TLR3), and SU6668 (inhibitor of TBK-1). 2-amionopurine represented the strongest inhibitor, while bafilomycine A and SU6668 only partially inhibited Poly (I:C) activation of mDCs.*

5.5. Differential IL-8 induction by various TLR ligands in cultured human keratinocytes

In order to characterize the functional relevance of TLRs in epithelial cells of the skin, primary keratinocytes and HaCaT cells were stimulated with a panel of defined microbial and synthetic TLR ligands. Subsequently, the induction of the proinflammatory chemokine IL-8, which is under regulation of NF-κB, was quantified by IL-8 ELISA (Figure 11). The TLR2 ligand PGN was able to activate both cell types, although HaCaT cells reacted more pronounced to PGN (Figure 11). On the contrary, Pam3Cys, another known ligand for TLR2 strongly stimulated primary keratinocytes, whereas HaCaT cells reacted to a lower extent (Figure 11). The bacterial protein flagellin, a ligand for TLR5, also stimulated both cell types (Figure 11).

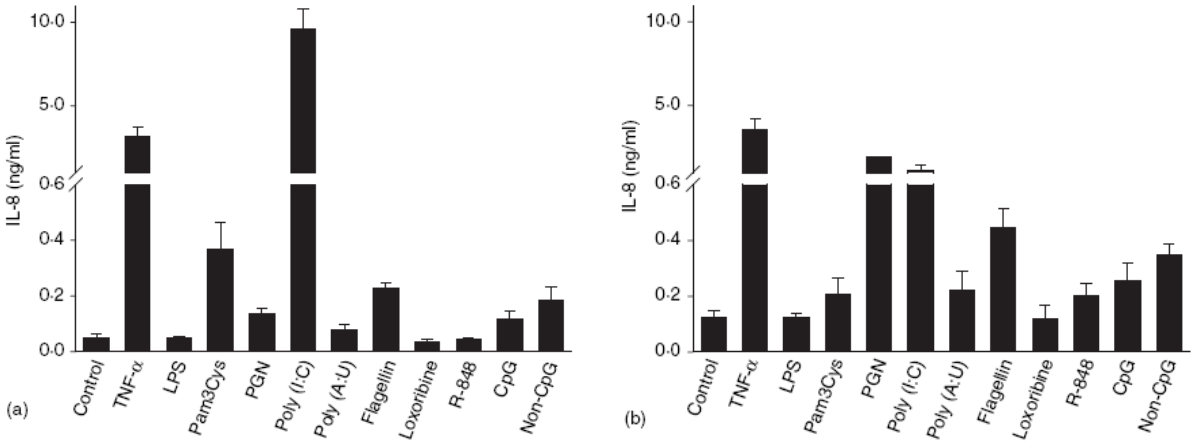


***Figure 11**. IL-8 production of primary human keratinocytes and HaCaT cells after stimulation with various TLR ligands. Primary keratinocytes (a) and HaCaT cells (b) were cultured in 96-well-plates and stimulated with the following ligands at indicated final concentrations: TNF-α (50 ng/ml), LPS from E. coli 0127:B8 (100 ng/ml), Pam3Cys (5 µg/ml), PGN (10 µg/ml), Poly (I:C) and Poly (A:U) (20 µg/ml each), recombinant flagellin (10 µg/ml), loxoribine (1 mM), R-848 (1 µg/ml), CpG and non-CpG-ODN (1 µM each). Normal cell culture medium was used as control. The concentration of secreted IL-8 in the medium after 24 hr of TLR ligand stimulation was measured by ELISA. Columns show the mean ± standard deviation of six independent wells. Shown is one representative IL-8 stimulation experiment out of four. Significant differences versus the control incubation (P< 0.001) were obtained for the following TLR ligands: TNF-α, Pam3Cys, PGN, Poly (I:C), flagellin, CpG and non-CpG-ODN (primary keratinocytes); TNF-α, PGN, Poly (I:C) and flagellin (HaCaT cells).*

The TLR3 ligand Poly (I:C), a synthetic analogue of double-stranded viral RNA, lead to the strongest activation of primary (Figure 10a) and HaCaT (Figure 11b) keratinocytes, whereas poly (A:U) as non specific control substance did not induce relevant amounts of IL-8. RNAse treatment of Poly (I:C) completely abolished the activation of primary keratinocytes and HaCaT cells demonstrating an RNA-mediated cell stimulatory mechanism (Figure 12). The expression of TLR3 protein, which is located intracellularly, changed upon keratinocyte activation with Poly (I:C) to a plasma membrane-like pattern (Figure 12, insert).

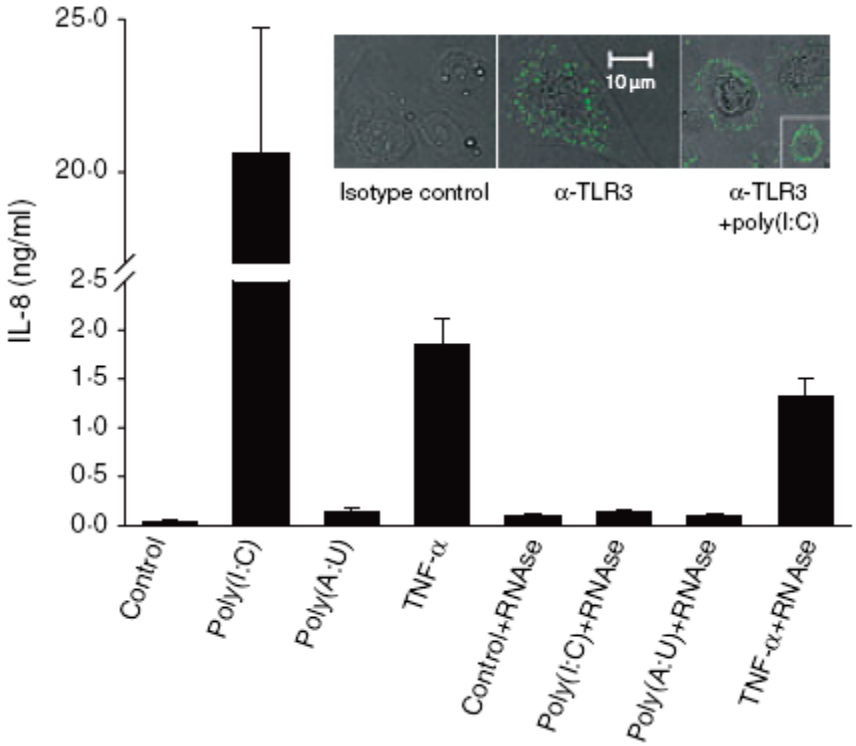


***Figure 12**. Analysis of TLR3 expression (insert) and of IL-8 production of primary human keratinocytes after stimulation with the dsRNA analogue Poly (I:C). Primary keratinocytes were tested for specificity of TLR3 activation by Poly (I:C) using RNAse A digest. Medium (control), Poly (I:C), and poly (A:U) were incubated overnight with RNAse A at a concentration of 20 lg/ml and were subsequently used for stimulation of keratinocytes. Medium (control), Poly (I:C) and poly (A:U) were also used without prior RNAse treatment. TNF-α (50 ng/ml) was used as another positive stimulus for IL-8 induction in keratinocytes with (TNF-α + RNAse) and without RNAse treatment. The concentration of secreted IL-8 in the medium after 24 hr of ligand stimulation was measured by ELISA. Columns show the*

mean ± standard deviation of six independent wells shown is one representative IL-8 stimulation experiment out of three. Insert: expression of TLR3 in primary keratinocytes under baseline conditions (α-TLR3) and after 16 h stimulation with Poly (I:C) (α-TLR3 + poly-(I:C)) as detected by goat polyclonal antibody directed against the amino terminus of TLR3. Non-immune goat IgG was used as control.

In primary keratinocytes, native flagellin from S. typhimurium induced a very strong IL-8 stimulation at lower concentrations than recombinant flagellin from B. burgdorferi. In HaCaT cells, the stimulatory potential of both flagellins was consistently lower than in primary keratinocytes (Figure 13).

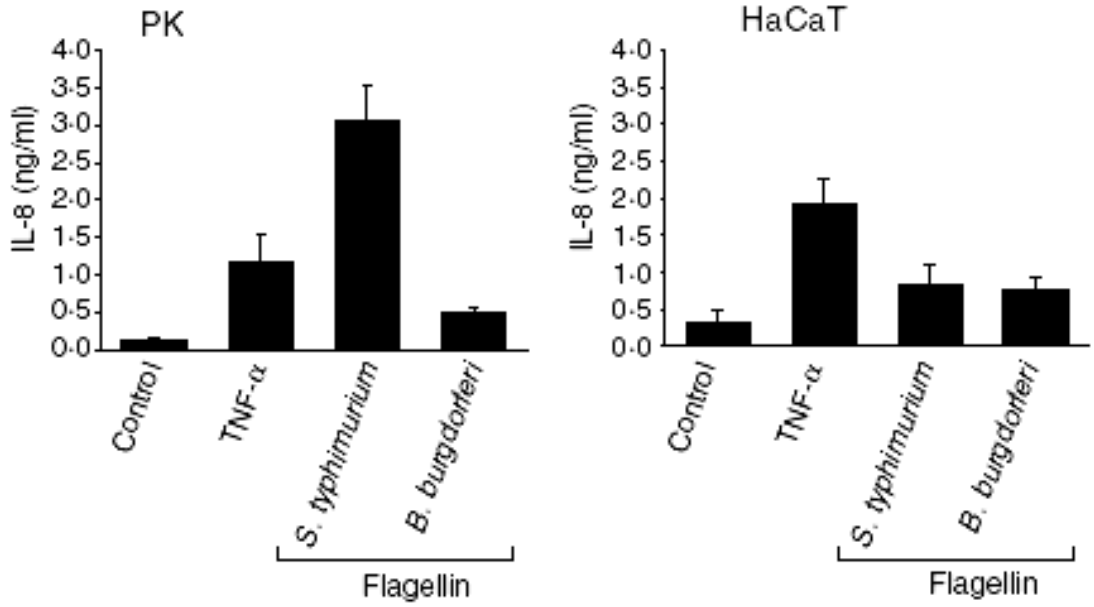


Figure 13. *Analysis of IL-8 production of primary human keratinocytes (PK) and HaCaT cells after stimulation with different flagellins. Two different flagellin preparations (B. burgdorferi; S. typhimurium) were tested on primary keratinocytes (a) and HaCaT cells (b). Recombinant flagellin from B. burgdorferi was used at a final concentration of 10 lg/ml, whereas native purified flagellin from S. typhimurium was used at a final concentration of 1 µg/ml. TNF-α (50 ng/ml) served as a positive stimulation control, medium alone (control) as a negative stimulation control. The concentration of secreted IL-8 in the medium after 24 hr of ligand stimulation was measured by ELISA. Columns show the mean ± standard deviation of six independent wells. Shown is one representative IL-8 stimulation experiment out of three.*

Other TLR ligands, like LPS, loxoribine, R-848, CpG and non-CpG DNA (Figure 2) did not induce a pronounced or specific IL-8 production as tested for different

concentrations and, in case of primary keratinocytes, using cells from various unrelated donors. a pronounced or specific IL-8 production as tested for different concentrations and, in case of primary keratinocytes, using cells from various unrelated donors.

5.6. NF-κB induction by TLR ligands in cultured human keratinocytes

For primary keratinocytes, the NF-κB activation and translocation from the cytoplasm into the nucleus was monitored in the RelA assay (Figure 14). Corresponding to the extent of TLR ligand-induced IL-8 secretion, Poly (I:C), flagellin, and TNF-α (positive control) induced the strongest nuclear translocation of RelA. The TLR2 ligands PGN and Pam3Cys also stimulated nuclear translocation of RelA (Figure 13), although to a lesser extent, whereas LPS (Figure 14) and all other ligands induced no nuclear RelA staining (data not shown).

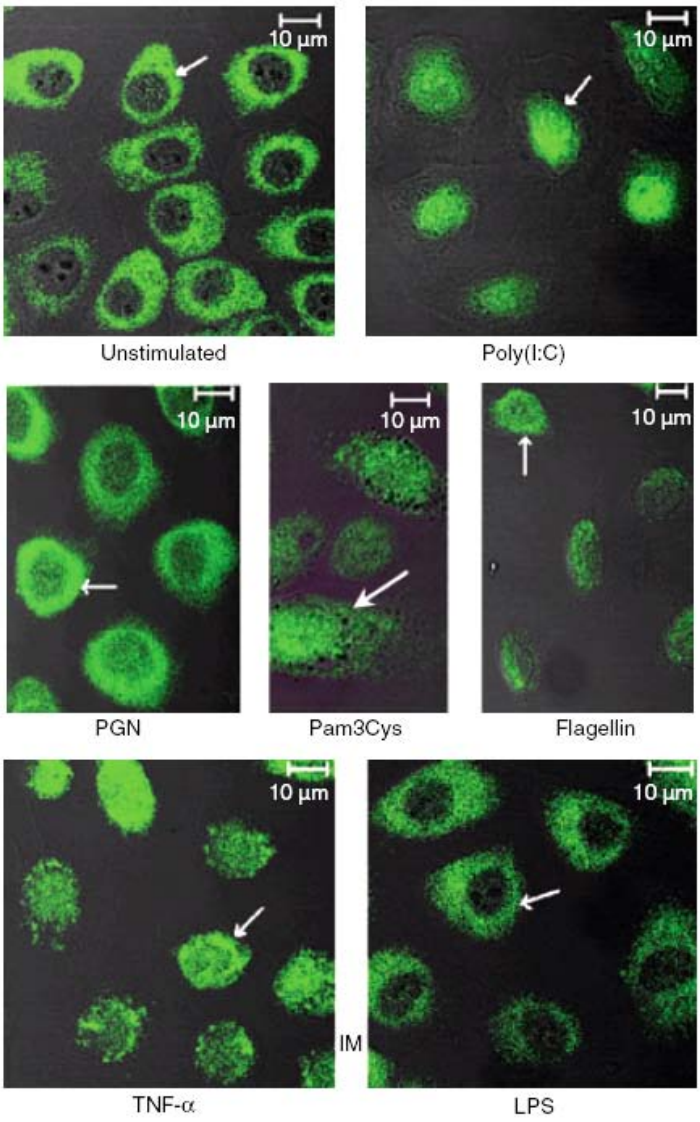


***Figure 14**. RelA assay for NF-κB translocation of primary human keratinocytes after stimulation with various TLR ligands. For RelA staining of primary human keratinocytes, cells were incubated for 4 hr with medium (unstimulated) as a negative control, with TNF-α (50 ng/ml) as a positive control, and with the TLR ligands Poly (I:C), PGN, Pam3Cys, flagellin, and LPS at the same concentrations used for the IL-8 assay (see legend to Figure 10). Incubation was stopped and cells were stained with a primary rabbit anti-p65 antibody followed by an FITC-labelled goat anti-rabbit antibody. Cells which are not reactive to the stimulus are represented by a cytoplasmic staining pattern, reactive cells by a nuclear one as denoted by arrows. Arrowheads point to cells representing a typical fluorescence pattern for each of the incubations.*

5.7. Role of LPS and TLR4 in cultured human keratinocytes

HaCaT cells express TLR4 at the mRNA level, but do not produce IL-8 upon stimulation with LPS. To investigate this feature, we analysed the possible induction of TLR4, CD14 and MD-2 mRNA through LPS stimulation by real-time PCR at various time points (Figure 6). In primary keratinocytes, TLR4 was not inducible by LPS, whereas in HaCaT cells an LPS -dependent up-regulation of TLR4 was found (Figure 15a). CD14 was mainly expressed in HaCaT cells, and a strong up-regulation after LPS stimulation was only seen in HaCaT cells (Figure 15b). MD-2, on the other hand, was expressed in primary keratinocytes, but only marginally in HaCaT cells. LPS stimulation lead to an insignificant increase of the detectable MD-2 signal in primary keratinocytes but not in HaCaT cells (Figure 14c). All LPS stimulation experiments were also performed with LPS from E. coli K235 (low in protein content) leading to identical results (data not shown). As positive stimulation control, human monocyte-derived dendritic cells were stimulated for 24 hr with 100ng/ml of each LPS preparation and a strong induction of the activation markers CD83, CD86, and HLA-DR was found in flow cytometry (Figure 15d).

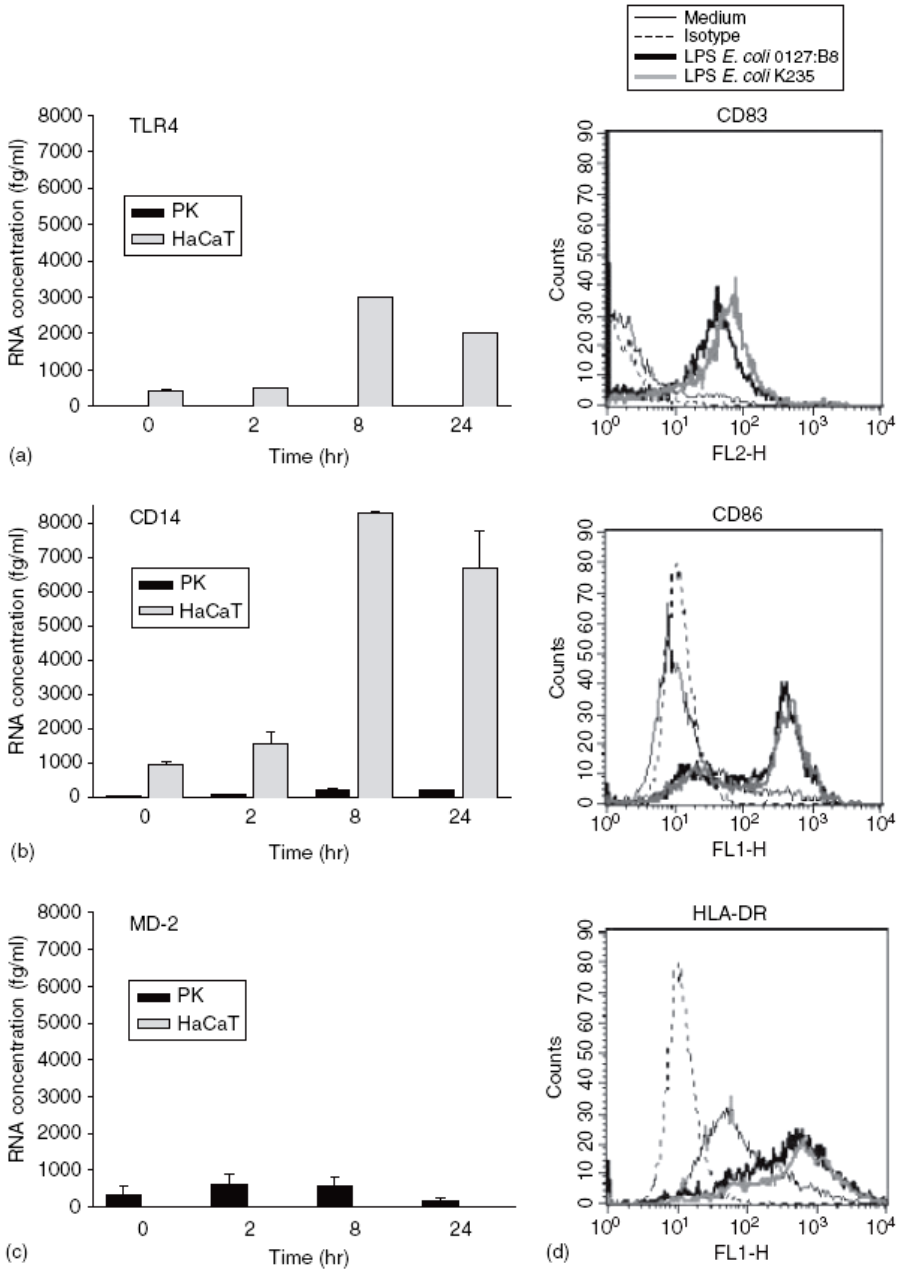


***Figure 15**. Induction of TLR4, CD14 and MD-2 by LPS in primary keratinocytes (PK) and HaCaT cells. Cells were cultured in six-wellplates stimulated with LPS (100 ng/ml) from E. coli 0127:B8. The LPS-inducible expression of TLR4 (a), CD14 (b) and MD-2 (c) was quantified after 0, 2, 8 and 24 hr of LPS stimulation. Real time PCR was performed as described in the legend to Figure 5. The activity of the LPS preparations used was demonstrated in human monocyte-derived dendritic cells (d). After 24 h incubation with 100 ng/ml LPS (LPS from E. coli 0127:B8 and LPS from E. coli K235), the cells were stained for the activation markers CD83, CD86, and MHC class II (HLA-DR) and analysed by flow cytometry.*

Even after continuous LPS stimulation of primary keratinocytes or HaCaT cells over a period of 4 days with LPS preparations from the two different sources, no significant amount of IL-8 was produced in either cell type at any timepoint (Figure 16).

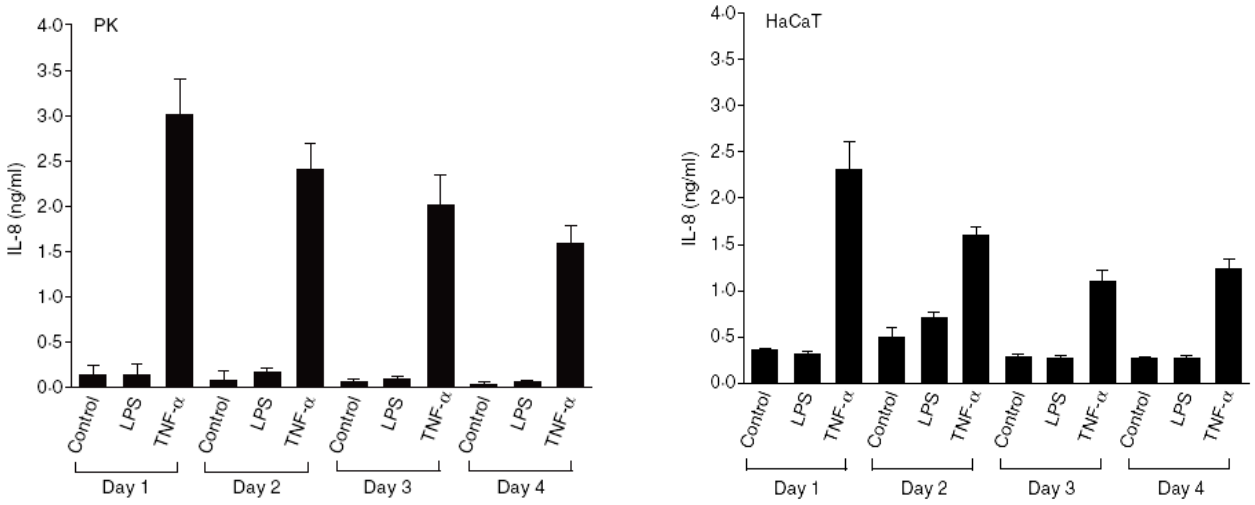


***Figure 16**. Induction of TLR4, CD14 and MD-2 by LPS in primary keratinocytes (PK) and HaCaT cells. Cells were cultured in six-wellplates stimulated with LPS (100 ng/ml) from E. coli 0127:B8. The LPS-inducible expression of TLR4 (a), CD14 (b) and MD-2 (c) was quantified after 0, 2, 8 and 24 hr of LPS stimulation. Real-time PCR was performed as described in the legend to Figure 5. The activity of the LPS preparations used was demonstrated in human monocyte-derived dendritic cells (d). After 24 h incubation with 100 ng/ml LPS (LPS from E. coli 0127:B8 and LPS from E. coli K235), the cells were stained for the activation markers CD83, CD86, and MHC class II (HLA-DR) and analysed by flow cytometry.*

5.8. Genome-wide analysis of changes in keratinocyte gene expression in response to Poly (I:C) stimulation

To assess the impact of viral infection on human keratinocyte gene expression, we performed a kinetic analysis of primary cells after stimulation with Poly (I:C), a synthetic ligand mimicking viral dsRNA. In two independent experiments, RNA harvested before and 2, 8 and 24 hours after stimulation was processed and analysed by Affymetrix HG-U133A oligonucleotide arrays that contain 22283 different

probe sets. Statistical analysis using a linear model showed that a total of 1539 probe sets was regulated over time (F-test p-value < 0.01). Figure 17*A* depicts the numbers of up- and down-regulated probe sets for the individual time points, showing that the transcriptional response to Poly (I:C) is relatively slow with very few changes after 2 h. After 8 h, the response is predominated by induction of gene expression, while after 24 h large sets of genes are up- or downregulated. To weed out probe sets regulated at low levels, we applied a filter for relative and absolute changes (see Materials and Methods) that reduced the number of regulated probe sets to 685. The kinetic patterns of induction and repression of these probe sets can be separated in different clusters using Self-Organizing Maps (Figure 17B). Genes down-regulated after Poly (I:C) fall in cluster 1 (repressed only after 24 h) and cluster 4 (down-regulation starts after 8 h). For up-regulated genes, cluster 5 contains the small number of transcripts induced already after 2 h, whereas cluster 6 comprises late Poly (I:C) targets. Clusters 2 and 3 separate genes that tend to peak in expression after 8 h or show continuing increases in expression over time.

To gain insight into the types of response at the different time points, we searched for over-representation of functional categories in the genes from the different clusters using Gene Ontology Biological Process terms (Table 3).

Probe Set ID	Gene.Symbol	0h	2h	8h	24h	sum.of.calls	F.p.value
203828_s_at	IL32	210	233	504	2240	2	0
204533_at	CXCL10	223	1898	12407	11946	6	0
204655_at	CCL5	71	170	2805	5950	6	0
210163_at	CXCL11	24	217	4496	4180	6	0
211122_s_at	CXCL11	26	236	3908	3813	6	0
220054_at	IL23A	250	272	363	2407	5	1,00E-05
1405_i_at	CCL5	73	146	2277	3485	6	2,00E-05
214974_x_at	CXCL5	36	55	131	499	7	2,00E-05

Probe Set ID	Gene.Symbol	0h	2h	8h	24h	sum.of.calls	F.p.value
211506_s_at	IL8	39	825	1278	4184	6	9,00E-05
205992_s_at	IL15	81	71	361	335	6	0,0001
205114_s_at	CCL3	73	84	393	3971	3	0,00023
220056_at	IL22RA1	120	127	252	249	8	0,00023
220322_at	IL1F9	139	1073	1205	4145	6	0,00038
216244_at	IL1RN	74	154	305	389	7	0,00043
215101_s_at	CXCL5	31	45	139	688	5	0,00045
202859_x_at	IL8	140	2408	3243	6290	6	0,00047
207850_at	CXCL3	44	807	254	486	6	0,0006
207375_s_at	IL15RA	168	167	405	435	4	0,00144
212659_s_at	IL1RN	1766	2599	4156	6193	8	0,00175
212195_at	IL6ST	140	203	532	449	8	0,00182
216243_s_at	IL1RN	2877	3925	6246	9391	8	0,00243
217371_s_at	IL15	26	26	45	45	4	0,00299
204103_at	CCL4	129	187	1347	2748	4	0,00339
209575_at	IL10RB	67	74	113	172	7	0,00398
205476_at	CCL20	57	1206	1518	2839	6	0,00414
204470_at	CXCL1	97	1052	897	1349	8	0,00577
211372_s_at	IL1R2	88	89	264	874	3	0,00632
210118_s_at	IL1A	1657	5050	2552	3674	8	0,0072
222223_s_at	IL1F5	238	288	245	879	4	0,00837
204116_at	IL2RG	245	252	258	381	2	0,00867
212657_s_at	IL1RN	3593	5765	8027	10396	8	0,00929
205403_at	IL1R2	121	121	643	2472	3	0,0101
206295_at	IL18	103	131	87	59	7	0,01787
209774_x_at	CXCL2	48	956	420	778	7	0,0593
203915_at	CXCL9	179	187	1238	661	6	0,10734
39402_at	IL1B	1971	4315	2626	3768	8	0,15485
205067_at	IL1B	3677	7112	4593	6684	8	0,16633
205227_at	IL1RAP	198	197	152	162	6	0,17163
201888_s_at	IL13RA1	156	147	163	188	4	0,19484
205798_at	IL7R	132	130	403	557	3	0,19656
212196_at	IL6ST	171	198	408	351	6	0,19994

Probe Set ID	Gene.Symbol	0h	2h	8h	24h	sum.of.calls	F.p.value
220663_at	IL1RAPL1	116	115	94	123	5	0,22081
211612_s_at	IL13RA1	175	185	229	299	8	0,24932
204864_s_at	IL6ST	106	93	170	193	8	0,36282
205207_at	IL6	79	126	310	427	7	0,39675
216598_s_at	CCL2	138	222	926	577	2	0,40162
207901_at	IL12B	25	21	22	22	3	0,40451
210904_s_at	IL13RA1	256	265	350	369	8	0,45173
207160_at	IL12A	39	38	38	46	2	0,50055
201887_at	IL13RA1	301	357	417	392	8	0,5381
206336_at	CXCL6	28	31	29	46	4	0,5621
205926_at	IL27RA	99	97	92	109	2	0,59514
205945_at	IL6R	138	179	99	207	8	0,6269
211000_s_at	IL6ST	92	89	156	182	2	0,666
207526_s_at	IL1RL1	166	141	256	316	7	0,76321
204863_s_at	IL6ST	48	40	73	81	2	0,79299
203233_at	IL4R	716	732	735	645	3	0,80181
64440_at	IL17RC	460	439	442	472	8	0,80824
203666_at	CXCL12	220	207	215	214	4	0,94538
206172_at	IL13RA2	572	516	320	291	4	0,9933
209827_s_at	IL16	213	214	214	214	2	0,99985

Table 3: *Chemokines and cytokines up-regulated in human keratinocytes after Poly (I:C) stimulation.*

Up-regulated genes are highly enriched for “immune response” functions, including the category “response to virus” for cluster 3 genes and “chemotaxis” for cluster 6 (later response) genes. In addition to the chemokines CCL5 and CXCL10 that are shown in Figure 17B, many other chemokines, as well as interleukins, or their receptors were up-regulated (for an overview see Table 3). Up-regulation of genes involved in antigen-presentation is also evident and includes HLA class I molecules, TAP1 and TAP2. The high score for the term Jak Stat cascade for cluster

3 genes likely is caused by the many IFN-regulated genes that are induced by Poly (I:C) in this data set (Table 4, showing that from a list of 65 manually curated IFN target genes expressed in the keratinocytes analysed here, 38 are regulated with F-test p-value < 0.01). Members of the NF-κB cascade were found enriched both in up- and down-regulated genes, suggesting complex regulation of this signaling pathway. Genes with a function in apoptosis were also found in all clusters but especially enriched in cluster 2. Poly (I:C) caused down-regulation of genes involved in basic cellular processes such as translation and nucleotide metabolism.

Probe Set ID	Gene.Symbol	0h	2h	8h	24h	sum.of.calls	F.p.value
204655_at	CCL5	71	170	2805	5950	6	0
203275_at	IRF2	186	185	821	513	7	0
208436_s_at	IRF7	274	295	2270	4112	8	0
204698_at	ISG20	212	369	3401	6241	4	0
33304_at	ISG20	271	384	3386	6549	5	0
204994_at	MX2	224	205	1730	3556	4	0
202659_at	PSMB10	208	211	685	1397	4	0
202307_s_at	TAP1	292	429	2537	3474	4	0
1405_i_at	CCL5	73	146	2277	3485	6	2,00E-05
202086_at	MX1	866	890	7129	8695	8	2,00E-05
206553_at	OAS2	268	258	896	1400	7	2,00E-05
209040_s_at	PSMB8	277	293	941	1258	4	3,00E-05
211075_s_at	CD47	717	772	1674	1874	8	4,00E-05
202531_at	IRF1	215	1064	1355	1382	6	5,00E-05
202638_s_at	ICAM1	122	451	1775	4277	6	6,00E-05
201422_at	IFI30	810	801	1371	2377	8	7,00E-05
201642_at	IFNGR2	1646	2171	2921	4901	8	7,00E-05
203595_s_at	IFIT5	206	233	982	982	7	9,00E-05

Probe Set ID	Gene.Symbol	0h	2h	8h	24h	sum.of.calls	F.p.value
209619_at	CD74	263	249	448	952	6	0,00012
208966_x_at	IFI16	846	917	2253	1989	8	0,00012
200923_at	LGALS3BP	1299	1390	2049	4929	7	0,00014
200749_at	RAN	426	547	499	200	8	0,00018
200814_at	PSME1	1608	1676	2259	3672	8	0,00019
209124_at	MYD88	1623	1822	5027	4341	8	0,0002
202637_s_at	ICAM1	349	666	2244	4812	5	0,00024
204972_at	OAS2	526	565	2832	4266	8	0,00038
205220_at	GPR109B	283	1735	4070	4299	7	0,00045
215485_s_at	ICAM1	295	409	979	2656	4	0,00067
203882_at	ISGF3G	1155	1276	2400	2739	8	0,00083
206332_s_at	IFI16	541	607	1636	1263	8	0,00085
201317_s_at	PSMA2	1269	1451	2205	2625	8	0,00095
209969_s_at	STAT1	278	250	1083	1778	6	0,00146
208173_at	IFNB1	83	465	250	92	3	0,0021
209878_s_at	RELA	677	729	1197	1378	8	0,00264
209139_s_at	PRKRA	356	453	324	149	8	0,00331
200887_s_at	STAT1	976	1065	2941	4845	8	0,00357
200750_s_at	RAN	2641	2873	2651	1491	8	0,0052
203596_s_at	IFIT5	161	159	363	397	4	0,00676
202146_at	IFRD1	123	144	147	75	8	0,0129
209323_at	PRKRIR	167	196	143	123	8	0,01312
209100_at	IFRD2	886	835	721	430	7	0,01427
201783_s_at	RELA	965	1073	1604	1719	8	0,01462
201464_x_at	JUN	535	1645	1182	1257	7	0,01579
205641_s_at	TRADD	692	644	824	1238	3	0,01668
204211_x_at	EIF2AK2	577	541	963	1192	6	0,01705
1729_at	TRADD	424	377	546	818	7	0,0208
201465_s_at	JUN	139	460	385	524	2	0,0286

Probe Set ID	Gene.Symbol	0h	2h	8h	24h	sum.of.calls	F.p.value
201316_at	PSMA2	138	131	204	173	7	0,04538
202727_s_at	IFNGR1	491	659	785	693	8	0,06098
205027_s_at	MAP3K8	19	25	33	30	6	0,07856
208965_s_at	IFI16	605	530	1675	1583	8	0,08031
201952_at	ALCAM	872	932	1531	1170	8	0,10603
203915_at	CXCL9	179	187	1238	661	6	0,10734
211676_s_at	IFNGR1	340	456	642	498	8	0,11542
201951_at	ALCAM	316	344	620	511	8	0,15139
204786_s_at	IFNAR2	63	63	69	82	2	0,21749
202147_s_at	IFRD1	157	174	190	125	4	0,253
201466_s_at	JUN	91	194	165	146	7	0,25328
200989_at	HIF1A	1013	1396	1629	1323	8	0,28131
200820_at	PSMD8	2559	2670	2531	2060	8	0,28634
213281_at	JUN	72	109	97	102	8	0,3354
216598_s_at	CCL2	138	222	926	577	2	0,40162
205469_s_at	IRF5	373	342	363	380	6	0,79852
202621_at	IRF3	620	643	753	695	8	0,80188
202670_at	MAP2K1	1262	1322	1483	1212	8	0,90229

Table 4: *List of 65 manually curated IFN target genes expressed in keratinocytes after Poly (I:C) stimulation.*

The molecular components for the cellular recognition of viral dsRNA have been identified in detail in the last years. Table 4 depicts the expression values and – where applicable – regulation of PRR, adapter molecules and interferon regulatory factors. Among the Toll-like receptors, only TLR1, TLR2 and TLR3 were found expressed, with TLR2 and TLR3 showing regulation. TLR7-9, all involved in sensing of nucleic acids were absent or below the limit of detection by microarray technology although TLR7 can be detected in Poly (I:C) stimulated keratinocytes using real-time

PCR (Figure 21). The essential adaptor proteins of TLR signaling, MyD88 and TRIF, are expressed in the primary keratinocytes. Looking at non-TLR sensors of viral ligands, both RIG-I and PKR were found to be expressed and up-regulated by Poly (I:C) in keratinocytes, whereas MDA5 was only found present at low levels after stimulation. Among the IRFs, we found constitutive expression of IRF3 and inducible expression of IRF1, IRF2 and IRF7. Together with the early induction of IFNB by Poly (I:C) (Figure 16B, cluster 5), this pattern of expression would be consistent with IRF3-mediated IFNB transcription, that then activates the expression of IRF1 and IRF7 to start an amplification loop increasing the expression of IFN-regulated genes via the Stat1/Stat2 pathway.

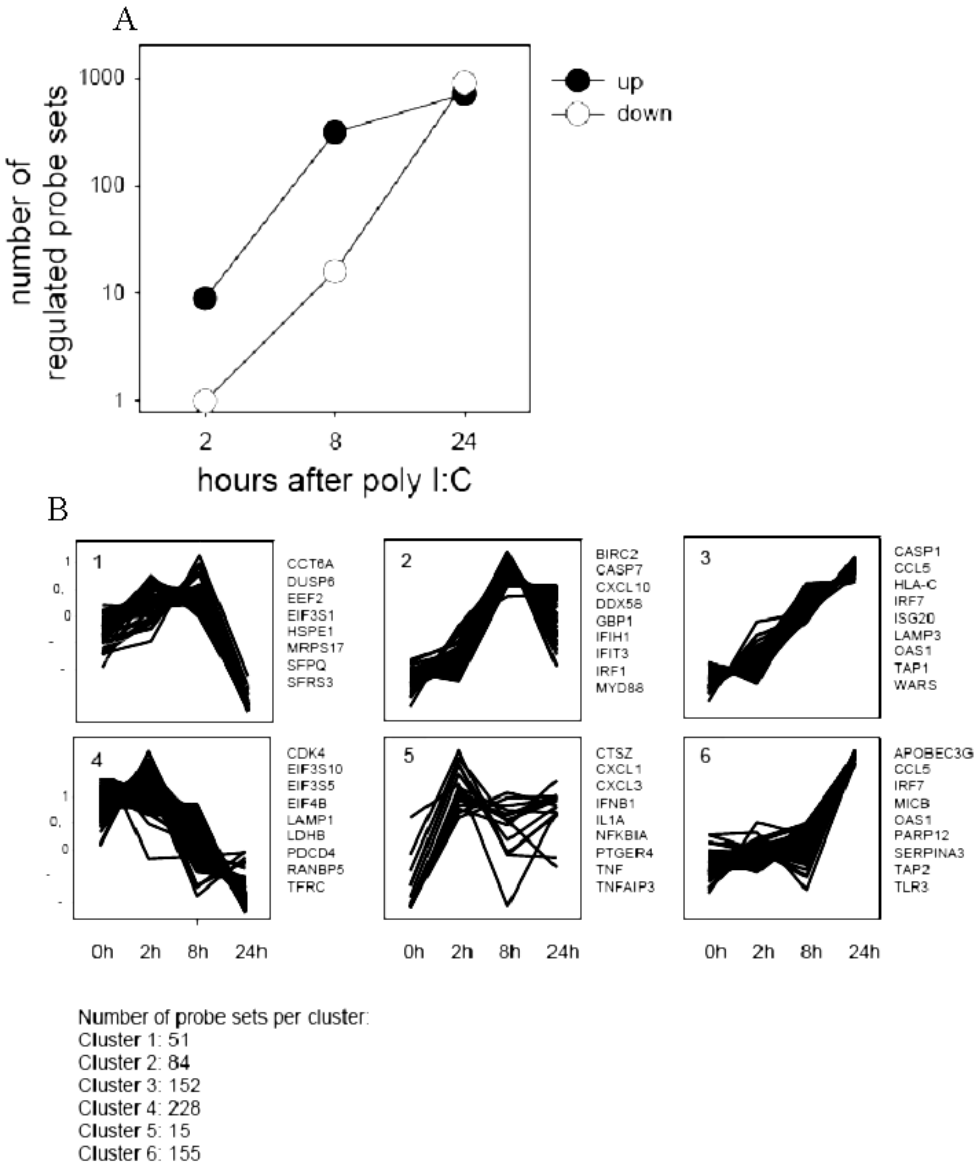


***Figure 17**. Overall changes in gene expression in keratinocytes following stimulation with Poly (I:C). (A) The number of probe sets that were significantly up- or down-regulated (Limma p-values <0.05) for the different time points. (B) Grouping of 685 regulated probe sets (defined as stated in Methods section) in clusters using self-organizing maps (SOM) according to patterns in kinetic changes. The y-axis unit is z-scores, indicating relative expression over time. The number of probe sets in each cluster is indicated. Examples of gene symbols are depicted for each cluster.*

5.9. All dsRNA receptors are expressed in primary keratinocytes

To further confirm the microarray results, we applied quantitative real-time PCR analysis which also showed mRNA expression for TLR3, PKR, RIG-I, and

MDA5 in unstimulated primary human keratinocytes (Figure 18A and 18B). To rule out individual variances between donors we used preparations of at least 9 different foreskins with reproducible results. We also analyzed the other receptors of the TLR family recognizing nucleic acids and confirmed our previous results regarding expression of TLR7, 8, and 9 (Mempel et al., 2003). Unstimulated cultured human keratinocytes did neither express TLR7, 8, or 9 in the microarray screen nor in the more sensitive real-time PCR approach (data not shown). Upon stimulation of cultured keratinocytes with Poly (I:C) for 36 h, we observed up-regulation of the different receptors at a variable degree with MDA5 showing the strongest (40-fold) and PKR showing the lowest (3-fold) induction (figure 18A). These results were paralleled on the level of protein expression for TLR3, PKR, RIG-I and MDA5. We applied fluorescent immunostaining and confocal microscopy to identify the cellular expression of all dsRNA sensing proteins in keratinocytes (Figure 18D). All, TLR3, PKR, RIG-I and MDA5 were strongly up-regulated upon Poly (I:C) stimulation in keratinocytes. To investigate the possible cross talks between PKR and TLR3 or RIG-I/MDA5 molecules, we applied co-staining of these receptors in both un-stimulated and stimulated keratinocytes with Poly (I:C). Of interest, staining for TLR3 in confocal microscopy revealed a strict intracellular localization which is different from other non-immune cell types like human fibroblasts (Jiang et al., 2002). TLR3 staining showed an irregular patch-like intracellular distribution suggestive of endosome-associated localization. Indeed, co-staining experiments with Alexa Fluor 594-conjugated dextran molecules which are known to be loaded into the endosome (Brandhorst et al., 2006) confirmed localization of TLR3 to this compartment (Figure 18C). In contrast, PKR which showed no co-staining with fluorescent dextrane (not shown) displayed a cytoplasmic distribution without preference for endosomal

structures thus confirming PKR as a receptor for cytosol-targeted Poly (I:C) and TLR3 as recognition molecule within the endosome (Figure 18C).

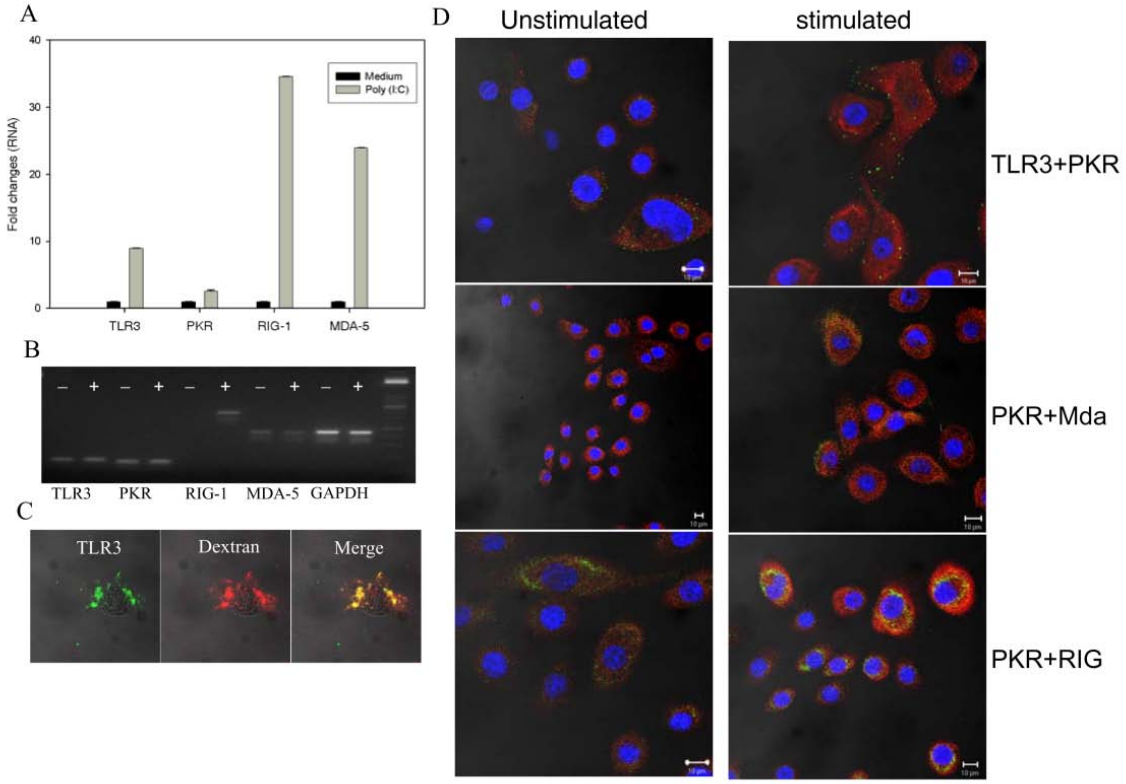


Figure 18. *Human keratinocytes express the whole panel of double-stranded RNA (dsRNA) sensing molecules. Baseline and Poly (I:C)-inducible expression of the dsRNA sensing molecules TLR3, PKR, RIG-I and MDA5 in cultured normal human keratinocytes. (A) Gene expression level of TLR3, PKR, RIG-I and MDA5 in human keratinocytes after stimulation with 20 µg/ml Poly (I:C) for 36 h or without stimulation (medium control) evaluated by quantitative real-time PCR (QPCR). QPCR was performed in duplicates with 5 replicate samples. (B) Gel electrophoresis of QPCR products from (A). QPCR products of TLR3, PKR, RIG-I and MDA5 were loaded on a 2% agarose gel. (+) indicates QPCR products after Poly (I:C) stimulation; (-) indicates unstimulated medium controls. The intensity of amplicon bands is compared to GAPDH. (C) Intracellular co-localization of TLR3 (green fluorescence) and Alexa594-labeled dextrane (red fluorescence). Cultured human primary keratinocytes were first loaded with dextrane for 1 h which was followed by stimulation with Poly (I:C) (20 µg/ml). (D) Immunofluorescence co-staining with specific antibodies against TLR3, RIG-I, MDA5 and PKR demonstrates Poly (I:C)-inducible intracellular expression of all four receptors in human keratinocytes. Keratinocytes were cultivated to 70% confluency on glass chamber slides. After incubation with Poly (I:C) or without (unstimulated, medium control), cells were fixed, permeabilized, and then co-stained with antibodies against TLR3, RIG-I, and MDA5, respectively (green fluorescence) and PKR (red fluorescence). Immunostaining was followed by nuclear staining with TOPRO3 (10 mM) for 20 min. Samples were analyzed under a confocal microscope.*

5.10. The major dsRNA recognizing and signaling pathways are functional in primary human keratinocytes

Gene expression analysis of human keratinocytes after Poly (I:C) stimulation has strongly suggested the involvement of not only pro-inflammatory and immune response signaling pathways under the control of NF-κB, but also of pathways under transcriptional control of IRF3/IRF7 (Figure 20; Tables 3 and 4). In order to look more specifically for dsRNA recognition signaling pathways in keratinocytes, we first screened for the phosphorylation of downstream kinases leading to NF-κB induction. As shown in Figure 19A and 19B, stimulation with Poly (I:C) led to the phosphorylation of P38 and JNK 2 kinase, both of which are known to be induced by the TLR3 and PKR pathways (Toshchakov et al., 2003; Sen and Sarkar, 2005). Consistent with the finding of phosphorylation of up-stream kinases in the NF-κB pathway, Poly (I:C) induced a complete nuclear translocation of p65 with subsequent mRNA and protein induction of the NF-κB-dependent chemokine IL-8 (Figure 20*A*-*C*). In immune cells, it has been demonstrated that Poly (I:C), besides NF-κB activation, is also able to induce through a TBK-1-dependent pathway the phosphorylation of IRF3 (Fitzgerald et al., 2003; Matsui et al., 2006). We demonstrated TBK-1 phosphorylation due to Poly (I:C) stimulation in human keratinocytes (Figure 19*C*). Congruent with the finding of TBK-1 phosphorylation Poly (I:C) stimulation also induced efficient nuclear translocation of IRF3 together with induction of IFN-ß (Figure 20*D*-*F*).

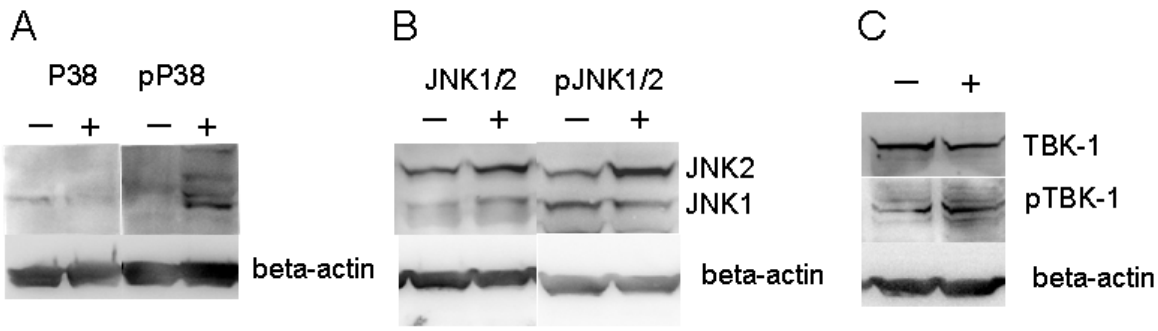
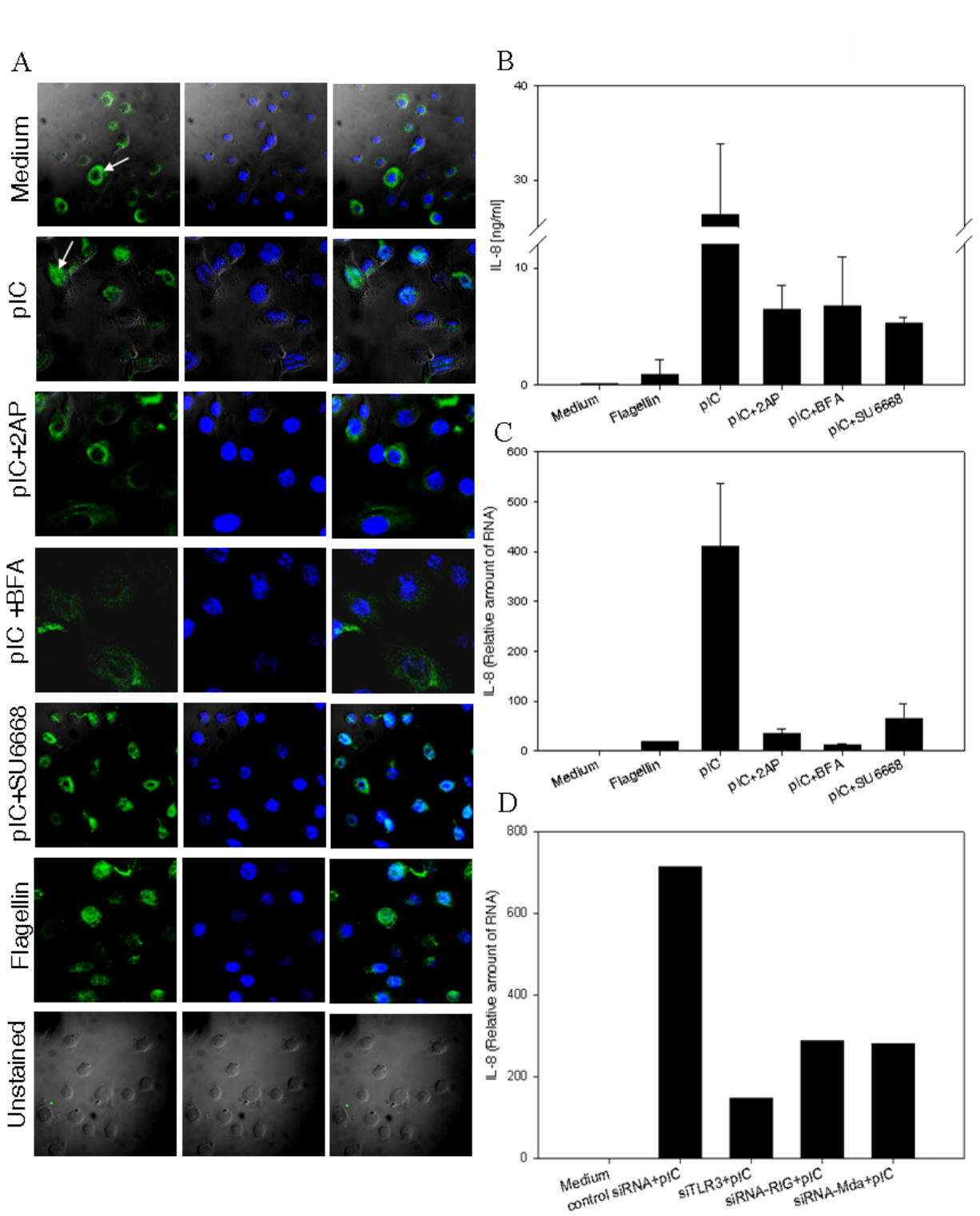


Figure 19. *Western blot analysis of P38, JNK1/2 and TBK-1 phosphorylation in keratinocytes stimulated with Poly (I:C). Stimulation of cultured human keratinocytes with the double-stranded RNA analogue Poly (I:C) induces protein phosphorylation in both, the NF-κB and the IRF3 pathway. The following kinases were analyzed: P38, JNK and TBK-1. Human keratinocytes were incubated with 20 µg/ml Poly (I:C) for 1 h. The phosphorylation of (A) P38, (B) JNK1/2, and (C) TBK-1 was confirmed by immunoblotting using antibodies specific for (A) P38 and pP38; (B) JNK1/2 and pJNK1/2; (C) TBK-1 and pTBK-1. (+) indicates Western blots after Poly (I:C) stimulation; (-) indicates unstimulated medium controls.*

5.11. Inhibition of dsRNA signalling pathways

To determine the effect of the various Poly (I:C)-induced signalling pathways in keratinocytes on the overall inflammation and immune response in more detail, we used inhibitors that target PKR, TLR3, and TBK-1. After recognition of dsRNA, PKR is phosphorylated to exert its activity on eIF2α and this phosphorylation can be blocked by addition of 2-aminopurine (2AP), a selective inhibitor of PKR (Medzhitov et al., 1997) which interestingly, not only abrogated NF-κB translocation (Figure 20*A*) and accordingly, massively reduced IL-8 induction (Figure 20*B* and 20*C*) but also strongly decreased IFN-β production (Figure 20*F* and 20*G*) and IRF3 translocation (Figure 20*E*). Thus, the proper function of PKR seems indispensable for both, the NF-κB and the IRF3/IRF7 pathway. To characterize the effect of inhibiting TLR3 signalling, we used the endosomal acidification inhibitor bafilomycin A (BFA), which prevents the adequate function of TLR3 within the endosomal compartment (de et al., 2005). BFA completely blocked p65-translocation (Figure 20*A*) and IL-8

production (Figure 20*B* and 20*C*) but, in contrast to 2AP, induced an enhanced IRF3 translocation (Figure 20*E*) and up-regulation of IFN-β on the mRNA and protein levels (Figure 20*F* and 20*G*). Therefore, the dominate function of TLR3 in keratinocytes is more likely to induce NF-κB-regulated than IRF3/IRF7-regulated genes and TLR3 seems not to be essential for IFN-β induction. As no specific inhibitor for RIG-I and MDA5 is available we took advantage of the common downstream kinase TBK-1 used by the RIG-I/MDA5 pathway for which the specific inhibitor SU6668 was available (Godl et al., 2005). The TBK-1 inhibitor SU6668 very effectively blocked IRF3 translocation (Figure 20*E*) and induction of IFN-β (Figure 20*F* and 20*G*) whereas it had a partially inhibiting effect on NF-κB translocation and IL-8 production (Figure 20*A*-*C*). It is therefore evident that primary human keratinocytes use all three dsRNA recognizing pathways to a different extent, thereby targeting different cytokines and chemokines. To demonstrate the specificity of the effect of the various inhibitors of dsRNA signalling on Poly (I:C) stimulation, we tested the TLR5 ligand flagellin in addition to Poly (I:C) in all our experiments. Compared to Poly (I:C), flagellin also induces significant p65 translocation (Figure 20*A*) (Mempel et al., 2003) but is a weaker inducer of IL-8 production (Figure 20*B* and 20*C*). None of the applied inhibitors affected flagellin-induced p65 translocation or IL-8 production (data not shown). Additionally, flagellin did neither induce IRF3 translocation nor IFN-β production in human keratinocytes (Figure 20*F*-*G*). To further extend the inhibitory experiments we used specific siRNA for each of the three signalling pathways (Figure 20D and 20H) demonstrating a similar result as seen with the chemical inhibitors.


A
Medium
pIC
pIC+2AP
pIC +BFA
pIC+SU6668
Flagellin
Unstained
B
IL-8 [ng/ml]
40
30
10
0
Medium
Flagellin
pIC
pIC+2AP
pIC+BFA
pIC+SU6668
C
IL-8 (Relative amount of RNA)
600
500
400
300
200
100
0
Medium
Flagellin
pIC
pIC+2AP
pIC+BFA
pIC+SU6668
D
IL-8 (Relative amount of RNA)
800
600
400
200
0
Medium
control siRNA+pIC
siTLR3+pIC
siRNA-RIG+pIC
siRNA-Mda+pIC

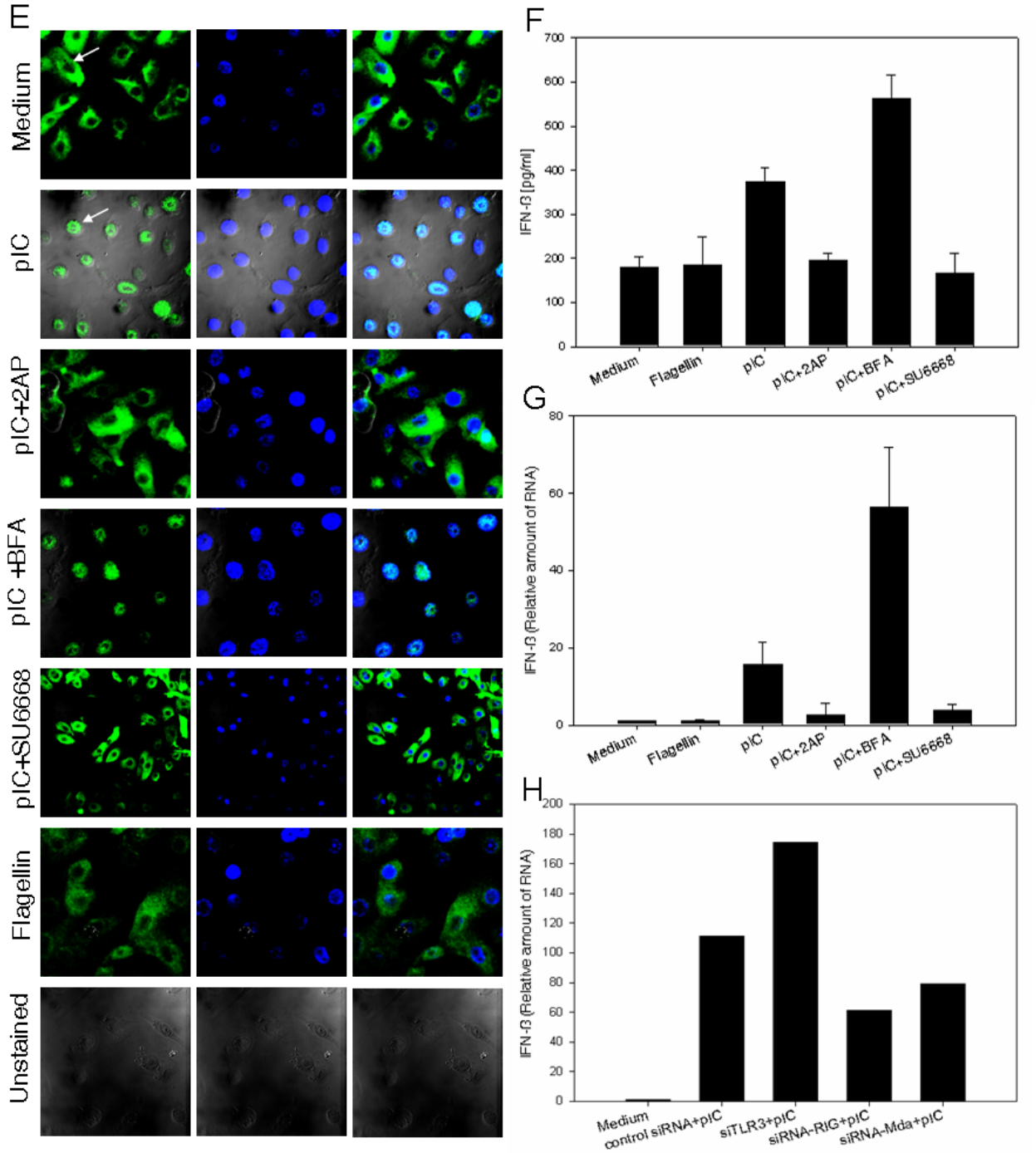


Figure 20. *Poly (I:C)-stimulated activation of the transcription factors NF-kB and IRF3, and induction of IL-8 and IFN-ß in cultured human keratinocytes in the presence or absence of inhibitors for TLR3 (bafilomycine A1; BFA), PKR (2-aminopurine; 2-AP), and TBK-1 (SU6668) as well as three relevant siRNAs for TLR3, RIG-I and MDA5. Primary human keratinocytes were cultured in 6-well plates in the presence of Poly (I:C) (pIC) (20 µg/ml) for 4 h after a 45 min pre-incubation with the inhibitors 2-AP (5 mM), BFA (100 nM) and SU6668 (5 µM). (A, D) To determine the effect of the dsRNA signalling pathway inhibitors 2-AP, BFA,*

and SU6668 on either RelA (A) or IRF3 (E) staining of primary human keratinocytes, cells were incubated with medium (unstimulated) as a negative control, or with Poly (I:C) without inhibitor as a positive control, or with one of the inhibitors as indicated. Cells were stained with a primary rabbit anti-p65 antibody (RelA) or a mouse monoclonal anti-IRF3 antibody followed by an Alexa488-labelled chicken anti-rabbit or anti-mouse antibody, respectively, followed by nuclear staining with TOPRO3 (10 mM) for 20 min. Cells non-reactive to the Poly (I:C) stimulus are characterized by a cytoplasmic staining pattern, reactive cells by a nuclear staining pattern. Arrowheads point to cells representing a typical fluorescence pattern for each of the reactivities. The TLR5 ligand flagellin was also investigated. (B- D) Influence of the dsRNA signalling pathway inhibitors 2-AP, BFA, and SU6668 and corresponding siRNAs on Poly (I:C)-stimulated secretion and expression of IL-8 in cultured human keratinocytes. (B) IL-8 secretion as measured by ELISA. Columns show the mean ± SEM of three representative experiments. (C, D) IL-8 mRNA as measured by quantitative real-time PCR (QPCR). PCR reactions were performed in duplicates and columns show the mean ± SEM of three representative experiments (C) or one representative experiment out of three (D). (F-H) Influence of the dsRNA signalling pathway inhibitors 2-AP, BFA, and SU6668 and related siRNAs on Poly (I:C)-stimulated secretion and expression of IFN-ß in cultured human keratinocytes. (F) IFN-ß secretion as measured by ELISA. Columns show the mean ± SEM of three representative experiments. (G, H) IFN-ß mRNA as measured by qPCR. Columns in (G) show the mean ± SEM of four experiments or one representative experiment out of three (H).

5.12. TLR7, the receptor for imidazoquinolines and single-stranded RNA, is functionally up-regulated in Poly (I:C)-stimulated keratinocytes

Our findings so far have demonstrated a significant proportion of regulated genes in human keratinocytes after Poly (I:C) stimulation which results in the expression of crucial NF-κB-dependent chemokines such as IL-8 and of IRF3-controlled effector cytokines such as IFN-β (Figure 17 and 20). Amongst the differentially regulated genes following Poly (I:C) stimulation were all known molecular components involved in the recognition of viral dsRNA (TLR3, PKR, MDA5, RIG-I) (Figure 18, Table 4). To investigate whether Poly (I:C) stimulation also regulates other receptors involved in innate antiviral immunity, we analyzed the expression of keratinocyte TLR7 in more detail. TLR7 was not found to be expressed in unstimulated keratinocytes. Although, after Poly (I:C) stimulation, TLR7 expression in keratinocytes was under the detection

limit of the Affymetrix array (in contrast to immune cells which express higher and easily detectable amounts of TLR7 mRNA, data not shown), according to the more sensitive real-time PCR results we observed a dramatic up-regulation of TLR7 in human primary keratinocytes after 36 h incubation with Poly (I:C) but not with the TLR5 ligand flagellin (Figure 21).

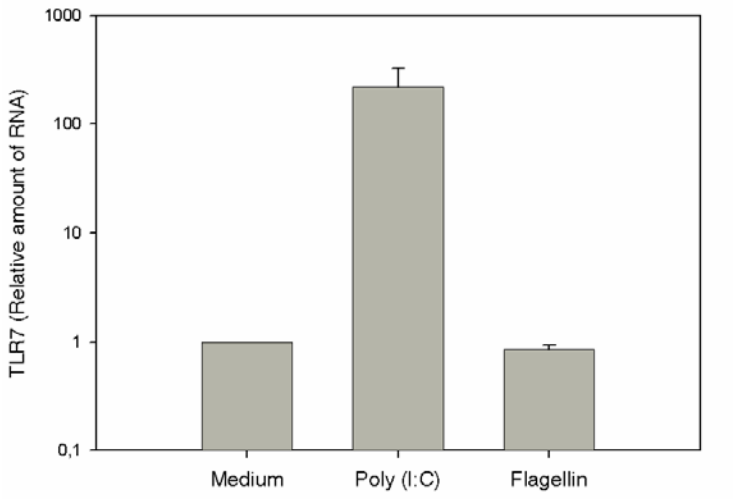


***Figure 21**. Stimulation of cultured human keratinocytes with the dsRNA analogue Poly (I:C) induces expression of TLR7. Primary human keratinocytes were stimulated with 20 µg/ml Poly (I:C) or 10 µg/ml flagellin for 36 h. Untreated cells were used as a control (medium control). The gene expression level of TLR7 was quantitated by real-time PCR. Data were calculated against GAPDH. PCR was performed in duplicates and columns show the mean ± SEM of five representative experiments.*

Consistent with the finding of lack of TLR7 mRNA expression in undifferentiated cultured primary keratinocytes derived from healthy skin which most closely resemble the basal epidermal cell layer, we observed expression of TLR7 in immunofluorescence staining of cryostat sections from normal human skin with an anti-TLR7 polyclonal antibody mainly in suprabasal but not in basal layers of human epidermis (Figure 22*A*). These results which indicate a differential expression of TLR7 in epidermal layers were mirrored and confirmed by real-time PCR performed from freshly isolated fractionated basal and supra-basal cell layers of normal human

epidermis showing an increased TLR7 mRNA level in suprabasal as compared to basal cell layers (Figure 22C). An even stronger TLR7 signal as in normal human epidermis was obtained with biopsies from human papilloma virus (HPV)-infected skin tissue – so called condyloma lesions – both, in immunofluorescence staining with anti-TLR7 antibody and in real-time PCR (Figure 22*B* and 22C). To demonstrate the quality and efficiency of the epidermal cell separation into basal and suprabasal fractions, we investigated mRNA expression of the two keratinocyte differentiation markers involucrin and transglutaminase-1, which were found to be strongly expressed only in the suprabasal cell layer, and of the basal keratinocyte marker β1-integrin, which was found to be expressed only in the basal cell layer (Figure 22*D*). Vice versa, no expression of involucrin and tranglutaminase-1 was found in the basal cell layer, whereas β1-integrin was absent from suprabasal cell layers.

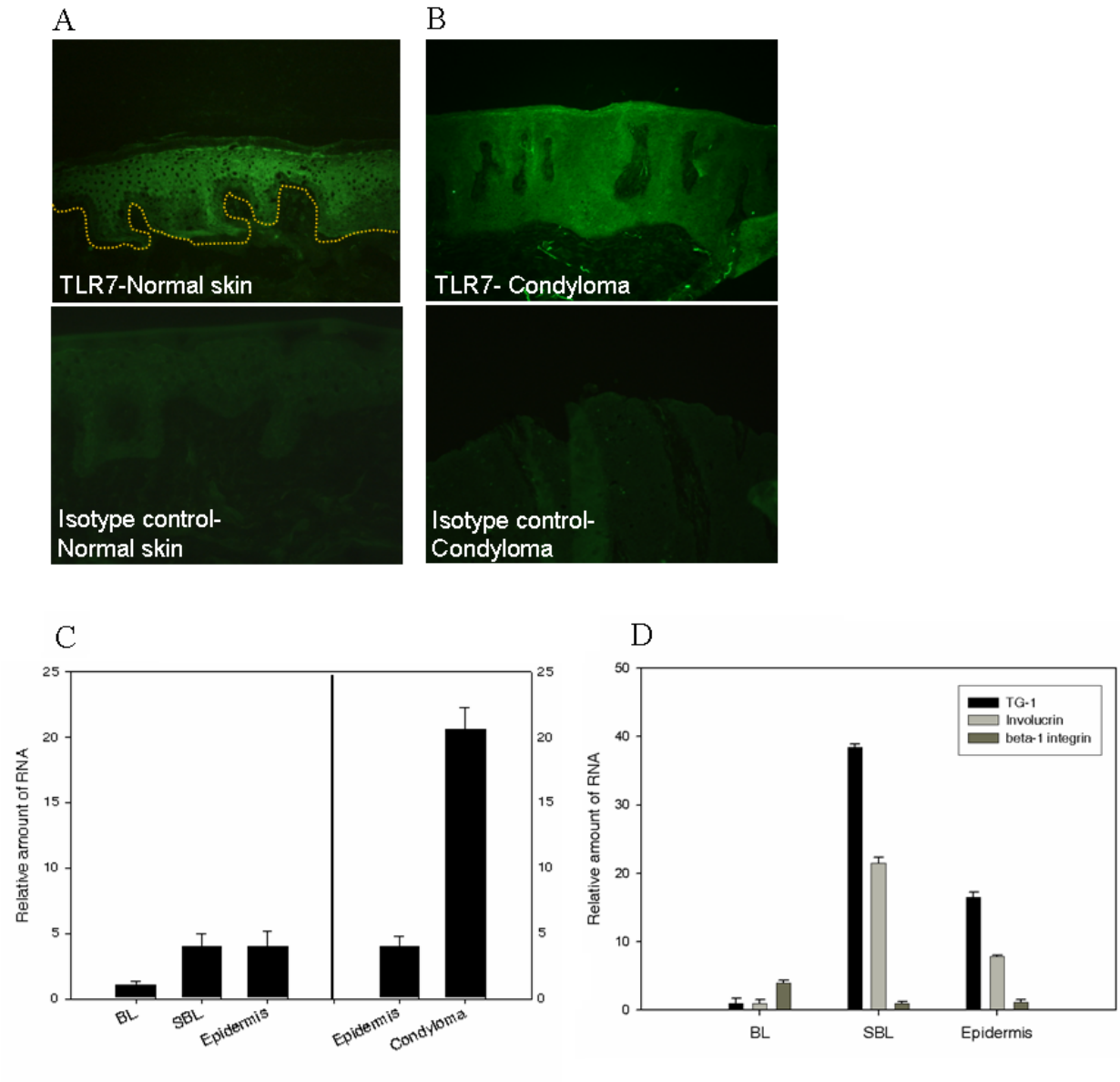


Figure 22. *TLR7 expression in normal and in human papilloma virus (HPV)-infected human epidermis. (A, B) Expression pattern of TLR7 in healthy human skin and in condyloma-derived HPV-infected skin tissue. TLR7 immunostaining was performed on cryopreserved tissue sections. The dotted line indicates the basement membrane zone at the dermo-epidermal junction. (C) Whole cell populations isolated from healthy epidermis were divided into basal (BL) and supra-basal (SBL) layer cells. The gene expression of TLR7 was evaluated using quantitative real-time PCR (QPCR). For comparison samples from whole epidermis and from condyloma biopsies were also analyzed. (D) To demonstrate the quality and efficiency of the fractionated cell isolation used in (C), the expression of involucrin and transglutaminase-1 as epidermal differentiation markers, and of β1-integrin as marker for basal layer cells was analyzed in QPCR either for whole epidermis or for basal (BL) and supra-basal (SBL) layer cells.*

We next sought to answer the question of functionality of TLR7 up-regulation following Poly (I:C) stimulation in keratinocytes,. To this end, we analyzed mRNA induction of the transcription factor IRF5, a central mediator of TLR7 signaling (Latz et al., 2004), in unstimulated cells, Poly (I:C)-stimulated cells and cells which were first stimulated with Poly (I:C) followed by stimulation with the TLR7 ligand gardiquimod. We observed that IRF5 was significantly (2.1 fold; *p*=0.03) induced only in cells treated with Poly (I:C) followed by stimulation with the TLR7 ligand gardiquimod (Figure 14). In contrast, Poly (I:C) alone never induce a significant upregulation of IRF5 at any time point (Figure 23; Table 4). Thus, stimulation with Poly (I:C) induces TLR7 expression in keratinocytes which renders them susceptible for subsequent stimulation by imidazoquinoline immune response modifiers with ligand specificity for TLR7.

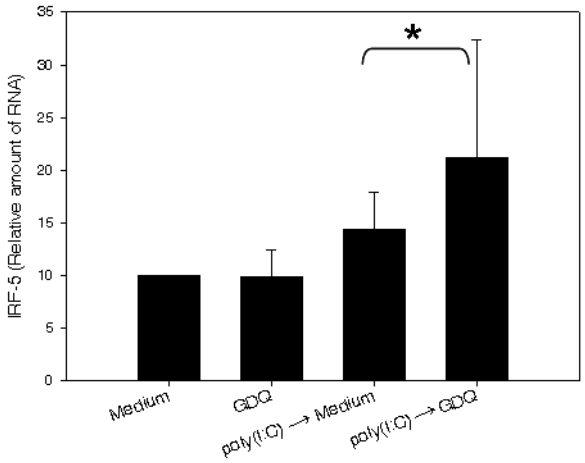


Figure 23. *Gene expression of IRF5 is induced in Poly (I:C)-stimulated human keratinocytes following incubation with the TLR7 agonist gardiquimod (GDQ). IRF5 was measured in keratinocytes treated under the following conditions: 20 µg/ml of Poly (I:C) for 24 h followed by 24 h medium or 1 mg/ml GDQ incubation; 1 mg/ml GDQ for 48 h; and medium (control) for 48 h. RNA was isolated after incubation, followed by cDNA synthesis and quantitative real-time PCR for IRF5. Columns show the mean ± SEM of nine experiments using keratinocytes from nine different donors. The asterisk indicates significant differences according to student's t-test between two modalities of incubation (p value= 0.03).*

6. Discussion

It was recently shown that TLR2, but not TLR4, is the specific receptor for S. aureus (Mempel et al., 2003). Upon ligation of TLR2 by S. aureus or its defined ligands, the NK-κB pathway is induced in human keratinocytes (Mempel et al., 2003). In this study, first we investigated the expression of TLR1–10 in epidermal keratinocytes and confirmed the functional relevance of keratinocyte TLR expression through stimulation with TLR-specific ligands. We provide evidence that human epidermal keratinocytes and the keratinocyte cell line HaCaT express several TLR family members. Antigen presenting cells (APCs) are one of the most important antigene interfaces in human body and are supposed to express a broad panel of PRPs, including TLRs.On the other hand, as it has been demonstrated that in vitro LCs may originate from monocytes (Geissmann et al., 1998). Therefore, in our study we analysed the response pattern of cultured mDCs driven from healthy and atopic individuals to various TLR-ligands. MDCs from atopic and non-atopic individuals showed expression of a broad panel of TLRs as previously reported this type of DC predominately produces type I TLRs (such as TLRs 2, 4, 5, and 6) which sense their ligands at the cell surface and transmit a signal to NF-κB by using the crucial adaptor molecule MyD88 (Hemmi and Akira, 2005). Atopic patients showed a very similar pattern of TLR expression with a mildly higher transcription for all TLRs, especially TLR5. When stimulated by the microbial compounds LPS, PGN, flagellin, and Poly (I:C) we and others (Chen et al., 2006) observed a stronger upregulation of the co-stimulatory molecules CD80, CD83, and CD86 in atopic mDCs whereas healthy controls tended to stronger induce the MHC I and MHC II molecules. In addition to the induction of mDC maturation, TLR-ligands also induced the production of the pro-inflammatory cytokines IL-12 and TNF-α in atopic mDCs at a similar level as in

controls thereby providing not only the “second” signal of co-stimulation but also a “third” secreted signal which is necessary to fully prime naïve T-cells and which also favours the development of a TH-1 immune response which is important to re-direct the dominant TH-2 responses in the context of SIT protocols.

In freshly prepared human epidermis, cultured primary foreskin keratinocytes, and in the permanent keratinocyte cell line HaCaT, except for TLR7, -8 and -9, all TLRs are expressed. The comparison of the TLR expression profile of primary keratinocytes and HaCaT cells revealed widely comparable data for most of the TLRs, but indicated also specific differences for TLR1 and TLR4, which were differentially expressed. Furthermore, the orphan receptor TLR10, for which a ligand has yet to be defined, and TLR6 were found to be expressed either in both cell types (TLR10) or in primary keratinocytes (TLR6) by quantitative real-time PCR, which is known to be more sensitive than conventional RT–PCR (Dagher et al., 2004). In a previous study, our group could not detect expression of TLR6 and TLR10 in primary keratinocytes using conventional RT–PCR (Mempel et al., 2003). TLR11, which has been described as a receptor that prevents infection by uropathogenic bacteria in the mouse and is probably not expressed as a full-length protein in humans (Zhang et al., 2004) was not investigated in this study. Regarding the TLR2 subfamily (TLR1, -2, -6) functional expression on human keratinocytes was found for TLR1 and TLR2, which are both known to recognize bacterial cell wall components. Pam3Cys (Takeuchi et al., 2002) activated IL-8 secretion in primary keratinocytes to a higher degree than in HaCaT cells. Whereas PGN is recognized by TLR2 alone (Schwandner et al., 1999) Pam3Cys recognition is mediated by a heterodimer of TLR2 and TLR1 (Akira and Hemmi, 2003). Based on the higher extent of TLR1 mRNA expression in primary keratinocytes as compared to HaCaT cells, it can be speculated that primary keratinocytes are also able to form a higher number of

TLR1/TLR2 heterodimers than HaCaT cells which could explain the predominant reactivity of primary keratinocytes to Pam3Cys.

TLR3 constitutes a separate subfamily within the mammalian TLRs that is characterized by intracellular expression (Funami et al., 2004). Ligation of TLR3, which senses double-stranded RNA (Wagner, 2004) was the most prominent stimulus for IL-8 secretion in primary human keratinocytes. TLR3 activation was achieved by the TLR3 ligand Poly (I:C) (Alexopoulou et al., 2001) a synthetic analogue of double-stranded viral RNA. Incubation of keratinocytes with Poly (I:C) also induced a redistribution of TLR3 staining to a more plasma membrane-like staining pattern (see Figure 18). It will be interesting to differentiate whether the Poly (I:C) mediated effects can solely be attributed to TLR3 or are also dependent on double-stranded RNA-activated protein kinase, an interferon-β (IFN-β)-induced protein constitutively expressed in human keratinocytes (Kuyama et al., 2003). Recent findings of Lebre *et al*. (Lebre et al., 2003) underline the importance of double-stranded viral RNA reactivity in keratinocytes for subsequent IFN-γ-mediated immune reactions. Thus, human keratinocytes possibly sense viral infections through their functionally active TLR3 receptors which enable the initiation of an immediate innate and delayed adaptive immune response to viruses infecting the skin.

TLR4 was the first human homologue of Toll to be described (Medzhitov et al., 1997) and was subsequently characterized as a receptor for LPS signalling (Poltorak et al., 1998). In addition to LPS, TLR4 also recognizes other ligands such as the fusion protein of respiratory syncytial virus, stress-induced members of the endogenous heat-shock protein family, and taxol in the murine system (Wagner, 2004). TLR4 is the sole member of the TLR4 subfamily. Controversial results have recently been reported regarding the role of LPS reactivity mediated by TLR4 in

primary human keratinocytes (Song et al., 2002; Kawai, 2003). Some authors (Song et al., 2002; Pivarcsi et al., 2003; Pivarcsi et al., 2004) found TLR4 and CD14 to be expressed by primary and HaCaT keratinocytes and keratinocytes to be activated by LPS, whereas Kawai *et al*. (Kawai et al., 2002) showed no TLR4 and CD14 expression and no LPS reactivity in keratinocyte cultures. There is some evidence, however, that TLR4 is expressed on epidermal keratinocytes in normal human skin in the absence of CD14 expression (Kawai et al., 2002). On the other hand, TLR4 expression was shown to be dependent on keratinocyte differentiation (Pivarcsi et al., 2004). The functional consequence of this finding is still unclear. Our results strengthen the finding that TLR4 is neither constitutively expressed nor functional in cultured primary keratinocytes. HaCaT cells, on the other hand, express TLR4 and CD14 at the mRNA level, but nevertheless do not react to LPS, which might be due to the lack of MD-2 expression (Visintin et al., 2003). Recent studies clarified the early molecular events involved in TLR4-mediated LPS signalling. MD-2 was shown to enable TLR4 binding of LPS and the formation of stable receptor complexes. CD14 on the other hand enhances LPS binding to MD-2 (Hayashi et al., 2001). Thus, even in the presence of TLR4 expression, as it is the case in HaCaT cells, no functional LPS signalling can be expected without a sufficient expression of both, MD-2 and CD14.

TLR5, a separate subfamily of the mammalian Toll homologues, recognizes the bacterial motor protein flagellin through a conserved site on flagellin required for protofilament formation and bacterial motility (Hayashi et al., 2001; Smith, Jr. et al., 2003). Our data provide evidence that TLR5 was expressed both in primary human keratinocytes and in the permanent keratinocyte cell line HaCaT at the mRNA level and the corresponding ligand, flagellin, induced NF-κB translocation and IL-8

secretion. HaCaT cells were not as reactive towards flagellin as primary keratinocytes, which might correspond to the lower level of TLR5 mRNA expression in the cell line. The finding of TLR5 expression and reactivity in human epidermal keratinocytes is of particular interest as skin infecting bacteria such as *B. burgdorferi*, which causes migratory erythema during the course of Lyme disease, are known to produce flagellin (Wallich et al., 1990). The specific recognition of the bacterial component flagellin through keratinocyte TLR5 and the flagellin-induced stimulation of epithelial IL-8 production could thus enable human skin to react to invading flagellated bacteria. Surprisingly, our experiments with primary keratinocytes showed an even more pronounced induction of IL-8 after stimulation with flagellin from *S. typhimurium*, which is not a common skin pathogen. However, typical skin lesions are known to occur during the course of enteric fever (Kollisch et al., 2005). Regarding the preparation of the two flagellins used in our study, it must be noted that *S. typhimurium* flagellin was a purified native protein, whereas flagellin from *B. burgdorferi* was a recombinant protein produced in *E. coli*. Furthermore, *B. burgdorferi* flagellin has an identity score of only 56·4% and 44·4% with *S. typhimurium* flagellin in the conserved flagellin sites recognized by TLR5 (Smith et al., 2003). Therefore, no final conclusions on the differential activities of the two different flagellins are justified. However, our data provide substantial evidence for a strong overall reactivity of human keratinocytes to flagellin from different bacterial sources.Members of the TLR9 subfamily (TLR7, 8, 9) of mammalian Toll homologues primarily sense pathogen-derived RNA and DNA motifs generated intracellularly in the infectious process (Wagner, 2004). In addition, synthetic ligands with TLR7 and TLR8 agonistic activity such as imiquimod, R848, and loxoribine have been described. In our experiments, the synthetic immunomodulators R-848 (specificity for TLR7 and TLR8) (Hemmi et al., 2002; Jurk et al., 2002) and loxoribine (specificity for

TLR7) (Heil et al., 2003; Lee et al., 2003) did not cause any IL-8 secretion or nuclear RelA staining, which corresponds well with the lack of TLR7 and TLR8 mRNA expression in primary keratinocytes and HaCaT cells. However, Schön *et al*. (Schon et al., 2003) could show an apoptotic effect of the R-848 analogue imiquimod (TLR7 ligand) in tumour-derived keratinocytes, but the possible involvement of TLR7 was not investigated in their study. Interestingly, the TLR7 ligand imiquimod now is increasingly used for the treatment of various skin disorders where keratinocyte transformations occur, such as in genital and common warts, in actinic keratosis, and more recently also in basal cell carcinoma (Tyring et al., 2002) . As we did not observe a significant basal expression of TLR7 in cultured normal keratinocytes, corresponding to the lack of irritation caused by imiquimod on normal human skin, the possible induction and up-regulation of TLR7 as a target of imiquimod in papillomavirus-infected or transformed keratinocytes should be investigated. As for TLR9 and reactivity to immunostimulatory DNA sequences (Hemmi et al., 2000; Bauer et al., 2001), recent studies demonstrated activation of keratinocytes by CpG-ODN without investigating TLR9 expression in particular (Mirmohammadsadegh et al., 2002). In multiple experiments using cells from different keratinocyte donors, we could not find any specific effect of CpG-ODN at concentrations up to 5 µM. Only nonspecific activation of primary keratinocytes both by CpG-ODN and non-CpG-ODN was obtained, which can not be explained by TLR9-mediated DNA sequence-specific effects. Because no TLR9 mRNA expression was observed with primers optimized for quantitative real-time PCR, it is not unexpected that no specific CpG-ODN effect was detectable. The non-specific CpG-ODN effects could be caused by the polyanion character of the ODN at higher concentrations. Moreover, it was recently demonstrated that the TLR9–CpG interaction only occurs after TLR9 recruitment from the endoplasmatic reticulum to a tubular lysosomal compartment, a subcellular

structure typically found in professional antigen-presenting cells but not in epithelial cells (Latz et al., 2004).

These data illustrate the diversity of TLR-mediated pattern recognition pathways present in human epidermal keratinocytes, which indicates that human skin may initiate a first line response to a variety of pathogen-derived components. With few exceptions such as in the case of TLR4, a strong correlation of the TLR mRNA expression pattern and the functional reactivity of primary keratinocytes and HaCaT cells to the various TLR ligands was found. However, differences observed in the constitutive and/or functional expression of some TLRs and TLR cofactor molecules between primary keratinocytes and HaCaT cells also demonstrate the importance of using primary cells in addition to immortalized cell lines, when investigating epithelial TLR expression and stimulation patterns, and possible functional consequences thereof. This study provides evidence for a TLR expression and response profile of normal human keratinocytes which extends beyond the role of TLR2 described for responses to *S. aureus* (Mempel et al., 2003). The variety in TLR expression may even indicate a role for human keratinocytes as sentinels of skin homeostasis. Further studies will have to elucidate the particular role and signalling response mediated by the various TLRs expressed by human epidermal keratinocytes and the relative importance of these TLRs for the innate cutaneous immune response.

The findings presented here strongly suggested that keratinocytes are also involved in building a large functional innate immune barrier against viral and bacterial pathogens. Our present results further demonstrate that keratinocytes efficiently respond to the viral dsRNA equivalent Poly (I:C) by expressing all known dsRNA sensing molecules (PKR, TLR3, RIG-I, MDA5) together with the downstream signalling pathways in a functional and differentially regulated way. The mechanism

by which dsRNA signalling in human keratinocytes promoted cellular responses involved NF-κB- and IRF3-dependent differential regulation of genes and their products known to be involved in antiviral immune responses, chemotaxis, innate immunity, and antigen presentation. We also demonstrated that stimulation with the viral dsRNA analogue Poly (I:C) drives human keratinocytes to express functional TLR7, the receptor for single-stranded viral RNA, which is not found in unstimulated cells. This finding may explain why keratinocytes in virally infected epidermis can serve as target cells for imidazoquinolines, which are ligands for TLR7 and effective immune response modifying drugs for the selective therapy of skin epithelial infections caused by human papilloma viruses without affecting healthy skin (Hengge and Ruzicka, 2004). Consistent with these *in vitro* studies we observed significant up-regulation of epidermal TLR7 in biopsies from papilloma virus-infected human skin (condylomas). The biological significance of this finding has to be further evaluated as we saw an only modest induction of IRF-5 while TLR7 was strongly up-regulated. In our hands, the most probable explanation is the lack of up-regulation for crucial adaptor molecules such as IRAK-4 and TRIF-1 which showed no induction in the gene array experiments.

It is not clear at the moment to which extent the capacity of keratinocytes to respond to viral pathogen associated molecular patterns reflects the overall response of human skin to viral challenges. In this context it is interesting that the major population of epidermal antigen presenting cells, e. g. Langerhans cells, are also equipped with a variety of antiviral response receptors (Quesniaux et al., 2004). As we included only foreskin-derived keratinocytes in our studies we were not able to analyze a possible interaction in detail, however, future work on the complex network of immune-responsive cells in the skin might answer the questions of cellular

interaction or cell types preferentially responding to epidermotropic viruses in more detail.

The parallel presence of all dsRNA recognition molecules in human keratinocytes in a functional and differentially regulated way is a novel and unexpected finding (Picard et al., 2003). Although NF-κB activation following stimulation with Poly (I:C) has also been observed in other cells of the epithelial lineage, such as in human respiratory (Guillot et al., 2005; Rudd et al., 2005; Tissari et al., 2005), reproductive (Schaefer et al., 2005), uterine (Schaefer et al., 2004), and intestinal (Cario and Podolsky, 2000; Vijay-Kumar et al., 2005) epithelium, this has been generally attributed to the expression and function of TLR3. Apart from TLR3-dependent NF-κB activation after dsRNA stimulation, IRF3 signals resulting in type I IFN production have also been demonstrated as a consequence of TLR3 ligand binding in normal human keratinocytes using a model that did not integrate the combined function of PKR, RIG-I, or MDA5 (Picard et al., 2003). As we have shown here, RIG-I and MDA5 are expressed and regulated after dsRNA stimulation in normal human keratinocytes and recognition of Poly (I:C) by these molecules promotes mainly IFN-β production through a TBK-1- and IRF3-dependent pathway and less activation of NF-κB-regulated genes. TBK-1 as common downstream kinase for both DExD/H-box-containing RNA helicases has been shown to play a key role in IRF3 activation and production of type I IFN through RIG-I and MDA5 in other cell types (Fitzgerald et al., 2003; Hemmi et al., 2004). We observed that inhibition of TBK-1 blocks IFN-β production after Poly (I:C) stimulation in human keratinocytes almost completely whereas it has only minor influence on the NF-κB regulated response. Thus, in human keratinocytes the cytosolic proteins RIG-I and MDA5 appear to be crucial elements for IRF3 activation upon stimulation with viral dsRNA.

Our experiments, however, can not rule out that there is a preferential binding of Poly (I:C) to MDA5 in human keratinocytes as it has been reported in the murine system (Kato et al., 2006). TLR3 on the other hand, as we have demonstrated here, is located in endosomal compartments in human keratinocytes and plays a more important role in the induction of NF-κB-regulated responses after Poly (I:C) stimulation. In other cell types it is generally accepted that ligation of TLR3 causes activation and translocation of NF-κB through the TRAF6 signalling pathway together with phosphorylation of IRF3 through TBK-1 (Hemmi et al., 2004). We found that in human keratinocytes, inhibition of TLR3 activity blocks NF-κB translocation and its further activation drastically, whereas IRF3 production was unchanged or even increased. These results are in line with a recent report showing a dominant pro-inflammatory response through TLR3 and a comparable pro-inflammatory and anti-viral response through RIG-I after Influenza A virus infection in human bronchial epithelial cells (Le et al., 2007). Moreover, programmed cell death induction in Mammalian reovirus infected cells also seems to depend on a functional RIG-I- IRF3 axis while RIG-I seems to be dispensable for NF-κB induced apoptosis (Holm et al., 2007). A possible explanation for this finding of increased IRF3 activity after endosomal inhibition of TLR3 can be derived from the results of Sato and colleagues who have characterized the binding domain for TRAF6 with TRIF (Hemmi et al., 2004). This binding domain is located in close vicinity to the binding domain of TBK-1 making sterical hindrances possible. In cases of TRAF6 recruitment to TRIF, a NF-κB dominated response is induced whereas in cases of TLR3/TRAF6 inhibition, more TBK-1 activated through the other dsRNA recognition molecules RIG-I, MDA5, and PKR might be available for the IRF3 inducing pathway. In contrast to the TLR3- and the MDA5/RIG-I-mediated dsRNA signalling pathways which show differential activation of either the NF-κB or the IRF3 transcription factor in keratinocytes, there

appears to be no such preference in transcription factor activation in the PKR-mediated pathway. Inhibition of PKR in human keratinocytes blocks both, the NF-κB and the IRF3 activation pathway very efficiently. From these data we would suggest that PKR plays a central role in the recognition of dsRNA in human keratinocytes, as inhibition of none of the other dsRNA recognition pathways has the ability to block NF-κB and type I IFN responses together and as strongly as the PKR inhibitor 2-AP.

Apart from regulating immune function and viral defense genes, the dsRNA analogue Poly (I:C) also induced genes with a function in apoptosis, especially those enriched in a cluster of genes with maximal up-regulation at 8 h (see Figure 17*B* and Table 2). These findings suggest that the early coordinated removal of virally infected keratinocytes through the onset of an apoptotic program appears to be, together with the active production of proinflammatory and effector chemokines and cytokines, another important aspect of the antiviral defense strategy of human epidermal keratinocytes.

We conclude based on the presented experiments that keratinocytes not only give rise to the “bricks and the mortar” of the *Stratum corneum*, a large physical barrier against viral and other pathogens, but also express a full antiviral defence program consisting of four dsRNA recognition molecules together with the relevant downstream kinases leading to NF-κB- and IRF3-dependent chemokine and cytokine production. Furthermore, stimulation of keratinocytes with dsRNA induced up-regulation of TLR7, another antiviral receptor of the innate immune system with specificity for single-stranded RNA and for the antiviral imidazoquinoline drugs. Taken together, our results have identified a complex and fully functional program of innate antiviral immunity in human keratinocytes that, together with the expression of antimicrobial peptides (Schauber and Gallo, 2007) and the network of epidermal Langerhans cells (Suzuki et al., 2000) protects human skin from viral attack. This

functional property of keratinocytes can contribute to the development of selective T cell receptors via maturation and functional polarization of dendritic cells (Lebre et al., 2003).

7. References

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8. List of figures

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10. Acknowledgment

My last remaining task is to acknowledge all those people that have contributed to the work described in this thesis. It is an impossible task, given the many people that have helped to design, implement, apply, criticize and sponsor the work. I am going to try anyway, and if your name is not listed, rest assured that my gratitude is not less than for those listed below.

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11. Summary

The human skin represents the first line of defense against potentially hazardous environmental threats like microbial pathogens. This central role of the innate immune defense is performed by the group of ‘‘pathogen-associated pattern recognition receptors,’’ among which the group of Toll-like receptors (TLRs) has evolved as the most essential during the last years. As keratinocytes comprise the majority of the cellular population in human skin, their functional TLRs expression profile has been investigated and compared to dendritic cells (DC), the first cellular immune compartment of the epidermis. This investigation revealed that both keratinocytes and DCs express TLR1, 2, 3, 5 and 6 which all respond to TLRs specific ligands. Surprisingly, in contrast to DCs, keratinocytes express TLR7 which can be a possible explanation for the positive effects of TLR7 ligands as anti-viral therapeutics. Moreover, the study of molecular mechanisms involved in keratinocytes response to viral infections demonstrates that all different receptors of double stranded RNA including TLR3, RIG-I, MDA5 and PKR are expressed and functionally active in RNA-virus infection mimicked by Poly (I:C). Using inhibitors for the various dsRNA signaling pathways (mainly NF-κB and IRF3 pathways), I demonstrated that in human keratinocytes, TLR3 seems to be necessary for NF-κB but not IRF3 activation, whereas RIG-I and MDA5 are crucial for IRF3 activation. PKR is essential for the dsRNA response in both signaling pathways and thus represents the central antiviral receptor for dsRNA stimulation. This study emphasizes the central role of keratinocytes play as an active arm of the body´s first defense in encountering microbes. In summary, this thesis uncovers the yet barely investigated responses of keratinocytes to viral infection by elucidating the selective role of the receptors involved.

12. Curriculum vitae

B E H N A M N A D E R I K A L A L I

PERSONAL INFORMATION

- Date of birth 5 June 1972
- Place of birth Mashad, Iran
- Family Status Married, 1 child
- Nationality Iranian

EDUCATION

- **2007- Now:** scholarship in Klinikum rechts der Isar, Technical University of Munich, Germany, Granted by German Ministry of Economy and StMWIVT Bayern.
- **2003-2007:** PhD student in laboratories of Prof. Dr. M. Ollert, Department of Dermatology and Allergy, Technical University of Munich, Germany. Supervised by Prof. Dr. H. Parlar, Institute of chemistry and technical analysis, Technical University of Munich, Germany

  Title of thesis: Interaction of Human Primary Keratinocytes with Toll Like Receptors ligands and Resulting Pro-inflammatory Signals.
- **1999-2002:** Master of science in Medical Biotechnology, Tarbiat Modares University, Tehran, Iran, Average: 18.19 from 20

  Title of thesis: Possibilities of down regulation of Cytochrom P450 1A1 gene expression in mRNA levels by DHEA and IL-1 in Rat hepatoma cells. (practical section of this thesis has been performed in the lab of Prof. Kettrup and Prof. Schramm at the Institute of Ecological Chemistry in GSF, Munich, Germany)
- **1997-1999:** Military service at the military hospital
- **1995-1997:** Bachelor of science in Medical laboratory technology, Mashad university of medical sciences, Mashad, Iran, Average: 18,02 from 20
- **1992-1994:** Associate diploma in Medical laboratory technology, Mashad university of medical sciences, Mashad, Iran, Average: 16,73 from 20

- **1986-1990:** High school diploma in empirical sciences, Shahid Hekmat high school, Mashad, Iran, Average: 16.95 from 20

PUBLICATIONS AND PRESENTATIONS

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