

Review article

How epithelial cells detect danger: aiding the immune response

The epithelial layer occupies a strategic important location between an organisms' interior and exterior environment. Although as such it forms a physical barrier between both environments, it became clear that the role of the epithelium extends far beyond this rather passive role. Through specialized receptors and other more general mechanisms, the epithelial layer is not only able to sense changes in its environment but also to actively respond to these changes. These responses allow the epithelium to contribute to wound and tissue repair, to the defense against micro-organisms, and to the control and regulation of the locale immune response. In this review, we focus on signals acting on epithelium from the exterior environment, how these signals are processed and identify research challenges.

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Sensing the environment

Considering the role of the epithelium in sensing changes in its environment, we need to take into account that many different kinds of epithelial surfaces exist. The environment of the epithelial cells lining the gastro-intestine tract will differ significantly from the environments encountered by airway epithelium or the epithelial layer of the urinary tract. This may well have consequences for the types and expression levels of epithelial receptors or their response. Where bladder epithelial cells respond efficiently to, for instance, lipopolysaccharide (LPS) (1), intestinal epithelial cells are relative tolerant (2). Even within the airways there is a large variation: the epithelial cells lining the lower respiratory tract are from a different embryonic origin than the cells lining the upper respiratory tract and within the nasal cavity one can find specialized olfactory epithelium alongside pseudo-stratified ciliated epithelium and 'stratified' epithelium. Similarly there are differences in the epithelium from the ileum, colon or other parts of the gastro-intestine tract. Moreover, numerous experiments on the role of epithelium in sensing its environment have been performed with (carcinoma) cell lines derived from local tissues. We can only stress that, although data obtained in these experiments is highly useful, it may not necessary reflect the true *in vivo* situation. Several mechanisms are known through which the epithelium can sense its environment. These comprise of both cell surface and intracellular receptors for which activation leads to a signal cascade and an altered gene expression profile.

Cell surface and intracellular receptors

The receptors of the innate immunity have only a limited specificity and can be activated by interaction with common proteins motives or pathogen-associated molecular patterns (PAMPs) found in micro-organisms. Inadvertent activation by host or microbial factors of these pattern recognition receptors or the inability to be activated underlies the pathogenesis of some common diseases underlining the relevance of these receptors in maintaining cellular homeostasis.

Toll-like receptors

The Toll-like receptors (TLR) are probably the best studied group of pattern recognition receptors. These receptors are the mammalian homolog of the *Drosophila* Toll receptor that in this organism was held responsible for activation of host-defense mechanisms in response to infection (3). Although in *Drosophila* the Toll receptor family is more likely to play a role during embryonic development (4, 5), transcriptional activation of some anti-microbial peptides is also dependent on Toll activation (6).

In most vertebrates, the Toll family comprises about 10 family members (7) with a highly conserved intracellular signaling domain that resembles the signaling domain found in the mammalian IL-1 receptor (8). After activation of the receptor, this Toll/IL-1 receptor (TIR) domain interacts with different adaptor molecules that through

Table 1. Ligands for Toll-like receptors

Receptor	Ligand	Source
TLR1	Triacyl lipoproteins	Bacteria and mycobacteria
TLR2	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Yeast
	Lipopeptides	Mycobacteria
	Lipoarabinomannan	Mycobacteria
	Lipopolysaccharide	Gram-negative bacteria
	Peptidoglycan	Gram-positive bacteria
TLR3	Viral dsRNA	Virus
TLR4	Fibrinogen	Endogenous
	HSP-60	Endogenous
	HSP-70	Endogenous
	LPS	Gram-negative bacteria
	Taxol	Plants
	Mannan	Yeast
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	Mycoplasma
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Yeast
TLR7/8	ssRNA	Virus
	Imidazoquinoline	Synthetic
TLR9	CpG-containing DNA	Bacteria and virus

This table lists known ligands for the various Toll-like receptors and their source.

activation of NF- κ B and/or IFN-regulatory factors (IRF) lead to transcription activation of a broad panel of genes. The homology between Toll-like family members also extends to the extracellular part of the receptor (9). Multiple leucine-rich repeats (between 19 and 25) and a single membrane proximal cysteine motive are involved in specific binding to a wide variety of microbial and endogenous ligands (10) (Table 1). Unclear is how such conserved domains in Toll-like family members are able to recognize different ligands specifically, also given that hydrophobic interactions seem to be a prominent factor (11). Two aspects may help in the receptor specificity. Firstly, there is a distinct intracellular distribution of the receptors (Fig. 1, modified from figure on the website of <http://www.invivogen.com>). TLR-1, 2, 4 and 6 are found at the cell surface where they mostly interact with bacterial cell wall components, whereas TLR-3, 8 and 9 that interact with viral or bacterial nucleic acids are found in intracellular compartments. Secondly, some of the specificity may come from the additional involvement of membrane bound or soluble factors interacting with the ligands, like MD2 (12, 13) and CD14 (14, 15) for LPS-mediated activation of TLR-4.

At the moment, there are still some doubts about some of the ligand specificities of the LTR receptors (16). The general issue is the purity of the ligands. For TLR-4, a number of host factors (HSP60, HSP70, Surfactant Protein-A) have been described that could activate the signaling cascade (17–19). However, it is technically very difficult to prove that this effect is not because of some minute contamination with LPS, as this is such a powerful ligand for TLR-4. Similarly, it would seem that

the reported action of LPS on the TLR-2 receptor in TLR-4 knockout experiments was an effect of a minor contamination of a TLR-2 ligand in the LPS sample (20, 21). Moreover, the activation of TLR-2 by peptidoglycans has also been called into question, as some experiments have shown that lipid proteins, that are present as contaminants in peptidoglycan extracts, are responsible for the observed activation (22).

TLR-mediated signaling

After binding of their ligands, TLRs are capable of differentially activating distinct downstream signaling events via several adaptor proteins (Fig. 1). The first path leads to activation of transcription factor NF- κ B in a MyD88-dependent way. The cytoplasmic TIR domain of the receptor forms a platform for the recruitment and activation of the adaptor molecule MyD88 and the kinases of the IL-1R-associated kinase (IRAK) family (23–25). Following IRAK-4 autophosphorylation and activation of the IRAK-1/2-complex (26), the TRAF6 adaptor protein interacts with a second kinase complex with TAK1 that ultimately leads to the inactivation of the NF- κ B inhibitor I κ B (27–30). After activation of NF- κ B, the transcription factor is no longer retained in the cytoplasm and is able to translocate to the nucleus where it activates transcription of a variety of cytokines/chemokines (TNF- α , CXCL10, IFN- γ and IL-1, -6, -8, -10 and -12) and other genes like COX-2 and SOCS. For TLR-2 and -4, signaling this pathway is dependent on TIRAP (31), whereas this signaling pathway for TLR-3, -7 and -9 is independent of TIRAP and induces type I interferons via IRF-5 and -7 (32).

The second, MyD88-independent, pathway also targets NF- κ B through the receptor bound adaptor molecule TRIF (33, 34), although the precise mechanism has not been resolved. This TRIF-dependent pathway also activates IFN-regulatory factor 3 (IRF3) leading to the induction of IFN- β . For TLR-4, this pathway acts through TRIF and TRAM (35, 36), whereas for TLR-3 only TRIF is required.

Natural occurring mutations in key signaling molecules will have consequences for the ability to respond to micro-organisms, although in all cases the deficiency has not been linked specifically to epithelial TLR expression. A number of families with IRAK-4 deficiency have been described that suffer from recurrent bacterial infections (37, 38). Given that all TLRs can signal through IRAK-4 this would be hardly surprising and, as both for TLR3 and TLR4 TRIF mediated signaling to IRF3 remains intact, the response to viral infections remains unaffected. Similar observations have been reported for mutations in TLR4 or TLR2. In the case of TLR2, a specific polymorphism (R753N) has been linked to increased susceptibility to tuberculosis (39). Interestingly, a different mutation associated with TLR2, upstream of the

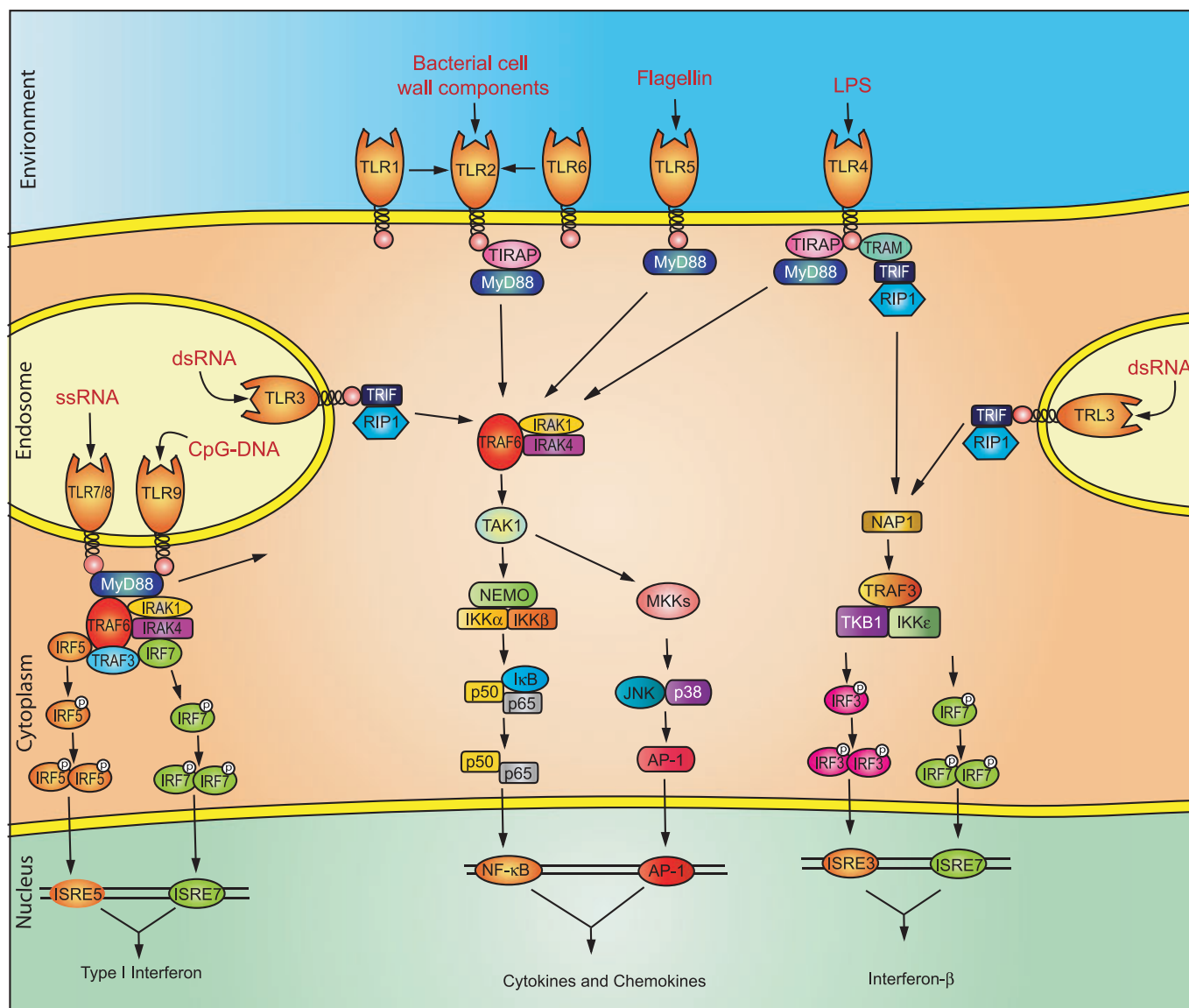


Figure 1. TLR signaling. Here different TLRs are shown in their respective cellular location; the signaling pathway leading to transcription factor activation is schematically drawn. Modified from http://www.invivogen.com/sscat.php?ID=13&ID_cat=2.

coding region, affects the development of asthma (40). Only children from farmers with this mutation show a reduced prevalence of asthma, linking TLR2 to the protective effects seen in rural populations vs urban populations (41).

Airway epithelial expression of TLRs

There is very limited data on the expression pattern of the TLRs in the upper airway and even less functional data. *In situ* hybridization (42) and immunohistochemistry (43) showed expression of TLR-2 and TLR-4 in nasal epithelium, but the expression of the other TLRs has

not been investigated. Variable expression for TLR-2 and TLR-4 on the RNA level was evident in tissue samples from nasal polyposis, chronic rhinosinusitis, cystic fibrosis and healthy controls (43). However, as this RT-PCR was done on tissues it cannot be firmly established that the variable expression is a consequence of differential expression in the nasal epithelium.

Significant more data is available for lung epithelium (44–48) both on the expression level, as well as their functional activity. Primary small airway epithelial cells on the mRNA level express TLR-1 through TLR-6, whereas TLR-6 through TLR-10 could not be detected (48). This distribution is partly reflected in the bronchial epithelium cell line BEAS-2 that showed relative high

mRNA levels for TLR-1 through -6, but that did also express TLR-7 through -10, albeit on a lower level than for TLR-1 through -6 (47). In both lung cell types, specific stimulation of TLR-3 with dsRNA leads to transcription induction of a broad range of genes (46, 47). These include chemokines and cytokines (IL-6, IL-8, GM-CSF, RANTES, TNF- α and I-TAC), components of the TLR-signaling cascade (MyD88, TRIF, IRAK-2) and components that affect the extracellular matrix (MMP-1, -8, -9, -10, -13). The changes induced by dsRNA in the TLR-signaling cascade are particularly interesting. Although some of the mRNAs (MyD88, TRIF, IRAK-2) are up-regulated, while other remain unaffected (TRAM, TIRAP, IRAK-1, IRAK-3), this is only partly confirmed on the protein level. The clear exceptions being TRIF and IRAK-1 that are down-regulated at the protein level with mRNA levels going up for TRIP or remaining constant for IRAK-1 (46).

Adding to the complexity of the TLR-signaling cascade is that the expression of the TLRs themselves is affected by external stimuli. Experimental models of viral or bacterial infection show functional changes to the TLR expression repertoire. After infection of epithelial cells with respiratory syncytial virus (RSV), the expression of TLR4 is strongly up-regulated, increasing the responsiveness of epithelial cells to LPS (49). This is not only a consequence of the up-regulation of the receptor but also of the adaptor molecule MD-2 (49), that like sCD14 (15) is required for an optimal response in epithelial cells (50). This suggests that a mechanism is in place whereby viral infections induce an activated alertness in epithelium for bacterial infections. Viral infections may also influence TLR3 (51, 52), by which they may also directly affect anti-viral responses. Similar observations have been made with isolated proteins that have been identified as molecular patterns of micro-organisms (46). Firstly, stimulation of TLR-3 with dsRNA significantly up-regulates TLR-1 through 3, an effect that can also be observed when TLR-5 on primary epithelial cells is stimulated with Flagellin. This is no general effect on TLR gene regulation as dsRNA down-regulates TLR-5 and -6, while Flagellin does not affect these genes. Interestingly, activation of the heterodimer TLR-2/6 through Zymosan strongly down-regulates most TLRs with only TLR-2 and -5 remaining unaffected. TLR expression is not only affected by receptor agonist but also by 'inflammatory' or 'allergic' mediators (46). Where the inflammatory mediators IL-1 β , TNF- α and IFN- γ have a limited effect when given on their own, a strong induction of TLR-2 and -4 is seen when epithelial cells are exposed to either IL-1 β or TNF- α in combination with IFN- γ . A similar picture emerges for IL-4 and IL-13, which by themselves have a limited effect on TLR-1 and -2 expression levels, but in combination with TNF- α strongly up-regulate the expression of these receptors. Overall it would seem that TNF- α potentiates both a Th1-driven (IFN- γ) as well as a Th2-driven (IL-4, IL-13) response.

Protease-activated receptors

Blood clotting and platelet activation involves a complex interplay between multiple proteases that through their action either activate or inactivate other proteases and/or cells. In this process protease-activated receptors (PARs) have been described and investigated first. Later research interests focus on PARs involved in injury and wound healing, but recently the role of PAR in inflammation is gaining more and more attention. Multiple (cellular) sources, in addition to the coagulation enzymes, have been described to produce proteases that act through PARs. These include: trypsins (II, IV) produced by the pancreas, endothelial cells or epithelium; mast cell proteases (tryptase, chymase); leukocyte proteases (cathepsin G, proteinase-3); or proteases from bacteria (gingipains-R), mites (DerP1, P3, P9) and fungi (pen C 13) (53).

To date, four distinct PARs have been cloned that all belong to the serpentine or 7-transmembrane type of receptors (54–59). Serpentine receptors are the most common found cell surface receptors and they share a common signal pathway that involves receptor coupled heterotrimeric G proteins. These G proteins in turn directly regulate the activity of different intracellular enzymes (guanylylcyclase, adenylylcyclase, phospholipase C) or indirectly affect the activity of calcium-dependent enzymes by increasing intracellular Ca⁺⁺ levels via calcium channels in the plasmamembrane and/or in the endoplasmatic reticulum. Three of the receptors (PAR-1, -2 and -3) are located in a small region (5q13) on chromosome 5 and share a two exon genomic organization with PAR-4 located on chromosome 19(p12) (60–62). A unique mechanism that uses the intrinsic enzyme activity of proteases to detect their presence underlies the activation of the PARs [Fig. 2, modified from Reed (63)]. Each of the receptors contains a ligand binding domain, encoded within the small first exon, which can be activated through interaction of a peptide sequence that is contained within the N-terminal part of the receptor itself. In the inactive state, the N-terminal extension of the receptor protein prevents the interaction of the internal peptide sequence with the ligand binding domain of the receptor. This N-terminal extension can be clipped off through the action of an extracellular protease, allowing the tethered ligand to activate the receptor (55).

Several factors contribute to the specificity of the individual receptors. The first level is the primary sequence around the cleavage site that allows only some proteases to clip off the N-terminal extension (Table 2). A broad collection of both endogenous (e.g. tryptase) and exogenous (e.g. allergens) proteases has now been described to act through PARs. The second level involves initial binding of the protease to either a distinct extracellular domain in the receptor itself or the initial interaction of the protease with another membrane-bound protein. Initial binding α -thrombin to the hirudin-like domain in PAR-1 explains the higher

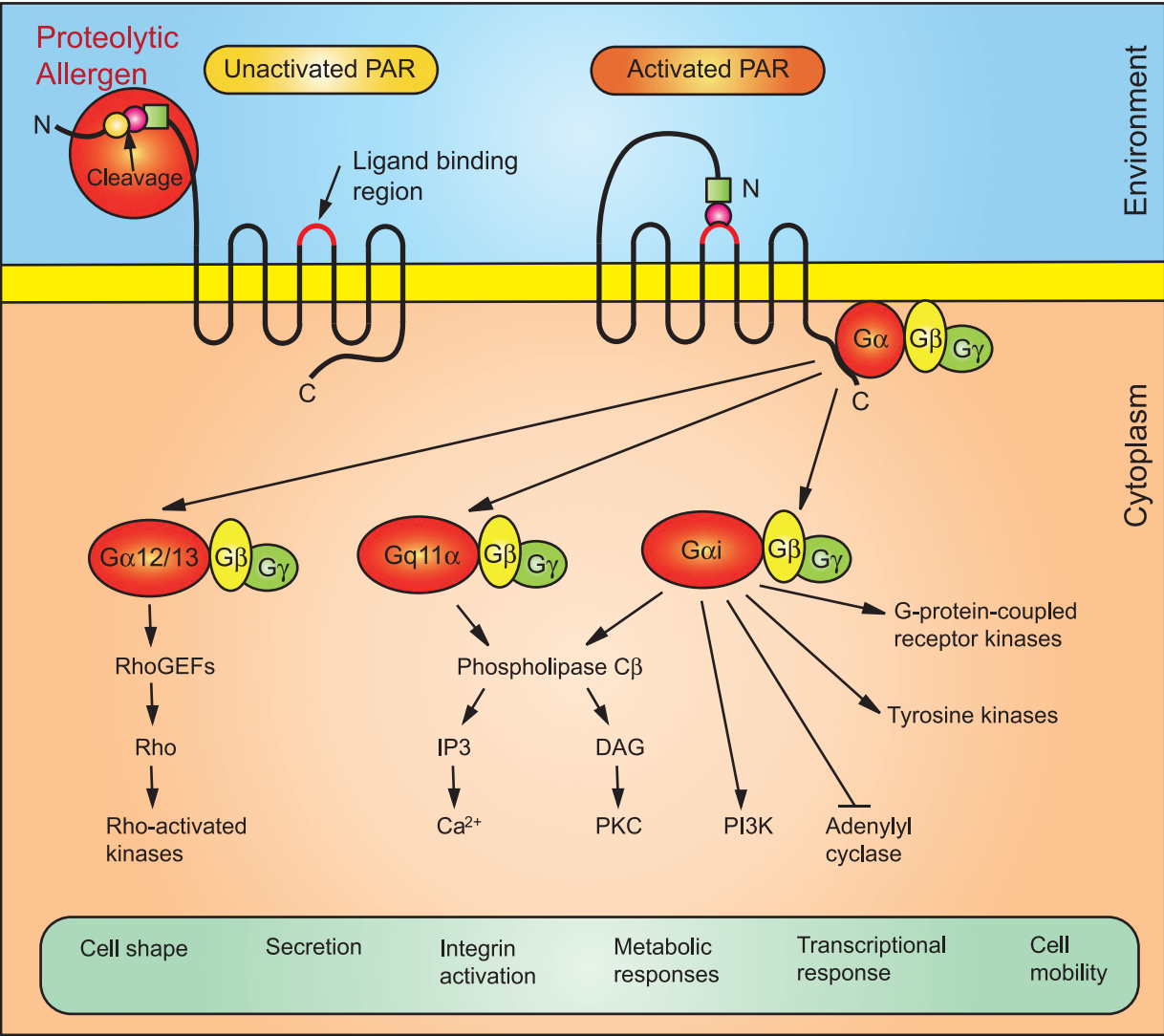


Figure 2. PAR signaling here it shows how PAR is activated and how different adapter molecules lead to different signaling pathways. Modified from Reed and Kita. The role of protease activation of inflammation in allergic respiratory diseases, *J Allergy Clin Immunol* 2004; 114: 997–1008. With permission from Elsevier.

Table 2. PAR receptors and activators

	PAR1	PAR2	PAR3	PAR4
Activating proteases (endogenous)	Thrombin Coagulation factor Xa Chymotrypsin	Trypsin Coagulation factor Xa Mast cell tryptase Proteinase-3 Elastase Cathepsin-G	Thrombin	Thrombin Trypsin
Activating proteases (exogenous)		Der P1 Pen C13 Cockroach allergen Fungal allergens; Aspergillus and Alternaria		
Agonist peptides	TFLLR-NH2	SLIGRL-NH2	TFRGAP-NH2	GYPGQV-NH2

This table shows which proteases can activate the different PAR receptors, exogenous proteases have been described only to activate PAR2. In addition it also shows what sequence of the N-terminal tail is recognized by the receptor.

potentiating activity of α -thrombin in comparison with γ -thrombin that is not able to interact with this domain (64, 65). This hirundin-like domain is not only present in PAR-1 but can also be found in PAR-3 (57). Interaction of the clotting factor FVIIa with the membrane bound tissue factor (TF) is essential for FVIIa's ability to activate PAR-2, although proteases like trypsin or tryptase can activate PAR-2 without needing to interact with TF first (66). Interestingly, there is even collaboration between the PAR receptors with PAR-3 acting as co-stimulatory factor for PAR-4 (67, 68). Three other factors that regulate the signaling capabilities of the PARs are (1) glycosylation around the proteolytic site, which may inhibit cleavage (69), (2) cleavage of the N-terminal region of the receptor at a more proximal site that also removes the tethered ligand (70, 71) and (3) secreted protease inhibitors. *In vitro* experiments have shown that peptides derived from the tethered ligand sequence can be used to either stimulate or to antagonize the receptors. Currently, it does not seem likely that this mechanism occurs *in vivo*, although some data show that peptide analogs may trigger signal cascades in a PAR receptor independent way (72–74).

PAR-mediated signaling

Activation of the serpentine receptors initiates the exchange of GDP for GTP bound to heterotrimeric G proteins. This leads to signaling through two pathways via the $G\alpha$ and the $G\beta\gamma$ subunits, respectively. The $G\alpha$ subunit can interact with at least three different enzymes: adenylylcyclase, guanylylcyclase and phospholipase C. The first two cyclases regulate the intracellular concentration of the second messengers cAMP and cGMP, the activated lipase cleaves the lipid plasmamembrane component phosphoinositide to form inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). These four second messengers activate further downstream processes that involve distinct kinases. Activation is either direct through cAMP, cGMP, and DAG or indirect through Ca^{2+} that is released by IP3 from the endoplasmic reticulum and mitochondrial stores (75). Different cell types may differentially respond to identical extracellular signals as distinct varieties of $G\alpha$ subunits exist, that either couple to different downstream enzymes or that inhibit rather than stimulate a given pathway. Signaling events downstream of the PAR receptors in epithelial cells have not been fully elucidated. This is partly because of our incomplete understanding of which $G\alpha$ couples to which effector enzymes in epithelial cells, as this coupling is not necessarily identical in all cell types.

The best studied receptor signaling cascade is that of PAR-1, a receptor that in endothelial cells, fibroblasts and platelets can interact with three different $G\alpha$'s. The first is $G_{q11\alpha}$ that links the receptor to phospholipase

C- β 1 leading to the production of the second messengers IP3 and DAG from their membrane bound precursor phosphatidylinositol (76). Specific receptors for IP3 on the endoplasmic reticulum and on mitochondrial stores lead to a transient Ca^{2+} spike, that through calcium-binding proteins like calmodulin activate calcium-dependent kinases. Parallel to the calcium-dependent pathway, DAG activates PKC- α directly. In addition to their direct effects, both kinases also cross-talk to the MEK/ERK pathway. Although it is not clear what the relative contribution of the individual mediators in this $G_{q11\alpha}$ activated pathway is, the whole cascade is important in the control of blood clotting, as knock-out mice for $G_{q11\alpha}$ display increased bleeding times (77). The second group of G proteins that couples to PAR-1 is $G_{12\alpha}$ and $G_{13\alpha}$. These G proteins couple to a cascade of guanine-nucleotide exchange factors (GEFs) of the Rho and Rac-family. In endothelial cells, the Rho pathway through Rho-kinase is involved in maintaining the structural integrity of the blood vessels, and the Rac pathway through myosin light-chain kinase is involved in cytoskeleton rearrangements. In addition to the activating pathways, PAR-1 can also signal via $G_i\alpha$, an inhibitory G protein. This pathway leads to down-regulation of adenylyl cyclase and a subsequent drop in intracellular cAMP levels. This process could be especially relevant in inflammatory cells, as decreased intracellular cAMP levels are associated with transcriptional activation of chemokines and cytokines.

In addition to G-coupled protein signaling, it has been described that PAR 2 can use a G-protein independent pathway, involving β -arrestins. It is believed that signaling via β -arrestins is involved in scaffolding of proteins that are involved in cell migration and actin assembly. This occurs via a β -arrestin-dependent dephosphorylation and activation of the actin filament-severing protein (cofilin). This PAR-2-evoked cofilin dephosphorylation requires both the activity of a cofilin-specific phosphatase (chronophin) and inhibition of LIM kinase (LIMK) activity (78).

Epithelial expression of PARs

The expression and function of PARs in the airway epithelium and in the different cells therein, has been studied to some extent, but the exact function in the inflammatory response remains unclear. In comparison with the issue of potential endogenous activators in the Toll-like receptors, this is a nonissue for the PARs. 'Inflammatory proteases' like tryptase and chymase (released upon activation of mast cells) are clear candidates to affect the inflammatory or allergic response. Moreover, as mutations in the protease inhibitors ADAM33 (79) or SPINK5 (80) have a clear clinical phenotype an involvement of PARs is suggested. PAR expression has been characterized in airway

epithelial cells. Interestingly, airway epithelial cells express all PAR-1 through -4, but only for PAR-1, -2 and -4 did activation lead to cytokine production, where PAR-3 agonist peptide did not evoke a response (81). Although PAR-3 can act as a co-receptor for PAR-4 it is not clear whether the absence of PAR-3 activation has any consequences for the activity of PAR-4. This is partly the result of the overlapping protease specificities of the receptors, as activators of PAR-4 (thrombin and trypsin) also activate PAR-1 (thrombin) and PAR-2 (trypsin).

Not only are the PARs expressed, they are also functional and may play a role in inflammation as their activation leads to cytokine production and release of PGE₂ (81). The best studied protagonist is PAR-2, as this receptor has been associated with activation by allergens. Activation of PAR-2 by proteases is associated with house dust mite allergen (82), cockroach allergen (83), trypsin (84) or PAR-2 activating peptides (74) and has been reported for many different epithelial cell lines or primary cells (81, 85). The extend of these responses nor their similarity is not fully clear, as in many studies only a limited set of outcome parameters (mostly IL-6 and IL-8) are reported. The link between epithelial PAR expression and clinical relevance has been addressed in a few studies. Inhibition of Factor Xa (a known activator of PAR-2) by fondaparinux, resulted in the reduction of airway hyperresponsiveness and a decreased epithelial mucin production *in vivo*. The later aspect was reflected *in vitro*, where Factor Xa enhanced AREG expression and mucin production in H292 cells (86). In this mouse ovalbumine (OVA)-model, no effect was seen on the influx of inflammatory cells. These outcomes are different from a similar model in rabbit sensitized for the major parietaria allergen (Par j1), where pretreatment with PAR-activating peptide reduces inflammation and relieves bronchoconstriction (87). Currently, it is not clear where these differences originate from but it is interesting to note that activating PAR peptides have been reported to exert an activity independent of the PAR receptor (72;73;73). A second study showing an epithelial-mediated protective effect of PAR activation (88) also involved the use of a PAR-activating peptide.

The expression levels of PARs are also affected by disease. Already in 2001, Knight et al. (89) described that in the lung epithelium of asthmatics the expression of PAR-2 is higher than in healthy individuals. Whether this reflects a protective mechanism or is part of the pathophysiology is unclear. More recently, an interest has been taken in nasal PAR expression. It has been reported that nasal epithelial cells express PAR-2 and that in allergic individuals this expression is higher. Furthermore, the increased PAR expression coincides with an increased number of eosinophils (90). In 2007, Lee et al. (91) confirmed these findings in a similar study in Korea. And more recently, a study has been published by Rudack

et al. (2007) (92) where they suggest that PAR-2 plays an active role in inflammation mechanisms of chronic rhinosinusitis. They showed an NF- κ B dependent expression and the release of CXC chemokines (GRO- α , IL8) but no regulation of CC chemokines (eotaxin/CCL11, released upon activation T-cell secreted (RANTES)/CCL5, Thymus and activation-regulated chemokine (TARC)/CCL17). These data show an expression on epithelial cells throughout the airway system and moreover this expression seems to be dependant on the inflammatory status of the individual. Whether this is the cause or consequence of this inflammation can be debated, but its presence and role in the immune response seems evident.

Cytoplasmic receptors

A second class of receptors by which epithelial cells can detect the presence of micro-organisms can be found in the cytoplasm, although one could admit that this distinction is somewhat artificial. Some of the TLRs (TLR3, and 7-9) are found in membrane vesicles inside the cytoplasm and are activated when (parts of) micro-organisms are taken up by a cell. Moreover, micro-organisms can produce toxins that can interfere with intracellular signaling events by binding to cytoplasmic enzymes, such as Pertussis toxin (PTx), which inactivates G_{i/o} type G proteins (93). These observations show that it is hard to define the concept of the 'cytoplasmic receptor'. In this paragraph, we will focus on those interactions that contribute to the defense mechanisms of the epithelial cell, rather than interactions that seem beneficial for the micro-organism.

The family of NOD and leucine-rich repeats containing receptors (NLRs; also known as nucleotide oligomerization domain with leucine-rich repeats (NOD-LLR), NACHT-LRR (NACHT is acronym of different proteins that have a nucleotide binding domain; NAIP, CIITA, HET-E and TP1), or CATERPILLER [CARD, transcription enhancer, R (purine-binding, Pyrin, lots of LLRs)] is a large family of cytoplasmic pattern recognition receptors, which contains more than 20 family members in mammals (94–96). Common for all NOD-LRR receptors is a central nucleotide-binding oligomerization domain (NACHT), an N-terminal effector-binding domain and C-terminal leucine-rich repeats (LRRs). The NLRs can be subdivided in subfamilies based on effector domains; NODs and IPAFs (ICE protease-activating factor) containing CARD (caspase recruitment domain) effector domains, NALPs (NACHT-LRR and Pyrin-domain-containing proteins) containing PYD (pyrin) effector domains and neuronal apoptosis inhibitor protein (NAIPs) containing three baculoviral IAP repeat domains as can be seen in Fig. 3. For many of these cytoplasmic receptors, the ligands are not known, but are most likely involved in recognition of cytoplasmic PAMPs and endogenous danger signals.

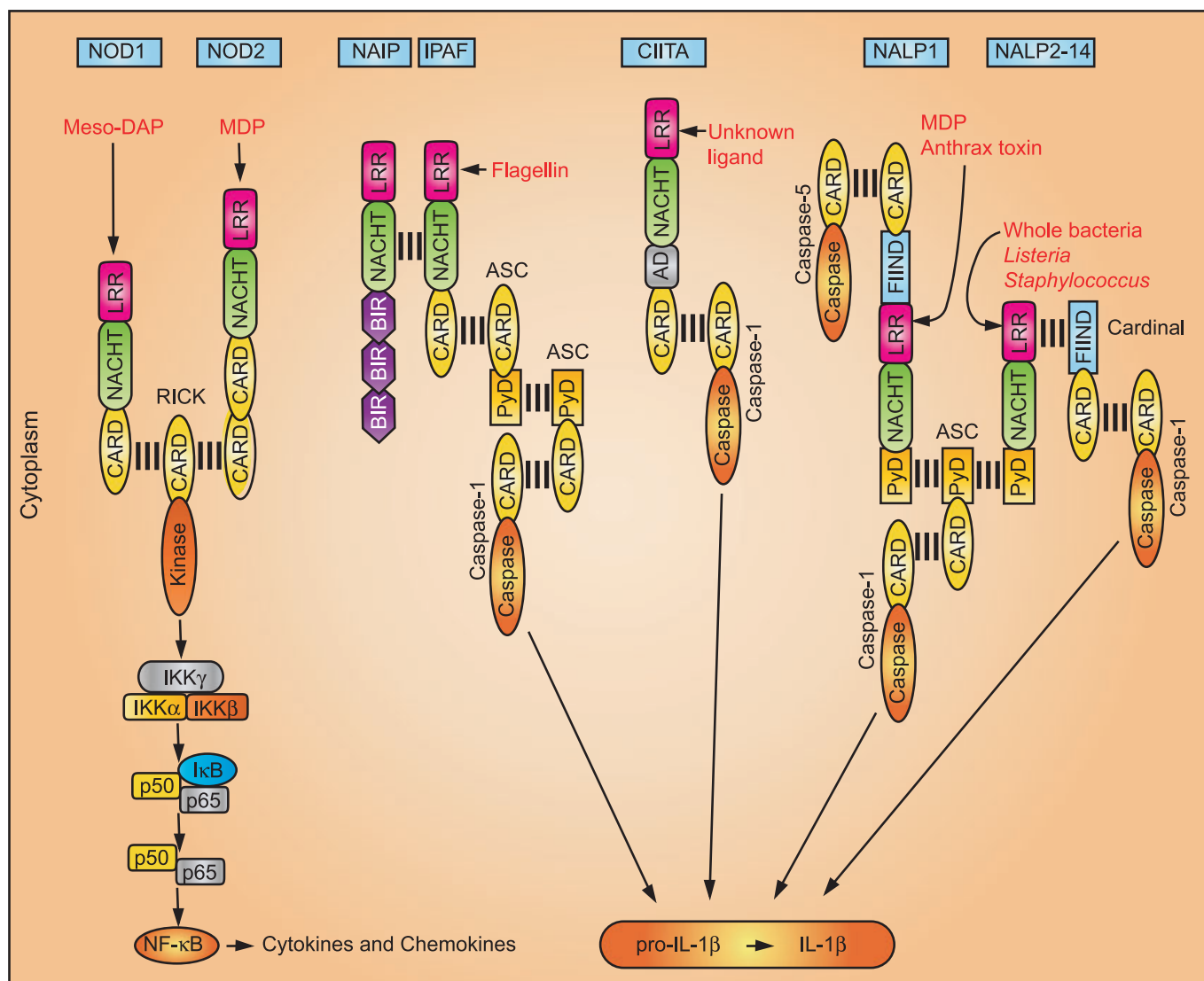


Figure 3. NLR signaling Figure showing different components of the inflammasome with the NOD-like-receptors (NOD1, NOD2, NAIP, IPAF, CIITA, NALP1 and NALP2-14), the adapter molecules (ASC and Cardinal) and the effector molecules (RICK and caspase-1 and -5).

NOD1- and NOD2-mediated signaling

NOD1 and NOD2 are receptors that can sense cytoplasmic microbial PAMPs. NOD1 has one and NOD2 has two CARD domains, and both receptors are involved in recognizing peptidoglycan fragments. NOD1 recognizes the peptide γ -D-glutamyl-meso-diaminopimelic acid (meso-DAP), which is found on gram-negative bacteria. NOD2 is the receptor for muramyl dipeptide (MDP) which is a peptidoglycan constituent of both gram-positive and gram-negative bacteria (96, 97). Upon recognition of the ligand, the CARD-containing serine/threonine kinase RICK is recruited via CARD-CARD interactions. This in turn mediates ubiquitination of IKK γ , which leads (partly) to NF- κ B activation (98). A

number of potential regulators of NF- κ B activity via NOD have been identified: TAK1, TRIP-6, GRIM-19 and ERBIN (99). In addition to activating NF- κ B, NODs can also activate MAP kinase pathways. NOD2 can activate p38 and ERKs, while NOD1 can activate JNK (100, 101).

NOD1 and NOD2 can act in synergy with various TLRs to enhance immune responses in antigen-presenting cells (99). In human monocytes and DCs, NOD1 and NOD2 agonists act cooperatively with LPS to stimulate the production of inflammatory cytokines (TNF α and IL-6) (102). There have also been synergistic effects described between NOD1 and NOD2 and TLRs 3, 4 and 9 (103). The interaction between NOD2 and TLR2 is not synergistic and it has even been reported that NOD2 antagonizes

TLR2 stimulated production of IL-12; however, this effect could not be repeated in another study (100, 104).

NALPs-mediated signaling

The NALP subfamily is the largest in the NLR family, it contains 14 members (NALP-1 to -14) and is characterized by the PYD effector domains (105). The function of the different NALPs is not well known; however, several NALPs form inflammasomes when activated. These inflammasomes are critical for the production of certain proinflammatory cytokines, such as IL-1 β and IL-18. Two distinct NALP inflammasomes have been identified: (i) the NALP1 inflammasome [comprising NALP1, the adaptor protein ASC (apoptosis-associated speck-like protein), caspase-1, and caspase-5] and (ii) the NALP2/3 inflammasome (comprising of ASC, Cardinal and caspase-1, in addition to NALP2 or NALP3) (106). Activated NALPs, recruit ASC via their PYD effector domains and this complex interacts with caspase-1 via a CARD–CARD interaction. In addition, NALP1 can recruit caspase-5 via its CARD domain. NALP2, lacking a CARD domain can recruit caspase-1 via a CARD-containing adaptor Cardinal (106–110). For activation of NALP1 cell rupture seems to be sufficient to induce inflammasome assembly (111). NALP3 seems more prone to activation by exogenous stimuli [PAMPs; bacterial RNA, (although not by LPS or LTA), antiviral compounds such as R848 and R837, and bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes*. In addition, also endogenous danger signals released by dying cells, such as uric acid crystals and extracellular ATP, can activate NALP3 (110, 112, 113).

The last subfamily of NLR is NAIPs, their signaling is similar to the other NLRs as they signal via a CARD domain to activate a caspase (in this case caspase-1) we will not go further into detail, also because there is very little known about the expression and the function of NAIPs in epithelial cells.

Epithelial expression of NLR receptors

Research on NOD in epithelial cells has focused mainly on the intestinal epithelium, this because mutations in the NOD gene are a strong risk factor for the development of Crohn's disease, the genotype-specific disease risk for heterozygous is 2.6 (95% CI 1.5–4.5) and for homozygous even 42.1 (4.3– ∞) in German and British populations (114). Research has shown that NOD signaling down-regulates the TLR-driven activation of cells by gut bacteria, so absence of a functional NOD signaling leads to increased NF- κ B activation, resulting in chronic inflammation (104). The expression of NOD1 and NOD2 has been shown in lung epithelial cells; however, these cells did not produce the inflammatory cytokines IL-6,

IL-8 or MCP-1 in response to NOD1 agonistic *meso*-DAP (γ -D-glutamyl-*meso*-diaminopimelic acid) or NOD2 agonistic muramyl dipeptide (MDP) (115). Whether these cytokines are the correct readout for NOD activation or whether NOD activation only acts through modulation of a TLR response is unclear. Until now, no report has been published where the expression of NOD1 or 2 has been shown in nasal epithelium, nor what their role there would be. However, seeing the widespread expression of NOD1 and NOD2 in various epithelia [tongue, salivary gland, pharyngeal, esophageal, intestinal, cervical, breast, lung and kidney epithelial cells (115)] it may very well also be expressed in nasal epithelium, where it could interact with TLR signaling in recognizing endogenous and exogenous danger signals. The epithelial NLR field is largely under explored as almost no data has been published on epithelial expression of the other two subfamilies of NLRs (NALPs and NAIPs).

Research opportunities

This review highlights many mechanisms available for epithelial cells to detect changes in its environment. Through a collection of receptors, the epithelium is able to respond to structural components of micro-organisms like bacteria, viruses and helminthes, or to enzymatic active components of potential allergens. If we would first focus on the external environment an already complex picture emerges. In everyday life, we are constantly exposed to many different environmental factors, each with its potential to activate epithelial cells. Although relative unique cascades are downstream of the receptors there seem to be only a few transcription factors acting as targets of these cascades. With NF- κ B such a prominent player in all signaling pathways, we would need to consider in more detail how signals are processed when more than one signal is present.

Most experiments up to now have focused on the effects of single stimuli, but this is a situation that in every day life may have limited value. Epithelial surfaces like the airways and gut are normally exposed to a multitude of (pathogenic) micro-organisms or other antigenic triggers which would all simultaneously activate their specific receptors. There could be a separation in time for processing of extracellular signals, but then we need to assume that only signals are processed where a pathogenic situation has occurred or where the structural integrity of the epithelium has been compromised. Examples would be the receptors located in endosomes or the cytoplasm that can only be activated when the epithelium has been infected, or cell surface receptors that are located more towards the basal side below the tight junctions of the epithelium. A second option of how epithelial cells may deal with multiple activating factors in its environment might well be related to our limited understanding of how signaling cascades target transcrip-

tion factors. Only few studies have studied the activation of more than one transcription complex with most studies investigating NF- κ B. This introduces a strong bias and would obscure the potential involvement of other transcription factors. However, having said this, it is not trivial to look at other transcription complexes as a source of specificity for responses to environmental signals. A complicating factor in this approach is that outcome will strongly depend on what readout is used for activation. For instance, Fig. 1 shows that in the TLR cascade the transcription activation of some genes (TNF α) may only depend on NF- κ B activation, some only on the IRF factors (INF α) and others on both (INF β). Moreover, this dependency may differ when a gene would be activated through another signaling pathway and could be further complicated when other transcription complexes are considered. Even within the NF- κ B pathway more attention should be paid to what specific form NF- κ B is activated, as multiple different subunits can interact to form distinct subtypes of NF- κ B transcription complexes. Traditional activation of transcription by the p65/p50 NF- κ B dimer can be seen for inflammatory cytokines, whereas the I κ B ζ /p50 dimer is required for IL-1R-mediated IL-6 induction (116). Remarkably, this same I κ B ζ /p50 dimer is not required for TNF α -mediated IL6 induction, and additionally inhibits rather than stimulates expression from the TNF α promoter (117). If we would also take into account that NF- κ B monomers can interact with IRF (118), Fos or Jun (119) monomers, an even more complex picture emerges. One option to deal with selection bias would be to look at global transcription patterns using microarrays, but this approach would need a strong focus. It could be applied to the investigation of the stimulatory activity of multiple environmental factors simultaneously, especially where one would be able to compare a 'normal' response vs the response in a 'diseased state'.

Next, we would like to consider the consequences of activation of all these known (and still unknown)

signaling cascades. There is little doubt that the response of the epithelium contributes to an effective defense against potential pathogens. Indeed as discussed, multiple natural occurring mutations in key signaling molecules affect the ability to efficiently respond to pathogens. Interestingly, here also our limited understanding is evident. Why do mutations affect the response to some bacteria and not others when they all signal through TLR-4 or only affect the response to some viruses when they all signal through TLR-3? Surely, it will be that other signaling cascades are involved that could differ between the responses for one micro-organism to the next, but it does highlight the complexity and the necessity of interacting signaling pathways. Where the effects of signaling mutants in the response on micro-organisms seem trivial, one unfortunate side-effect of these mutants is that some will contribute to a *de novo* immunological response to otherwise innocent (environmental) factors. When this factor is some protein from the organism itself we are faced with auto-immunity, when this factor is found in the environment we are faced with allergy. Two distinct mechanisms may lead to this *de novo* immunological response. Either it is a direct effect of the (environmental) factor on the epithelium, leading to a stronger response or the (environmental) factor is seen in the context of other (danger) signals, leading to a deviated response.

In our resume, we have strongly focused on the epithelium looking toward the external environment; however, this will not be the complete picture. Just like the involvement of Toll-like receptors in the development and differentiation of *Drosophila*, also the Toll-like receptors of higher eukaryotes are able to sample the internal environment. Heat shock factors as ligands for TLRs, mast cell tryptase as trigger for the PARs are just a few examples where it has become clear that the role of pattern recognition receptors extends far beyond the recognition of just pathogen-associated molecular patterns.

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