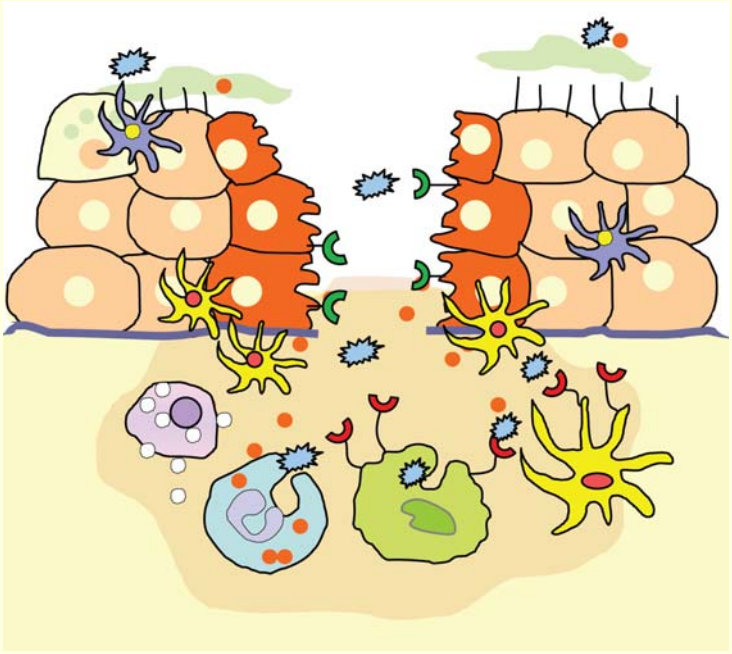
CO-OPERATIVE REGULATION OF EPITHELIAL HOMEOSTASIS AND IMMUNITY



by Renat R. Shaykhiev
University of Marburg

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Direktor: Prof. Dr. Claus Vogelmeier

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Renat R. Shaykhiev
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Im Namen Allahs, des Allerbarmes, des Barmherzigen.

In the name of Allah, Most Gracious, Most Merciful.

Во имя Аллаха, Милостивого, Милосердного!

БИСМИЛЛӘҺИР-РАХМӘНИР-РАХИМ

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SUMMARY

Epithelium of barrier organs plays a primary host defense function. In the lung, airway epithelium protects from respiratory pathogens routinely present in the air without development of excessive inflammation or tissue injury. We hypothesized that such a barrier function might be achieved due to a co-operation of immunoregulatory and tissue repair mechanisms. Consistent with such a concept, in the present study we identified multiple links between innate immunity and epithelial homeostasis using airway epithelium as a model.

First, we found that, at physiologically relevant concentrations, the endogenous antimicrobial peptide LL-37 exerts protective effects on airway mucosal cells by inducing epithelial cell (EC) migration, proliferation, wound closure, and by regulation of inflammatory responses of ECs and dendritic cells (DCs) induced by microbial signals. High concentrations of LL-37 (> 20 μg/ml) were toxic for airway ECs.

Second, innate immune recognition of microbial patterns by airway ECs initiated repair (cell migration, wound closure) and growth (cell proliferation, survival) events in epithelium independently on cells of myeloid origin. Microbial patterns signaling via toll-like receptor (TLR) – MyD88 – NF-kappa B pathway showed the most prominent effect on epithelial homeostasis. Epidermal growth factor receptor (EGFR) is involved in epithelial repair responses induced by LL-37 or epithelial TLR signals. Particular sensitivity of epithelial cancer cells to growth-promoting effects of TLR agonists suggests that epithelial TLRs might be involved in autonomous cancer cell growth.

Finally, in an *in vitro* model of interaction of DCs with differentiated airway epithelium, ECs were able to control DC activation by prevention of a direct contact with bacteria and / or due to the regulatory properties of soluble factors which are released by ECs. DCs “educated” within airway epithelial microenvironment were substantially less sensitive to stimulation with microbial factors. Prolonged presence of monocyte-derived DCs beneath airway epithelium significantly increased epithelial permeability, suggesting that bidirectional interactions between ECs and DCs exist and may potentially modulate epithelial barrier function and mucosal tissue homeostasis.

Taken together, epithelial homeostasis and innate immunity are closely connected, and their co-operative regulation is involved in the maintenance of tissue integrity and immune balance. Understanding of such a mechanism might be important for further progress in the development of novel therapeutic approaches to chronic diseases (asthma, interstitial lung diseases, inflammatory bowel disease, cancer and others), which are associated with concomitant immune dysregulation and epithelial tissue injury.

1. INTRODUCTION. *General concept of the work*

The cardinal feature of any biological system is its ability to live and die. Because of necessity to live in different and sometimes dangerous environments the organism has to develop a strategy of survival. Any biological strategy of living organism is aimed at the maintaining a stable, constant condition inside itself, called homeostasis. Since biological systems as well as their external environments are usually unstable, survival of living organisms ultimatively depends on their ability to resist environmental challenges and repair the damage caused by them. The first ability is usually linked to host defense that involves strategies to prevent, recognize and eliminate danger. The second ability represents a tendency of living systems to regenerate their physiological structure, providing tissue integrity and appropriate function. Although tissue repair usually depends on mechanisms different from host defense, these processes are interrelated, well-coordinated and, likely, evolved together as a general survival strategy in response to exogenous danger. For example, pathogens often induce damage to tissue they do infect. On the other hand, tissue injury is a factor predisposing to microbial invasion.

Recognition of a microbe by host defense system generates a number of positive feedbacks (inflammation, immune response) to provide an appropriate elimination of the pathogen. However, when the antigen is eliminated, negative feedbacks are necessary for "fine tuning" aggressive antimicrobial responses and prevention of secondary, endogenous danger, associated with concomitant inflammation and tissue damage. Repair mechanisms should be activated as soon as possible to prevent microbial invasion and dissemination, and also in the late stages, to reconstitute a normal structure when the immunological homeostasis is achieved. Therefore, a homeostatic strategy likely involves both co-operative regulation antimicrobial defense and tissue repair.

The mucosa is a primary site of exposure to various kinds of danger factors. As a physical barrier, it provides first line of resistance against microbes, and, when injured, generates a number of positive feedbacks towards immune and tissue repair systems. The function of mucosal barriers mostly depends on epithelial cells, which serve as a source of putative homeostatic signals in response to microbial challenge and tissue injury. Epithelial integrity is closely related to host defense function and may be significantly modulated by microbial factors and immune system. On the other hand, the epithelial barrier appears to be primary mechanism regulating immune homeostasis in tissues.

1.1. Innate immunity

1.1.1. General features of innate immunity

The ability to resist environmental challenges is a major feature of all living organisms. To be free of pathogens, the host must have a constitutive defense system able to recognize and eliminate them. Existence of such a mechanism is supported by a fact that microbes are encountered routinely in the life of organisms, but only some of them and only in some cases cause an infectious disease. The antimicrobial defenses may be of such a state of effectiveness that infection can be prevented entirely, for example, when a barrier function of the organ is intact. This first line of defense is provided by nonspecific factors, like, for example, a skin barrier, bactericidal secretions of glands, or mucociliary elevator of the mucosal surfaces. These mechanisms are constitutive; they protect the body from different kinds of danger regardless of its nature and, therefore, are called natural, or nonspecific, resistance.

However, this type of resistance is not sufficient, if microbes escape from, evade or even disrupt tissue barriers. If microbial invasion does occur, second-line defenses should be recruited immediately after the pathogen crossed the natural barriers and. On the other hand, should be able to recognize the presence of pathogen and discriminate it from self structures. This kind of defense response can be called innate immunity because it exists in the body from birth and is based on discrimination of “nonself” from “self”, that is a classical property of immune system (112). All organisms and all living cells possess innate mechanisms of pathogen recognition and elimination.

The innate immune response is generated against pathogens, but not against their antigens, is usually constitutive and do not result in immunological memory. These features distinguish innate immunity from the adaptive immunity (111) that is acquired, developed throughout the live of an organism, based on individual experience, always induced, antigen-specific, and long-lasting. Adaptive immunity, being a feature of only vertebrate organisms, can not be found in individual single cell but rather needs well-organized system of highly specialized cells (lymphocytes – T cells and B cells) able to recognize antigen specifically, generate antigen-specific effectors (antibodies and cytotoxic cells) and, finally, translate the information about encountered antigens into long-lasting immunological memory. Adaptive immunity takes several days to initiate all antigen-dependent processes and become protective in contrast to innate immune system, which is able to react immediately when the danger is present.

A key function of innate immune system lies in its capacity to provide signals essential for the development of adaptive immune responses to antigens (82). It is now

widely accepted that antigen-specific immune response may be achieved only if co-stimulatory molecules and cytokines are provided together with antigen, and these molecules are induced when the pathogen is first recognized by the cells of innate immune system (82,87). Many cells of the body that do not belong to the immune system in a classical sense are able to sense the presence of pathogens using innate antigen-nonspecific recognition mechanism (Figure 1). Some endogenous tissue-derived damage-associated molecular patterns may also be recognized by innate immune system (108).

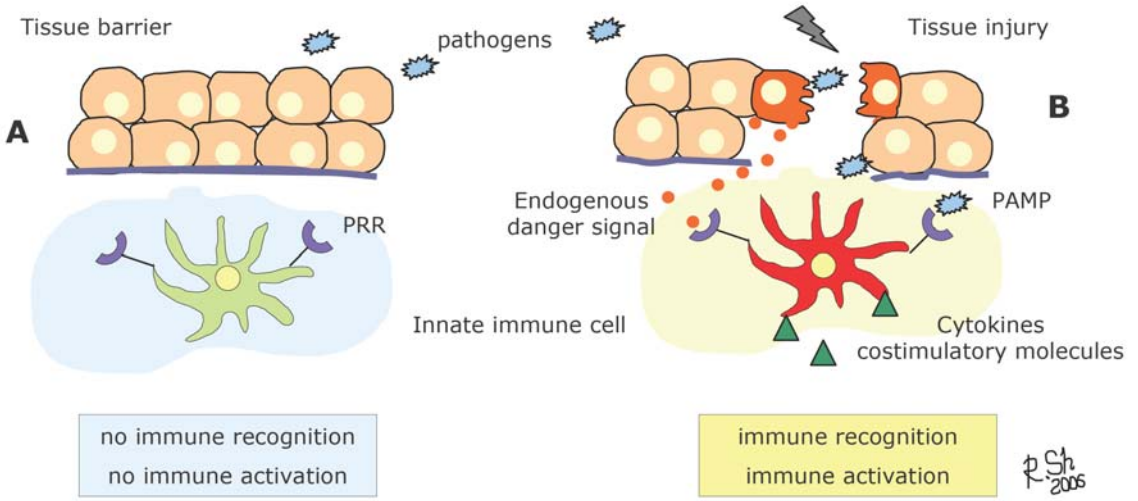


Figure 1. General strategy of innate immunity. (A) Innate immune cells associated with peripheral tissues are normally not activated when there is no tissue injury or/and pathogen that entered tissue barrier. However, once this happens (B), these cells are able to sense the presence of pathogen-associated molecular patterns (PAMP) or/and probably also endogenous danger signals, generated in tissue after injury, by pattern recognition receptors (PRRs). Innate immune recognition ultimately leads to upregulation of cytokines and costimulatory molecules necessary for inflammation and immune response.

The common mechanism, generated following recognition of dangerous signals, results in a rapid mobilization of inflammatory cascade, early production or activation of nonspecific broad-spectrum antimicrobial substances and, in a later phase, induction of adaptive immune response. Since most of these processes usually start immediately within primary site of contact with pathogens and injury, the role of peripheral tissues as a source of a plethora of innate immune cells and signals in the function of the whole immune system is now beginning to be more and more appreciated.

1.1.2. Innate immune recognition

As stated above, the innate immune system is able to recognize pathogens in a way different from adaptive immune system. Antigen-specific receptors of acquired immunity are exclusively expressed by T- and B-lymphocytes. During the early stages of differentiation they become genetically programmed to make a single kind of structurally unique receptor through a series of gene re-arrangements, making the repertoire of antigen receptors in the entire population of lymphocytes extremely large and diverse (29). In contrast, innate immune cells are not able to recognize every possible antigen (111). Being a first-line defense, the innate immune system can be activated immediately after contact with pathogens by recognition of invariant biochemical motifs shared by entire classes of pathogens. These motifs have been called pathogen-associated molecular patterns (PAMPs), and the receptors of innate immune system, able to identify them - pattern-recognition receptors (PRRs) (86). PRRs are encoded in the germline by non-rearranging genes and are present also in invertebrates.

Innate immune system utilizes different PRRs which belong to several families of proteins and can be expressed by virtually all cells of the body. There are soluble PRRs, such as the acute phase proteins mannan-binding lectin and C-reactive protein, which act as opsonins to neutralize and clear the pathogen through activation of the complement and phagocytic systems (87,111). Transmembrane PRRs include scavenger receptors, C-type lectins and the Toll-like receptor (TLR) family (87). There are also intracellular PRRs (like NOD proteins, endosomal TLRs and RIG-I-like proteins) (36) detecting pathogens that are internalized by and/or invade the host cells.

Toll-like receptors (TLRs) are the most extensively studied PRRs during the last decade. Toll, the founding member of the TLR family, was initially identified as a gene product essential for the development of embryonic dorsoventral polarity in *Drosophila* (6), and later was shown to be involved in antimicrobial defense of flies (104). In 1998 Toll-related protein was found in humans (144). It was quickly followed by discovery that the mammalian TLR4 recognizes LPS (137).

TLRs are widely expressed in many cell types. Most of the TLRs are expressed by the sentinel innate immune cells of hematopoietic origin, such as macrophages, and dendritic cells (DCs), and by the cells of adaptive immunity as well (87). Although innate immune recognition by nonhematopoietic cells, like epithelial, endothelial cells and fibroblasts, has some specificities, these structural cells do also express many, if not all, TLRs (3).

Thus far, 13 mammalian TLRs, 10 in humans and 13 in mice, have been identified: TLRs 1–9 are conserved among humans and mice, yet TLR10 is present only in humans and TLR11 is functional only in mice (22). The ligands of TLR2 are peptidoglycan (PGN), lipoteichoic acid (LTA) (193), lipopeptides and lipoproteins, zymosan and mannans (3). Recognition of these patterns usually requires heterodimer formation with TLR1 or TLR6 (22). TLR 3, 7-9 are intracellular: being located in the endosomes, they recognize nucleic acids of pathogens – dsRNA (TLR3) (4) and ssRNA of viruses (TLR7 and TLR8) (71) as well as bacterial DNA (TLR9) (74). TLR5 is a receptor for bacterial flagellin (69). As already mentioned, LPS is recognized by TLR4 (137), however, other ligands like the fusion protein of respiratory syncytial virus, the plant diterpene paclitaxel and some endogenous molecules (154) have also been found to activate this receptor (3). Biological roles, ligands and expression patterns of TLRs 10, 12, and 13 remain unclear.

TLRs are transmembrane proteins, which are composed of N-terminal leucine-rich repeats (LRRs) responsible for interaction with ligands, and a cytoplasmic Toll-IL-1 receptor (TIR) domain (87). Biochemical pathways of TLR signaling have been analyzed in a great detail (Figure 2) (130). In brief, after ligand binding by LRRs, the TIR domains recruit TIR-containing adaptor molecules, including myeloid differentiation primary response protein (MyD)88, MyD88 adaptor-like (Mal, also known as TIR domain-containing adaptor protein, TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). This recruits and activates kinases of the interleukin-1 receptor associated kinase (IRAK) family. Activated IRAK then recruits TRAF6 (tumor necrosis factor receptor-associated factor protein) that ultimately leads to activation of the IKK complex (3). The catalytic subunits of this complex phosphorylate IκBα, the inhibitory molecule that keeps the transcription factor nuclear factor kappaB (NF-κB) in an inactive state in the cytoplasm, and this then targets this molecule for ubiquitination and subsequent degradation by the proteasome. NF-κB is then free to translocate to the nucleus and drive the expression of genes, many of which are involved in the pro-inflammatory response (130). After recognition of viral nucleic acids, the interferon-regulatory factors (IRF) 7 and 3 are activated via MyD88- and TRIF-dependent pathways, respectively, necessary for type I interferon (IFN) production (3).

Although the general pathway of TLR activation by most PAMPs is similar, some of them require special accessory proteins. The presence of CD14 is necessary for recognition of LPS (190) and some TLR2 ligands (22). The additional requirement for MD-2 co-expression further complicates LPS recognition by TLR4 (157). CD36 is

important for effective recognition of diacylglycerides and probably other TLR2 ligands (22). TLR3 signals via the MyD88-independent pathway, which can also be used by TLR4 and mediated by TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta) resulting in IFN-beta induction (130).

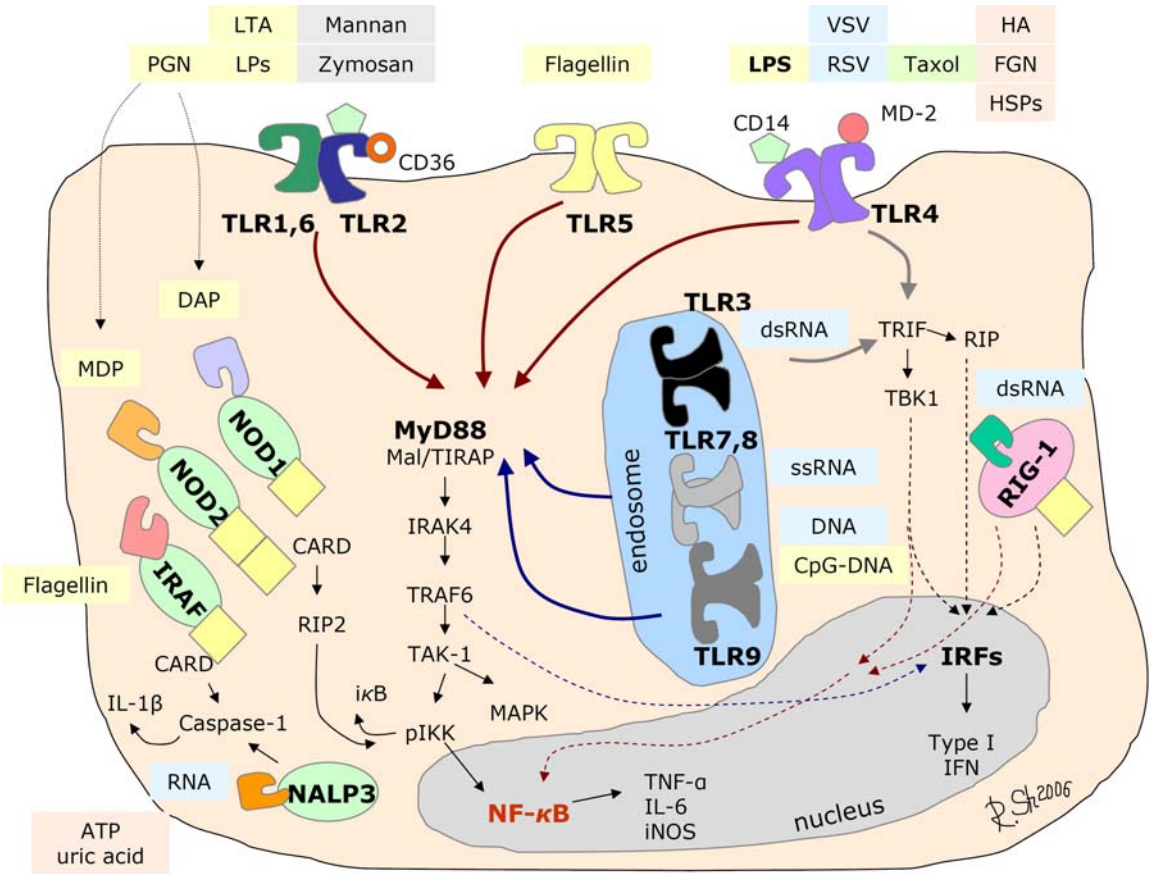


Figure 2. Mechanisms of innate immune recognition. Various molecular patterns of bacterial (yellow blocks), viral (blue blocks), fungal (grew), plant (green) and endogenous (red) origin are recognized by different PRRs of TLR, NLR (NODs, NALPs) and RLR (RIG) families. This results in activation of intracellular pathways necessary for induction of cytokines, antimicrobial peptides and other host defense factors.

Nucleotide oligomerization domain (NOD) proteins and **NOD-like receptors (NLRs)** are intracellular PRRs (Figure 2). They possess LRRs involved in ligand sensing; a nucleotide binding oligomerization domain (NOD); and signaling domain(s), such as caspase-activating and recruitment domain (CARD), PYRIN, or baculovirus inhibitor of apoptosis repeat (BIR) domains (114). NOD1 and NOD2 activate NF-κB through the recruitment and oligomerization of receptor-interacting protein (RIP) 2 resulting in activation of the IκB kinase complex (36). NOD proteins recognize PGN-derived

products: NOD1 detects g-D-glutamyl-meso-diaminopimelic acid (31), whereas muramyl dipeptide (MDP) is a NOD2 ligand (61).

Bacterial signaling via NODs induces activation of caspase-1, which catalyzes processing of pro-interleukin (IL)-1β to produce the mature cytokine. A complex of proteins responsible for these catalytic processes has been called inflammasome (106), which also includes the members of the NALP family, which are NOD-LRR / pyrin-domain-containing proteins. The ligands for NALP family members are currently unknown, except for NALP3, which is involved in the recognition of bacterial RNA, ATP, and uric-acid crystals (131).

RIG-I-like receptors (RLRs) are widely distributed intracellular PRRs recognizing viruses (Figure 2). They include retinoic acid inducible gene-1 (RIG-I) and melanoma differentiation-associated gene (MDA)5, which are sensors of dsRNA (3). Similar to the anti-viral TLR pathway, RLRs signal to IRFs inducing type I IFN production. Uncapped 5'-triphosphate viral RNA was recently identified as a specific RIG-I ligand (80).

A growing body of evidence indicates that the immune system can also recognize endogenous signals – dead cells and molecules released during tissue damage (108). It has been found that necrotic cells are able to induce maturation and T-cell priming activity of resting dendritic cells (DCs), whereas healthy cells do not (55). Consistently, uric acid released from necrotic cells was found to activate DCs and augment priming of $CD8^+$ T-cell responses (156). Of note, many endogenous danger signals are now shown to signal via receptors, originally described as PRRs. The fragments of hyaluronan produced after tissue injury interact with TLR4 and TLR2, and via this pathway initiate inflammation and tissue repair in acute lung injury (90). Also nonpathogenic commensal bacteria were found to be recognized by TLRs and induce protective effects during mucosal injury in a MyD88-dependent manner (140). Uric acid, released from necrotic cells, can be detected by NALP3 (107). These novel data suggest that recognition of endogenous factors by PRRs may be involved in non-immune tissue homeostasis.

1.1.3. Antimicrobial peptides and proteins

Innate immune system does not only recognize pathogens, but also inactivates them using antimicrobial peptides (AMPs) and proteins. The natural ability of normal tissues to possess antibacterial activity has been revealed in 1922 by Fleming, who has isolated the first tissue-derived bacteriolytic substance (now known as lysozyme) (53). Currently about 900 different AMPs and proteins have been described (28). Not only the number, but also the knowledge about the functions of AMPs has been increased to the moment.

Antimicrobial proteins are present at relatively high concentrations in host defense cells of myeloid origin, especially neutrophils, but also in epithelial cells (ECs) and tissue secretions. Some of them (like lysozyme, secretory phospholipase A2 or cathepsin G) are enzymes that lyse various microbial components. For example, lysozyme, which is produced by phagocytes and ECs, degrades bacterial PGNs by cleaving the glycosidic bond of *N*-acetyl glucosamine (51). Other antimicrobial proteins use nonenzymatic strategies. Lactoferrin, a highly abundant component of milk and mucosal secretions, binds to iron, an essential survival factor for many microbes, and also possesses bactericidal activity at the N-terminus (57). Bactericidal/permeability-increasing protein (BPI) selectively exerts multiple activities against gram-negative bacteria: cationic N-terminal peptide causes direct cytotoxicity and neutralizes LPS, while C-terminal domain binds to phagocytes and, therefore, may act as an opsonin (44).

AMPs are endogenous polypeptides of fewer than 100 amino acids that have antimicrobial activity at physiological concentrations under conditions prevailing in the tissues of origin or elevating there during pathologic conditions (56). Unlike the commonly called antibiotics, which in most cases are synthesized by special metabolic pathways, the amino acid sequence of AMPs is naturally encoded in the genetic material of the host organism (28). There are many different groups of AMPs, however, the most of them are cationic amphipathic peptides able to accumulate on, interact with and subsequently damage negatively charged microbial membranes (197). In addition, some AMPs can alter bacterial metabolic pathways, reduce cell-wall, nucleic acid, and protein synthesis, and inhibit enzymatic activity (28). In humans and other mammals, defensins and cathelicidins constitute the two main AMP families (191). Neutrophils and epithelial cells (ECs) are the major sources of mammalian AMPs (197).

Defensins are cysteine-rich, cationic peptides with β-sheet structures that are stabilized by three intramolecular disulphide bonds between the cysteine residues. Mammalian defensins are classified into three subfamilies, α-, β- and θ-defensins, which differ in their distribution of and disulphide links (56,153). Human α-defensin-1, -2, -3 and -4 are mainly expressed by neutrophils and, for this reason, are also called human neutrophil peptides (HNPs) (58). Human β-defensins (hBDs) are expressed mainly by ECs (56). hBD1 is constitutively expressed, whereas hBD2 and hBD3 can be induced by microbes or inflammatory cytokines (56,181,187). hBD4 is expressed in testes and gastric antrum, whereas hBD5 and hBD6 are restricted to the human epididymis (153).

AMPs of **cathelicidin** family contain an N-terminal signal peptide (preregion), a conserved cathelin-like domain (proregion), and a C-terminal microbicidal domain (hence the name “cathelicidin”) (196). Humans generate only one cathelicidin, called hCAP18 (103). Cathelicidins are usually stored in the granules of neutrophils as an inactive proform and undergo processing to mature peptide during or after secretion by appropriate proteases. For example, hCAP18 is cleaved by proteinase 3 (165) or elastase (64) to liberate its C-terminal antimicrobial domain, which is called "LL-37" because this peptide begins with two leucine residues and has 37 amino acid residues. hCAP18/LL-37 has also been found in various epithelia (14). Because neutrophil secondary granules readily degranulate to the extracellular space, cathelicidins can be found in inflammatory fluids at relatively high concentrations (32,147). Moreover, accumulation of neutrophils within mucosal tissues may activate epithelial cathelicidin by a proteolytic process mentioned above. The C-terminal AMPs of cathelicidins are microbicidal against a broad spectrum of microorganisms, including bacteria, fungi, and parasites (197). Similar to other cationic AMPs, the mechanism of cathelicidin-mediated microbial killing depends on the formation of ion channels or pores in the microbial cell membrane. LL-37 also possesses a potent endotoxin-neutralizing activity due to interaction with a negatively charged lipid A portion of the LPS molecule (103,121).

There is accumulating evidence for other host defense and immunoregulatory functions of AMPs. It has been shown that some of them may act as chemoattractants for inflammatory and immune cells: LL-37 via formyl-peptide receptor-like 1 (FPRL1) attracted neutrophils, monocytes and T cells (40), via formyl-peptide receptor (FPR) – eosinophils (175), via other mechanism – mast cells (126); hBD2 induced migration of immature DCs and memory T cells via CCR6 (192). AMPs may also be involved in tissue homeostasis: HNPs have been shown to increase airway epithelial repair (1), LL-37 can induce angiogenesis (100). Recent data suggest that some AMPs may play a regulatory role during inflammation (116,150).

1.1.4. Inflammation as a host defense response

Inflammation is a tissue response to infection, injury or irritation. The principal features of inflammation are (1) the presence of damage as a trigger of host response; (2) activation of host defense and immune mechanisms; and (3) healing of damaged tissues (124).

Inflammation starts with tissue injury (110) (Figure 3). Injury can be recognized at all levels of biological organization (75): as described above, innate recognition of danger is genetically encoded in every living cell in form of PRRs and, therefore, is not a unique prerogative of immune system.

Following injury, tissue cells release mediators with different defense functions: (1) AMPs to kill microbes immediately; (2) vasoactive substances (for example, histamine of mast cells) to increase the local blood flow and vascular permeability, and (3) cytokines to attract and activate inflammatory cells of haemotopoietic origin. For example, IL-8 is a major chemoattractant for neutrophils; GM-CSF increases survival of granulocytes; TNF-alpha activates virtually all cells involved in inflammatory process (170). Necrotic tissue cells as well as some substances released by lytic cells like uric acid and ATP may be recognized by innate immune system as danger signals as described in previous section.

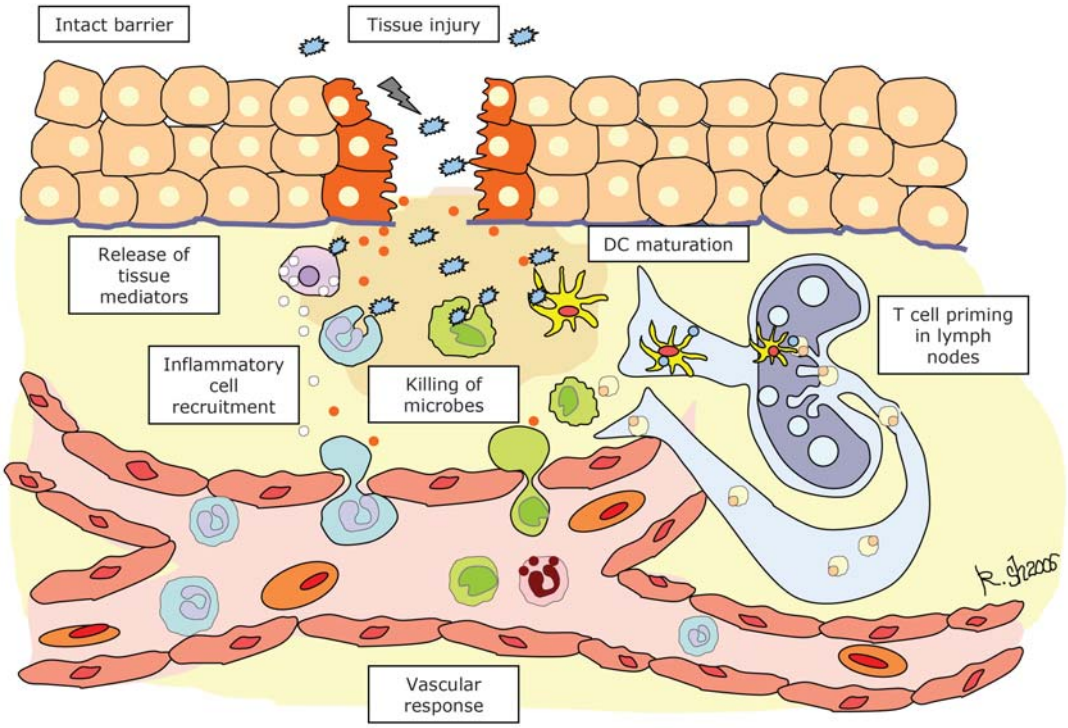


Figure 3. Inflammatory process. Inflammation is initiated by tissue injury, caused by physical damage to the tissue barrier or infection. Various mediators (including chemokines and vasoactive amines) are released by tissue cells (epithelial cells and mast cells /violet/) to increase vascular permeability and attract inflammatory cells from blood (neutrophils /blue/, monocytes /green/ or eosinophils /pink/), that migrate to the site of injury and kill microbes. Dendritic cells /yellow/, matured in the presence of pathogens, migrate into regional lymph node, where they present antigen to T cells and thereby prime specific immune response.

Leukocyte migration into damaged tissue is an essential feature of inflammation. Neutrophils (also called polymorphonuclear /PMN/ leukocytes) are the most abundant haemotopoietic cells during the early stages of nonspecific inflammation (124). Once neutrophil has migrated into the tissue, its primary role is to recognize, phagocytose and kill pathogens. Following binding to opsonized pathogens, neutrophils extend pseudopods that engulf the particle and take them up into the maturing phagosome (151). Killing by neutrophils occurs intracellularly (by AMPs, like α-defensins (58), and serine proteases, degranulated into phagosome, or reactive oxygen compounds generated upon activation of neutrophil membrane-bound NADPH oxidase enzyme complex (151) or extracellularly through a release of the AMPs (like cathelicidins) of specific granules (197).

Neutrophils are short-living cells. Following phagocytosis they die and then are removed and/or replaced by mononuclear inflammatory cells. Following migration into tissues, monocytes can differentiate to become macrophages or DCs (7). Being localized in tissues, these cells play a key role in inflammation as sensors of danger, initiators and regulators of host defense responses. They express particularly high levels of PRRs (87).

Phagocytosis of microbes is a fundamental function of macrophages, discovered by Mechnikov more than 100 years ago (110). Immature DCs are also potent phagocytes (17). An important consequence of phagocytosis by macrophages and, especially, DCs is processing the antigens for presentation on major histocompatibility complex (MHC) molecules to T cells, providing a link between inflammation and immunity, innate immunity and adaptive immunity (23).

Conversion of the host defense response from antimicrobial tissue-damaging processes to the anti-inflammatory processes is necessary to promote tissue repair at the late stages of inflammatory reaction (124). During the progression of inflammation, platelet-leukocyte interactions elicit the formation of lipoxins A_4 and B_4, which serve as "stop signals" by blocking the further recruitment of PMNs from the circulation (149). Following phagocytosis of apoptotic cells generated in inflamed tissue, macrophages produce TGF-β, a potent anti-inflammatory cytokine (75).

Inflammation should be well-balanced, since inappropriate activation of any component of the reaction can potentially result in a disease. Insufficient activity of inflammatory mediators and cells may lead to infection. On the other hand, prolonged and excessive local inflammation is associated development of chronic autoimmune diseases, tissue injury (117), pathologic angiogenesis, fibrosis and cancer (10,93).

1.1.5. Dendritic cells - link between innate and adaptive immunity

In 1968, for the first time, the cells with dendritic morphology have been found by Langerhans (102), but their general distribution and function were described by Steinman and Cohn about 100 years later (169), who called them "dendritic cells" (DC). DCs are unique not only due to their shape, but because of a specific nonredundant role they play in the immune system, i.e. their ability to prime naïve T cell responses (17).

DCs are characterized by a great developmental plasticity (Figure 4). At least three subsets of human DCs are known: (1) myeloid DCs; (2) Langerhans cells (LCs); and (3) plasmacytoid DCs (pDCs) (16). The first two can be generated from $CD11c^{+}HLA\text{-}DR^{+}$ precursor DCs, or $CD14^{+}CD11c^{+}$ monocyte precursors under different conditions (7). Their differentiation into defined DC subpopulations usually occurs after recruitment of immediate precursors to the effector sites under the influence of factors present in tissues. $CD11c^{-}$ pDCs can be derived from the $CD11c^{-}$ $IL\text{-}3R^{+}$ precursors and are likely of lymphoid origin (7).

All DCs that reside in the periphery have an immature phenotype, specialized for recognition and phagocytosis of the antigenic material, but not yet capable of stimulating naive T cells since they lack costimulatory molecules (16). Although precise mechanisms that keep DCs within tissue in an immature state are not known, a number of tissue factors have been proposed to play this regulatory function. During infection, the DC features change dramatically towards mature phenotype and acquisition the ability to prime naïve T cells (Figure 4). Within 1 h, DCs accumulate at the sites of antigen (Ag) deposition as demonstrated in bronchial epithelium after Ag inhalation (109). This is likely a result of recruitment of circulating DC precursors and also pre-existing immature DCs which express CCR6, whose ligand MIP3α is induced in the inflamed epithelia (43).

DC maturation is initiated in the periphery upon Ag encounter and inflammatory cytokines and completed during the DC–T cell interaction (16). Ags captured by DC are subsequently degraded in endosomes, and the generated polypeptides are transported into the MHC class II–rich compartments for their loading onto the nascent MHC class II molecules while DCs mature (17). DC maturation process is associated with several coordinated events such as (1) loss of phagocytic receptors and adhesive structures; (2) upregulation of costimulatory molecules CD80, CD86 and CD40; (3) change in class II MHC compartments; and (4) shift in a chemokine receptor profile with downregulation or desensitization of CCR1, CCR2, CCR6, necessary for escape from the local gradient of MIP3α, and upregulation of CCR7, which is critical for DC migration to the draining lymph nodes (43).

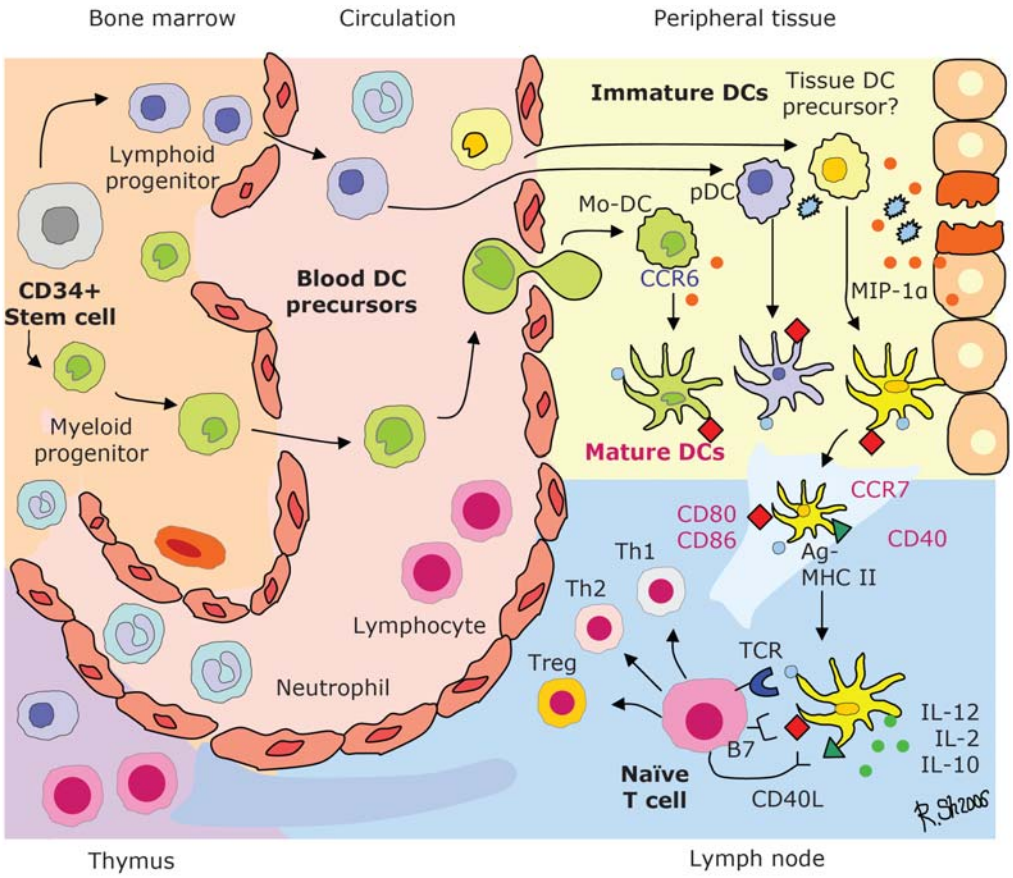


Figure 4. DC lifespan. Myeloid (myeloid DCs) or nonmyeloid (pDC) DC precursors migrate in peripheral tissues where they differentiate into immature DCs with high Ag-capture capability; possible local DC generation from resident unknown precursor is also depicted. Upon encounter microbial stimuli DCs primed and educated by local tissue factors capture and process Ag, starting to mature. Mature DCs expressing co-stimulatory molecules (CD80, CD86) and Ag-MHC class II complex migrate to the draining lymph nodes, where they activate T cells via Ag-specific mechanism supplemented with co-stimualtion and secretion of cytokines, which determine the T-cell differentiation into Th1, Th2 cells or T regs.

In the lymph nodes, DCs migrate to the T cell areas, seek out Ag-specific T cells and induce their activation and differentiation into effector cells (17). DCs provide three signals required for naïve T cell activation (Figure 4): (1) Ag presented by the MHC molecule to be recognized by TCR; (2) costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86), which trigger CD28 expressed on naive T cells; and CD40 that interacts with CD40L on T cells; and (3) cytokines like IL-12 (16). Depending on the density of the peptides presented, types of costimulatory molecules expressed and cytokines secreted by the DCs, naive $CD4^+$ T cells differentiate into Th1, Th2 (92) or T regulatory (Treg) cells (163).

TLR-dependent signals normally induce DC phenotype with Th1-priming function (i.e. high expression of costimulatory molecules and secretion of IL-12) (92). DCs matured in inflammatory environment but without TLR signals prime T cells lacking helper function (168). Tissue-derived factors like human thymic stromal lymphopoietin TSLP (166), prostaglandin (PG) E2 (91) and GM-CSF (21,50) promote Th2-priming DCs. DCs with IL-10(+) IL-12(-) cytokine production profile induce Tregs (163). Thus, DCs provide a direct link between innate and adaptive immunity and determine the character of immune response.

1.2. Epithelial homeostasis and host defense

1.2.1. Epithelial polarity and barrier function

The epithelium provides a tissue barrier for all kinds of external danger. Due to its proximity to the environment, epithelial tissue can be viewed as a first-line of host defense. In addition to a physical barrier function, epithelial cells (ECs) produce various bioactive molecules that inactivate microbes, regulate tissue homeostasis and modulate functions of other cells involved in the host defense. Epithelial barrier function is maintained by a well organized polarity so that the apical surface faces the lumen or outside of the organ and the basal surface faces the basal lamina and the blood vessels. Each pole is characterized by a distinct composition of proteins and lipids, defining different membrane domains with specific functions.

Apical surfaces of ECs contain cilia, necessary for cell motility, and microvilli that increase surface area important for transport of substances across the membrane. The lateral surface is characterized by different kinds of connections between the cells. Intercellular adhesion is primarily mediated by the apical junctional complex (5), which consists of tight and adherens junctions, and desmosomes, while gap junctions connect adjacent cells and allow the passage of small molecules for cell-to-cell communication.

The most apically located tight junctions (TJs) represent closely associated areas of two cells whose membranes join together forming a virtual impermeable barrier. They are composed of transmembrane proteins (occludins, claudins and junction adhesion molecule-1 (JAM-1)) and intracellular proteins (such as, for example, zona occludens (ZO) family proteins) (148). TJs prevent paracellular diffusion and seal the intercellular space between adjacent cells, creating a barrier against free diffusion of fluids, electrolytes and macromolecules as well as of luminal microorganisms and their products, and contribute to transepithelial resistance (TER) (148).

Adherent junctions (AJs) are subjacent to the TJs and required for their integrity (5). TJ assembly is initiated by homotypic interactions between E-cadherin, a major cell adhesion molecule, and nectin on the surface of adjacent cells. Through its cytoplasmic tail, E-cadherin binds intracellular proteins called catenins, which interact with the actin cytoskeleton. E-cadherin acts as a potent tumor suppressor by providing a firm adhesion between contacting ECs and preventing their migration and invasion of other tissues (49). Desmosomes also provide intercellular adhesive forces in polarized epithelia.

On the basolateral side, the membrane of polarized ECs adheres to the extracellular matrix mainly via integrins, a family of transmembrane proteins that induce the formation of multiprotein focal adhesion complexes. In focal adhesions, transmembrane receptors of the integrin family and a large set of adaptor proteins form the physical link between the extracellular substrate and the actin cytoskeleton important for regulation of cell migration (188).

Airway epithelium represents a barrier different from other barriers in the body. It protects simultaneously distinct tissue compartments equally important for homeostasis. First, it serves as a mechanical and chemical filter of the inspired air, providing sterility and safety of the alveolar compartment of the lung. The gas exchange process in alveoli requires the ventilation of a large volume of environmental air that potentially contains pathogens and dangerous particles. The latter must be captured and neutralized in the airways; otherwise, pulmonary infection will develop. Second, intact airway epithelium is primary regulator of the mucosal homeostasis, preventing the access for pathogens to the subepithelial and submucosal compartments that may result in excessive inflammation and / or generalized infection.

Airway epithelium is covered on its apical surface by a thin liquid layer called airway surface liquid (ASL) (Figure 5). The ASL is the first line of defense against inhaled pathogens and is mandatory for effective mucociliary clearance (98). Almost one century ago, Fleming observed that respiratory secretions possess bactericidal properties (53). Recently, Smith et al. (162) have translated this discovery into clinical lung science by showing that airway epithelium in cystic fibrosis (CF), a hereditary disease characterized by inflammation and bacterial colonization of the lung, fails to kill bacteria because of abnormal ASL.

The ASL consists of two layers, a mucus layer and a periciliary liquid layer (PCL), that are propelled upwards by coordinated ciliary beating (25). The PCL represents an aqueous solution with a height, equalling the height of extended cilia (about 7 μm), and a

relatively low viscosity enabling ciliary beating and cell surface lubrication (25). PCL is also considered to be an important contributor to the efficiency of cough clearance (98).

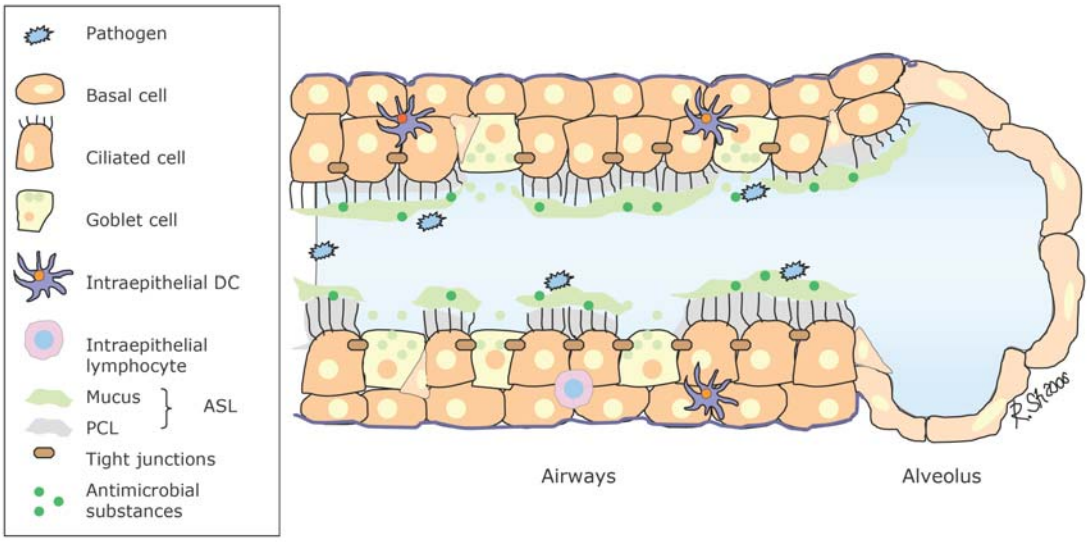


Figure 5. Airway epithelial barrier represents a physical border of ECs that resists damage and prevents pericellular diffusion of exogenous factors due to TJs which link the neighboring ECs at the apical zones. Defense function is maintained by the mucociliary escalator and antimicrobial substances present in the ASL that consists of mucus, produced by goblet cells and other secretory cells, and periciliary liquid layer (PCL). Intraepithelial DCs and lymphocytes provide a local sentinel function and are involved in the regulation of tissue homeostasis.

The second component of the ASL is mucus. It forms a gel-like aqueous layer in which soluble compounds are mixed with mucus polymers and aggregates of mucins, produced by goblet and secretory glandular cells, and other high molecular-weight glycoproteins and proteoglycans (145). MUC5AC and MUC5B are the major mucins of human respiratory mucosa (145). The diversity of the carbohydrate side chains within the mucin gel allows entrapment of a wide variety of particles, like bacteria, for clearance from the airway by means of the mucociliary transport (98). Many antimicrobial factors (like lactoferrin, lysozyme and defensins) accumulate in mucus (57). Under physiological conditions, the airway epithelium, by virtue of these substances, can inactivate bacteria and eliminate it from the airways within several hours (181).

Therefore, airway epithelium represents a tightly-organized mechanical barrier and maintains an effective antimicrobial environment to prevent microbial colonization and induction of inflammatory response.

1.2.2. Epithelial injury and repair

Epithelium covers virtually all barrier organs. It is continuously exposed to external environment, where microbes and secreted microbial products, potential allergens, toxic substances and irritants are present. Therefore, epithelial injury is not a rare event. Restoration of the barrier integrity after injury represents a fundamental defense function of epithelia. Epithelial repair process involves a stereotypical sequence of events – epithelial restitution and regeneration (136). Epithelial restitution starts immediately after injury and is characterized by rapid migration of cells towards the wound area in order to re-establish surface epithelial continuity. An increased migratory capacity of the cells is a result of the de-differentiation process after injury (94) and may be explained by the loss of contact inhibitory signals at the damaged area (49). Rapid restitution after injury limits fluid and electrolyte losses and prevents pathogens from the lumen getting into local and systemic immune compartments (136).

Various factors initiate the repair process. They include (a) epidermal growth factor (EGF) family members like EGF, transforming growth factor-alpha (TGF-α), heparin-binding EGF or amphiregulin (97); other growth factors like keratinocyte growth factor (KGF) (187), insulin-like growth factor-1 (IGF-1) (158), hepatocyte growth factor (HGF) (195); (b) inflammatory cytokines like IL-1 beta (59); (d) extracellular matrix proteins (fibronectin, fibrinogen) and matrix metalloproteinases (129); and other factors. Of note, many of these factors or their signaling mechanisms become active following tissue injury, providing a positive feedback control of epithelial homeostasis. An interesting example is signaling via EGFR (184). Its ligands are constitutively present at the apical pole of the cell. The steady-state signaling is prevented by the basolateral localization of EGFR. However, upon damage, when epithelial integrity is compromised, the receptor becomes accessible to the ligands and their interaction promotes the repair process (184).

Like in many other mucosal surfaces, epithelium of the airways has a great capacity to repair itself after injury, as demonstrated *in vitro* (94,194) (Figure 6) and *in vivo* (47). Immediately (within 5-15 min) after injury, secretory, ciliated and (probably also) basal ECs at the wound edge de-differentiate, spread and migrate across the denuded basement membrane without any external stimulation or non-EC participation at a speed of about 2-3 microns/min (47). Cell migration is controlled by the members the ras-related Rho family of small GTPases: Rho and Rac regulate the polymerization of actin to produce stress fibers and lamellipodia protrusions, respectively, while Cdc42 triggers the formation of filopodia, an actin-based structure found at the cell periphery (128).

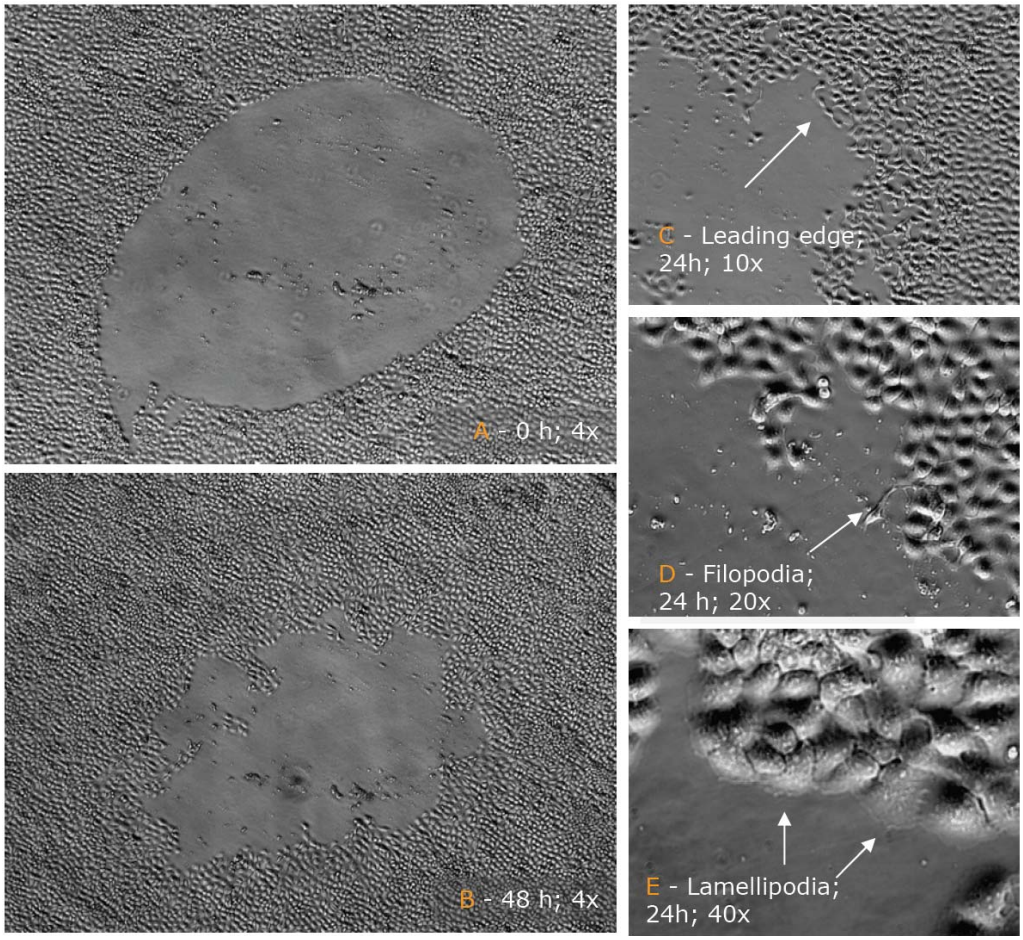


Figure 6. Airway epithelial repair *in vitro*. Damaged epithelium has an intrinsic capacity to repair after injury, as shown here by the reduction of the initial mechanically-induced wound area (A) after 48 h of culture (B) under serum-free conditions without adding of any exogenous growth-promoting stimuli. Micrographs made at 24 h after wounding (C-E) show that cell spreading and migration are the principal mechanisms of epithelial repair during the early post-wounding period. Formation of migration-related structures, filopodia (D) and lamellipodia (E), at the wound edges is shown. Cell migration under serum-free conditions may be, however, not sufficient to provide complete repair of relatively large epithelial defects, as shown in B. Thus, cell proliferation is necessary for entire epithelial regeneration. Data of Renat Shaykhiev.

Cell migration may be not sufficient for the complete epithelial repair (Figure 6B). The replacement of the damaged cellular elements by newly generated cells is also necessary, particularly, for repair of injuries with initially large wound areas. This may be achieved by means of epithelial cell proliferation, a process initiated normally after the majority of migratory cells have already covered a significant portion of the wound (94). Epithelial regeneration is dependent on the function of epithelial stem cells (141) which are extraordinarily resistant to any kind of damage and can be induced to enter DNA synthesis by wounding.

Increase of cell numbers in the damaged area facilitates formation cell-to-cell contacts necessary for contact inhibition of further proliferation and induction of cell differentiation (49). Re-differentiation of airway epithelium after injury can be revealed by *de novo* appearing secretory or ciliated cells in parallel with decrease of mitotic rate at the damaged area (94). At the molecular level, EC differentiation can be characterized by acquisition of the markers of polarity, including TJs and cadherins (5). Differentiation phase of repair is essential for the complete functional restoration of epithelium.

1.2.3. Innate immune functions of ECs

1.2.3.1. Innate immune recognition

It is now established that ECs of many organs express different kinds of functional PRRs (79). In the airway epithelium, TLRs have be found to be expressed at the mRNA (TLR1-TLR10) (119) and protein levels (TLR1-TLR5, TLR9) (63). Recent *in vivo* studies revealed the functional TLR2 (76) and TLR5 (198) in airway ECs. NOD1 and NOD2 proteins have also been found in airway ECs (132) and implicated in pathogenesis of lung infections caused by *P. aeruginosa* (178) and *Mycobacterium tuberculosis* (52), respectively. Some pathogens utilize simultaneously more than one PRR. For example, *Streptococcus pneumoniae* possesses a TLR2 ligand LTA and pneumolysin, which interacts with TLR4 (3). Different virulence factors of *P. aeruginosa* are able to interact with different PRRs – TLR2 (exoenzyme S, LTA), TLR4 (exoenzyme S, LPS) (46), TLR5 (198) and NOD1 (peptidoglycan) (178).

Some respiratory pathogens can be recognized by receptors not belonging to the PRR family. For instance, *Staphylococcus aureus* has been shown to interact with TNF-α receptor TNFR1 (62) and platelet-activating factor receptor (PAFR) (105). PAFR has also been implicated in the recognition of *S. pneumoniae* (37). Taken together, interaction of pathogens with airway ECs may activate multiple signaling pathways.

An important outcome of pathogen recognition by ECs is induction and release of proteins and peptides with host defense functions. Accordingly, microbial factors, which acivate TLR2 (76,187) and NOD2 (185) increase the expression of epithelial defensins. However, the ability of ECs to provide host defense response to invading pathogens independently on bone-marrow-derived cells represents a controversial issue (67,99).

Since airway epithelium constantly interacts with microbes present in inhaled air, persistent innate immune recognition may result in chronic inflammation. Under steady-state conditions, EC activation via PRRs is usually prevented (79). It has been shown for epithelial TLR4: in contrast to the cell surface expression of TLR4 in monocytes and

macrophages, intracellular expression of TLR4 has been documented in ECs, including airway epithelium (65). TLR5 is usually expressed on the basolateral epithelial surfaces (60), suggesting that this receptor is likely not accessible to luminal flagellin-containing bacteria, which, however, may activate the receptor, when epithelium is injured. This seems to be relevant for respiratory infection caused by *P. aeruginosa*, the major source of flagellin in cystic fibrosis, which selectively interacts with damaged airway ECs (135). Increased availability of TLR2 at the apical surfaces correlates with inflammatory responses seen in the CF airway mucosa (119).

Another mechanism of epithelial unresponsiveness to microbial factors may be lack of co-factors required for the appropriate recognition by PRRs. For example, ECs express low levels of CD14 (8) and MD-2 (89), which are essential for LPS recognition by TLR4. Upregulation of these accessory molecules was induced *in vitro* by cytokines TNF-α and IFN-γ (89), indicating that self-amplifying effect of inflammation at the mucosal surfaces may be due to the increase of epithelial responsiveness to microbial factors.

1.2.3.2. Epithelial antimicrobial proteins and peptides

Antimicrobial proteins and peptides (AMPs) represent a first-line innate effector mechanism to kill microbes directly due to interaction with their membranes or disrupting their metabolic status as described earlier. In the airway mucosa, antimicrobial factors from submucosal gland cells, ECs and neutrophils are accumulated within the ASL (181). Among them, the proteins lysozyme, lactoferrin and SLPI are the most abundant factors of airway secretions in health and in lung diseases such as asthma, chronic bronchitis, and CF (27). Surfactant proteins A and D arising from alveolar ECs bind bacteria and fungi, as well as microbial factors, and enhance pathogen clearance (189).

Cationic AMPs of the defensin and cathelicidin families contribute considerably to the lung defense (11,57). Importantly, they can be produced locally by airway ECs (11). Some AMPs like hBD1 (159) and cathelicidin LL-37 (14) are expressed in the airway epithelium constitutively. The production of other AMPs increases dramatically during infection and inflammation: for example, hBD2 can be induced via (TLR2)-MyD88-NF-kappaB pathway by microbial factors and inflammatory cytokines (76,159,187).

The levels of LL-37 increase mainly via activation resulting from proteolytic cleavage of the mature peptide from inactive precursor hCAP18/LL-37 by proteinase 3 (165) or neutrophil elastase (64). However, the exact mechanism of LL-37 activation in the airway epithelium remains unknown. Although transcriptional induction of LL-37 expression in the skin has been reported (54), there is no data supporting the existence of

a similar mechanism in the lung ECs. The levels of LL-37 were found to be increased in the sputum (164) and BALF (32) in CF lung disease, characterized by chronic neutrophil inflammation and colonization of airways with *P. aeruginosa*. Thus, activation of the peptide by proteases from neutrophils and bacteria is possible. However, the major source of LL-37 in the airways is likely neutrophils recruited in the lung during inflammation.

AMPs have a broad spectrum of activity against Gram-positive and Gram-negative bacteria as well as against fungi and enveloped viruses (11,180). The minimal inhibitory concentrations of the peptides are in the range 0.1–100 μg/ml (11). AMPs differ in their killing activity against particular pathogens: LL-37 is more potent than HNP1 against a variety of bacteria including *P. aeruginosa* (14), and HBD-2 is more effective than HBD-1 in killing *Escherichia coli* (159). Many AMPs work synergistically. Three factors found in human airways - lactoferrin, SLPI and LL-37 - have synergistic activity with lysozyme (14,160). In addition to antimicrobial activity, AMPs can induce various responses in host cells as described earlier.

The information about the activities of AMPs in the lung *in vivo* is limited. Cathelicidins are the only AMPs extensively studied *in vivo*. It has been shown that mice treated with intratracheal LL-37/hCAP-18 vector had a lower bacterial load and a smaller inflammatory response than did untreated mice following pulmonary challenge with *P. aeruginosa* (15). CRAMP, a mouse analog of LL-37, protects mice against necrotic skin infection caused by Group A *Streptococcus* (127) and urinary tract *E.coli* infection (34). The latter study provides evidence that EC-derived cathelicidin contributes substantially to mucosal protection from bacteria. Moreover, cathelicidin protects from sepsis due to the binding to and neutralization of endotoxin (15,35,103,122).

1.2.3.3. Epithelial contribution to inflammation

Interaction of ECs with pathogens is critical for the initiation of inflammation, when the tissue barrier is damaged and constitutive mechanisms are not sufficient to provide an appropriate defense without the help of other cells. In such a case ECs play a key role by recruiting the circulating haemotopoietic cells and regulation of their function (13,122).

Epithelial factors that regulate immune system may be divided into following groups based on their function: (1) chemokines; (2) other cytokines and soluble factors that regulate inflammatory cell activity and survival; (3) inducible EC surface adhesion molecules; (4) inducible AMPs; (5) dead ECs resulted from physiological turnover of epithelium or accumulate during tissue injury. Many of these factors, excluding dead cells, are simultaneously generated following innate immune recognition via PRRs.

Many respiratory pathogens after recognition by airway ECs induce the release of the chemotactic cytokine IL-8, which play an important role in lung inflammation by recruiting neutrophils (170). IL-8 belongs to the group of chemokines that contain structural/functional domain Glu-Leu-Arg, or the "ELR" motif. Blockade of CXCR2, a common receptor for ELR+ chemokines, in models of pneumonia results in reduced neutrophil infiltration and clearance of microbes in the lung, and increased mortality (113). Cytokines IL-6, GM-CSF, TNF-α, also produced by ECs during infection, activate PMNs, monocytes and DCs (13,42,170). Production of CCL20 by inflamed ECs is thought to be a key mechanism of immature DC migration to the tissues (43).

Induced expression of EC adhesion molecules plays an important role during inflammation. In one recent study, direct interaction of airway ECs with *Haemophilus influenzae* was sufficient for overexpression of epithelial intercellular adhesion molecule-1 (ICAM-1) (81). Although ICAM-1 is also expressed by endothelial cells, *in vivo* cell type specific inhibition studies revealed that epithelial ICAM-1 was specifically involved in bacterial clearance in addition to its effect on leukocyte migration and adhesion (177).

Increased expression and activity of AMPs in the lung during infection and inflammation may be an additional to killing immunomodulatory mechanism. As already mentioned, human cathelicidin LL-37 has been shown to be chemotactic for neutrophils, monocytes, T cells (40), to modify inflammatory responses of DCs and monocytes (39,116), and to modulate apoptosis of neutrophils (19,39,123).

Tissue damage usually results in a massive EC death via necrosis or apoptosis. For a long time necrosis has been suggested as the only kind of cell death with a pro-inflammatory consequence (55,75). However, some recent studies showed that under special conditions apoptotic cells can also be pro-inflammatory and immunogenic (88).

1.2.3.4. Interaction with immune cells

Some cells of immune system have been found to exist within epithelial layer, including intraepithelial DCs (IEDCs) and intraepithelial lymphocytes (IEL), indicating that local immune responses may depend on interaction between these cells and ECs.

IEDCs were identified in the epithelium of human large airways by the group of Holt in the late 80th (78). Later, the phenotype of lung IEDCs has been analyzed in detail: they were identified as CD1a+ Langerin+ cells, similar to Langerhans cells in skin (41). More recently, it has been shown that IEDCs extend their dendrites into the lumen of the airways and, therefore, are able to capture luminal Ags (84). Such a mechanism has earlier been described for intestinal IEDCs by Rescigno et al, who have found that

mucosal DCs can produce TJ proteins and form temporal junctions with ECs in order to penetrate epithelium without compromising its barrier function (142). Proteolytic cleavage of TJs may represent an alternative mechanism: Der p 1, one of the major allergens of the house dust mite has cysteine proteinase activity able to cleave the TJ protein occludin and allow access to DCs (186). Consistently, DCs were found to penetrate the nasal epithelial layer in patients with allergic rhinitis, but not in healthy subjects (172).

Although it has been established that lung DCs capture luminal microbial Ags and transport them to the thoracic lymph nodes (84,183), the character of immunity induced by IEDCs is not clear. Several studies revealed an immature (143) or semi-mature (84) phenotype of IEDCs, even after exposure to pathogens, suggesting that local priming of protective Th1-dependent immunity by such a DCs is unlikely. However, Th2-priming capacity of airway (21) and colon IEDCs (143) has been described. Since ECs and IEDCs represent close neighbors, influence of ECs on IEDCs seems to be possible. Some factors released by ECs including GM-CSF (21,50), thymic stromal lymphopoietin (TSLP) (143,166), and PGE2 (91) may educate DCs resulting in a phenotype that promotes Th2-type immunity.

IELs represent another population of immune cells located within epithelium. They express $\gamma\delta$ TCR and, therefore, suggested to recognize Ag in a different manner as well as perform a different set of functions than T cells with $\alpha\beta$ TCR (70). There is evidence that these cells may recognize self-antigens expressed by damaged ECs (85). IELs produce growth factors, like KGF and IGF-1 (101), which are involved in epithelial repair. In the lungs, IELs have been supposed to coordinate responses to pathogens that penetrate epithelial barrier. One study reported more severe lung injury after bacterial challenge in $\gamma\delta$ TCR $^{-/-}$ mice (95). More recently, $\gamma\delta$ T cells have been shown to control excessive inflammation during lung infection *in vivo* (182). Consistently with down-regulation of Th1-immunity, $\gamma\delta$ T cells might increase airway hyperreactivity and Th2-mediated inflammation (66). It would be important to know whether airway ECs educate IELs expressing $\gamma\delta$ TCR similarly to IEDCs to acquire Th2-promoting activity in asthma.

Not only IELs but also other lymphocytes may be modulated by ECs. It has been shown that stressed ECs express MHC class I genes, MICA and MICB, which can be specifically recognized by NK cells via the NKG2D receptor (20). Expression of NKG2D ligands by airway ECs has been shown to play a protective role during *P. aeruginosa* infection in a recent study (24). According to a recent evidence, Ag-specific memory T

cells can be maintained in the airways and inducible bronchus-associated lymphoid tissue, and contribute to the early phase recall responses independently on secondary lymphoid organs (118). It is critically important to understand the role of tissue structural cells and ECs, in particular, in generation and regulation of local immune responses.

2. STATEMENT OF THE PROBLEM AND HYPOTHESES

A common homeostatic strategy appears as intrinsic tendency of the body to sustain an effective antimicrobial protection and prevent tissue damage. Since infection is often associated with injury of the barrier organs, the optimal defense mechanism activated in such a dangerous situation should involve a coordinated regulation of immune and tissue repair mechanisms. Epithelium represents a key element of the host defense in mucosal tissues, since it is a primary site of interaction with pathogens and putative source of factors that mobilize and regulate the immune system. Epithelial integrity is an important factor that controls invasion of microbes and unwanted immune activation. Physiological relevance of such a regulatory mechanism has been demonstrated for many chronic inflammatory diseases, like asthma (68,77,97), chronic obstructive pulmonary disease (COPD) (134), interstitial lung diseases (152), inflammatory bowel disease (171), and cancer (93). These pathological conditions are characterized by persistent activation of the immune system that is associated with dysfunction of epithelial barrier.

In the present work, different kinds of interactions between epithelium and immune factors have been analyzed, using airway ECs as a model. The specific aim of this study was to delineate the particular mechanisms that mediate integration of innate immune and epithelial repair processes. The following hypotheses were tested:

1. Whether human endogenous cathelicidin antimicrobial peptide (AMP) LL-37, expressed by ECs (14) and activated during lung infection and inflammation (32,147), regulates airway epithelial repair, in addition to its direct bactericidal effect? Such a mechanism has recently been established for the neutrophil defensins (1).

2. Whether innate immune recognition of pathogens by ECs directly modulates epithelial growth and repair processes? Since microbial invasion of mucosal tissues is often associated with tissue injury (110) and, in particular, with damage to epithelial barrier (167), simultaneous induction of epithelial repair after pathogen recognition may represent an autonomous positive feedback mechanism for the maintenance of epithelial homeostasis during infectious tissue injury. No data currently exist in literature regarding this possibility.

3. Whether LL-37 modulates the responses of ECs and innate immune cells to microbial factors? Direct killing of microbes by the AMP at the mucosal surface might prevent simultaneous activation of undesirable inflammatory processes. Although it is known that LL-37 neutralizes endotoxin (103) and modulates activation of macrophages and monocytes by LPS and LTA (116,150), the data regarding DCs are very limited and controversial (18,39). No data exist regarding modulation of EC responses to TLR signals by LL-37. Regulation of responses to other ligands, like flagellin, is also not clear.

4. Whether airway ECs modulate DC activation? The immunoregulatory function of the intact epithelium is likely to keep the mucosal DCs, in an inactive state, preventing the development of inflammation and autoimmunity in response to a routine microbial stimulation. A plethora of factors derived from damaged or infected ECs may differently modulate the function of DCs. There are published data regarding intestinal ECs which can modulate DC function during their interaction (30,39,143). The present study was focused on the interaction between DCs with human differentiated airway epithelium.

3. MATERIALS AND METHODS

3.1. Cells and cell culture

3.1.1. Airway ECs

Primary human bronchial ECs (PBECs) were isolated from large airways resected during surgery and cultivated either as conventional submersed cultures or as air-liquid interface (ALI) cultures as described previously (12). The protocol was approved by the ethics committee of the University of Marburg and informed consent was obtained from the donors. For conventional cultures, airway epithelial growth medium (PromoCell, Heidelberg, Germany) was used. To generate and maintain differentiated PBECs, the cells were dissociated and plated at a density of 0.25 to 0.5×10^6 cells/cm^2 onto semipermeable 12 mm or 6 mm diameter 0,4 μm pore-sized Transwell inserts (Corning Costar Corporation, Cambridge, MA), pre-coated with 0.01% Type I collagen (Sigma, Taufkirchen, Germany). The culture (ALI) medium consisted of a 1:1 mixture of DMEM and Ham's F-12 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin and 2% Ultroser G serum substitute (BioSerpa S.A., Cergy-Saint-Christophe, France). Cells were grown at 37°C, 5% CO_2, the culture medium was changed every day. Their apical surface was exposed to air as soon as they reached confluence to establish the ALI. Differentiated PBECs were selected for experiments by measuring the transepithelial resistance (TER, R_t) using an epithelial ohmmeter (EVOM, World Precision Instruments, Sarasota, FL). Cultures were considered differentiated if the R_t was more than 500 Ω/cm^2.

The human bronchial mucoepidermoid carcinoma-derived cell line NCI-H292 (ATCC, Manassas, VA) was cultured at 37°C in 5% CO_2 in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (PAA Laboratories GmbH, Pasching, Austria) and containing 10 % heat-inactivated fetal bovine serum (FBS, Gibco). The culture medium was changed every 2 days. Cells were passaged every 4-5 days using 0.25 % trypsin and 0.1 % EDTA (PAA Laboratories GmbH).

3.1.2. Monocytes and DCs

Monocytes were isolated from peripheral blood mononuclear cells (PBMC). PBMC were isolated from a buffy coat made from 500 ml blood of a healthy volunteer donor. Pre-enrichment of PBMC was performed by density gradient centrifugation using Ficoll Separating Solution (density 1.077 g/ml) (Biochrom AG, Berlin, Germany). $CD14^+$ monocytes were isolated from PBMC by positive selection using a magnetic cell sorting (MACS) system (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's protocol. Isolated monocytes were maintained at 37°C in 5% CO_2 in 10% FBS – containing RPMI medium.

To generate immature DCs, MACS-isolated monocytes were seeded in petri dishes and cultured in RPMI 1640 medium containing 10% FBS (Invitrogen), 20 ng/ml IL-4 50 ng/ml (Strathmann Biotec, Hamburg, Germany); GM-CSF (Strathmann Biotec). On day 3, the fresh medium containing GM-CSF and IL-4 was added to the cells. After 7 days of culture, cells were harvested, washed once with PBS and used for the experiments.

3.1.3. Naïve $CD4^+$ T cells

PBMC were isolated from a buffi coat by density centrifugation, as mentioned above. Naïve $CD4^+$ T cells were isolated from PBMC using Naïve $CD4^+$ T Cell Isolation Kit (Miltenyi Biotech) by MACS-based depletion of non-T helper cells and memory T helper cells (negative selection) as per manufacturer's protocol and maintained at 37°C in 5% CO_2 in X-VIVO 15 serum-free medium (Cambrex Bio Science, Verviers, Belgium).

3.1.4. EC – DC co-culture

PBECs were cultured in 12- or 24-well plates on semipermeable 0,4 μm pore-sized Transwell inserts until differentiated, as described above. Differentiated epithelia with the R_t more than 500 Ω/cm^2 were selected.

Following models of EC-DC interaction were used in our experiments (Figure 7):

(1) *Epithelial education of DCs.* To test whether ECs provide factors that modulate DC phenotype and function in a steady-state and change their responsiveness to microbes or microbial products, 1-10 x 10^5 DCs were added to the lower chamber of the Transwell system. As a control, DCs were also plated out at the same concentration and volume in tissue culture plates without ECs. The small pore size of semipermeable membrane permitted diffusion of soluble factors from one chamber to another, but did not allow the cells and cell fragments migrate between the chambers. After 24 h of co-culture (called DC "education" by ECs) , PBECs were exposed to different microbial stimuli from the apical surface (1A), so that DCs could not be activated directly and only EC-derived factors might mediate possible changes in DC activation status.

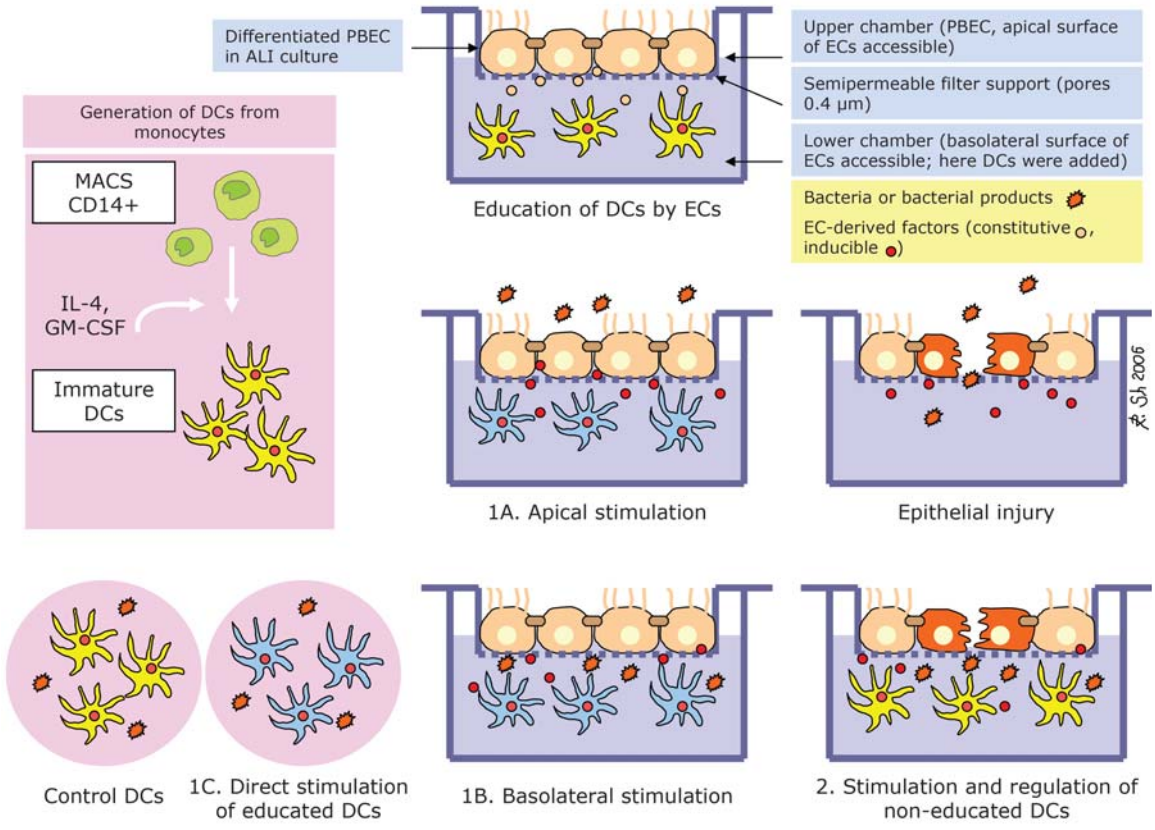


Figure 7. *In vitro* models of EC-DC interaction. PBEC were differentiated in an ALI culture system. Monocyte-derived DCs were added to the lower chamber and co-cultured with ECs during 24 h (epithelial education). Then educated DCs (blue) were stimulated indirectly via the apical side of ECs (1A) or directly – from the basolateral EC surface (1B) or without ECs (1C). To study the modulation of activation capacity of non-educated DCs (yellow), freshly generated immature DCs were added in the bottom chamber 16-24 h after stimulation of ECs with bacteria or bacterial products in the presence or absence of epithelial injury (2).

Alternatively, the stimuli were added into the lower compartment (1B), where they were able to interact directly with educated DCs. By comparing with non-educated DCs, the impact of epithelial education on DC activation capacity can be evaluated. However, after addition to the lower chamber, the stimuli can also activate ECs via their basolateral PRRs to produce soluble factors. Therefore, this later model can assess the influences of both education and regulation of DC activation by ECs. In other set of experiments (1C), educated DCs were harvested and then stimulated directly with microbial factors, so that no influence of ECs on the activation process of DCs was possible, and the changes of DCs maturation compared to the control DCs could be explained by epithelial education.

To ensure that epithelial factors that modulate DC phenotype and function are soluble, epithelial supernatants were collected from the basolateral surfaces of fully-differentiated PBEC and then used for DC education.

(2) *Epithelial modification of freshly generated non-educated DCs.* To model the interaction between ECs and freshly recruited non-educated DCs, monocyte-derived DCs were added into the lower chamber of the Transwell system. One day before, microbial stimuli were applied either from the apical or basolateral surface of ECs to mimic a mucosal inflammation, characterized by recruitment of DCs into the inflammatory site. In an additional set of experiments, epithelial monolayers were wounded mechanically by scraping using a sterile pipette tip before stimulation with bacteria or microbial products.

In all co-culture models, TER was monitored daily. After the 24 h co-culture period, DCs were harvested and prepared for the fluorescence activated cell sorting (FACS) analysis. Supernatants were collected for ELISA.

The following stimuli were used for EC-DC interaction experiments:

(1) live and heat-inactivated (30 min, 96°C) bacteria *P. aeruginosa* (strain PAO1): bacteria were grown to an OD_{600} of 0.8 in Luria-Bertani (LB) medium; 10 bacteria per EC or DC were applied for stimulation; at 1 h after incubation, bacteria were washed out and the medium was replaced with medium containing 100 μg/ml of gentamicin;

(2) microbial products: lipopolysaccharide (*E. coli* 0111:B4 LPS, 0.1 or 1 μg/ml, (Sigma-Aldrich Chemie GmbH, Munich, Germany), or *E. coli* 0111:B4 LPS Ultra-Pure (InvivoGen, San Diego, CA), or ultra pure Re-LPS from *Salmonella minessota* R595 (Alexis, Lausanne, Switzerland); flagellin from *Salmonella typhimurium,* 0.1 or 1 μg/ml (InvivoGen) or ultra-pure flagellin *Salmonella typhimurium* strain 14028 (Alexis); lipoteichoic acid (LTA from *Steptococcus pyogenes*, 1 μg/ml; Sigma-Aldrich).

3.2. Analysis of airway epithelial growth and repair

3.2.1. Wound closure assay

NCI-H292 and PBECs were grown to confluence in 6-well tissue culture plates as described above. In some experiments, the cells were serum starved for 18-24 h before being wounded. Three to ten circular wounds (from 1 to 3 mm in diameter) were scraped in each well with a sterile 10-µl pipette tip. The wounded monolayers were rinsed with an appropriate experimental medium to remove all cellular debris and then different stimuli were applied. The wound area was determined videomicroscopically with an inverted microscope (Axiovert 25; Carl Zeiss GmbH, Oberkochen, Germany), using the Evolution LC Megapixel FireWire Camera Kit bundled with Image-Pro Discovery software (Media Cybernetics, Inc., Silver Spring, MD) and a video monitor. Immediately after wound creation and at different post-wounding time-points (24 and 48 h) and expressed as percentage of the area measured immediately after wounding. After the image was acquired, the measurement data were converted to mm using a calibration image.

Various experimental conditions were used in wound closure assay depending on the purpose of particular study:

1) The effect of LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH) was evaluated under both serum-free and serum-containing conditions. The peptide was chemically synthesized (Charité, Humboldt-Universität, Berlin, Germany) and tested at different concentrations (0.1 - 50 µg/ml). A scrambled version of LL-37 (sLL-37; sequence RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL-COOH) and the chemically synthesized all-D-form of LL-37 (D-LL-37) were used in selected experiments at similar concentrations to exclude a nonspecific effect of LL-37.

2) The effect of different microbial patterns on epithelial wound closure has been assessed using serum-free conditions after 16-24 h of serum starvation. Different kinds of LPS (TLR4 ligand) preparations (*E. coli* 0111:B4 LPS, (Sigma-Aldrich), or *E. coli* 0111:B4 LPS Ultra-Pure (InvivoGen), or ultra pure Re-LPS from *Salmonella minessota* R595 (Alexis) were tested at concentrations from 0.01 to 20 µg/ml in the presence or absence of LPS-binding protein (LBP, 25-100 ng/ml, R&D Systems, Wiesbaden-Nordenstadt, Germany). In addition, the effect of LPS was studied in the presence of LL-37 (1-10 µg/ml) and also under 10% FBS-containing conditions. Among TLR2 ligands were LTA (*Steptococcus pyogenes*, 10-50 µg/ml, Sigma Aldrich), peptidoglycan (PGN, also ligand for NOD proteins) from *Bacillus subtilis* and *Staphylococcus aureus*, 1-10 µg/ml (InvivoGen), FSL-1 (Pam2CGDPKHPKSF), a synthetic lipoprotein derived from *Mycoplasma salivarium* activating the TLR2-TLR6 complex, 1 µg/ml (InvivoGen), heat-

inactivated *S. aureus* (10-100 bacteria per EC). Muramyl dipeptide (MDP; Bachem, Heidelberg, Germany), a synthetic NOD2 agonist, was used at concentration 10 μg/ml.

In all wound closure experiments, transforming growth factor alpha (TGF-α; Sigma-Aldrich; 20 ng/ml) was used as a positive control.

For inhibition studies, mechanically wounded NCI-H292 cells were exposed to inhibitors of MEK (PD98059, 25 μM; Alexis, Nottingham, UK), p38 MAPK (SB203580, 10 μM; Calbiochem, La Jolla, CA), P2X7 receptor (KN-62, 10 mM; Calbiochem), G protein – coupled receptor signaling (*Pertussis toxin*; PTx, 50 ng/ml; Sigma), NF-κB activation inhibitor (3nM or 10 nM; Calbiochem), or epidermal growth factor (EGF) receptor tyrosine kinase (AG1478, 1 μM; Calbiochem) 1 h before addition of stimuli. In an additional set of experiments, AG1478 were also added to the cells after wounding. To evaluate the role of FPRL1 as a possible receptor for LL-37, wounded monolayers were incubated with HRYLPM-COOH peptide, recently described as a full and selective activator of FPRL-1 (9). Because some inhibitors used in our study affect the principal signaling mechanisms essential for basal epithelial repair process, for some experiments we calculated the inhibition index (the "inhibition" area related to the remaining wound area at the given time-point). Such a parameter allows to compare contribution of a targeted pathway on the wound closure in differently stimulated groups when both of the groups are sensitive to the inhibitor.

To determine the involvement of TLR2 and MyD88 in the effect of microbial patterns on epithelial wound closure, the NCI-H292 cells (0.5-1 x 10^6 cells per well of the 6-well plate) were transfected with 5-15 nM of TLR2 siRNA (Hs_TLR2_1 HP siRNA or Hs_TLR2_2 HP siRNA, QIAGEN GmbH, Hilden, Germany), MyD88 siRNA (QIAGEN) or control siRNA (QIAGEN) at the same day as cell planting (according to the fast-forward protocol) using HiPerFect transfection reagent (QIAGEN). When the cells reach confluence (usually 24 or 48 h after transfection depending on the initial cell number), the medium was changed to the serum-free RPMI, and after 18-24 h of serum starvation, wound closure assay was performed. In some experiments, neutralizing monoclonal antibodies against cytokines (IL-1β, TNF-α; 1 μg/ml; R&D) were applied to the cells in order to evaluate the possible secondary autocrine effect caused by the release of these inflammatory cytokines.

To study the repair of differentiated airway epithelial cells, we mechanically damaged the ALI cultures by a sterile pipette tip, scraping off a ring of cells (from 2 to 3 mm in diameter) without damaging to the filter support. The stimuli were added in both

upper and bottom chambers of ALI culture system to provide the access to both apical and basolateral receptors of PBECs. Wound healing was assessed by the measurement of TER (*Rt*) at different time points. The results were expressed as *Rt* recovery indicating an increase of *Rt* (%) comparing to the immediate post-wounding value.

3.2.2. Analysis of EC migration

Chemotaxis of NCI-H292 cells was measured by a modified Boyden's chamber technique (26) using a 96-well multiwell chamber (Neuroprobe, Bethesda, MD). Culture medium containing one of the following stimuli was placed into the bottom wells: 1) LL-37 at concentrations from 1 to 20 μg/ml; 2) TGF-α (20 ng/ml) as positive control; 3) sLL-37; or 4) culture medium alone. The wells were covered with a PVP-free 8-μm-pore polycarbonate filter (Neuroprobe, Gaithersburg, MD) coated with 0.01 % collagen type I solution (Sigma Aldrich). Shortly before the experiment, the cells were detached using 0.05 % trypsin and 5 mM EDTA. 10^5 cells in 50 μl were placed into each of the top wells. Then the chamber was incubated for 6 h. After incubation, the filter was removed and cells on the top surface of the filter were removed by scraping. The migrated cells on the lower side of the filter were fixed by placing the filter in 100 % methanol overnight. The filter was stained with Haemalaun (Waldeck GmbH & Co. KG, Division Chroma, Münster, Germany) for 20 min and then washed in water. Cell migration was quantified and expressed as the number of stained cells on the lower surface of the filter in three random fields using a light microscope at × 20 magnification. The experiments were performed under both serum-free and serum-containing (10% FBS) culture conditions.

To assess the contribution of cell migration to the wound closure process induced by different stimuli used in our experiments, the wound edges were examined at different time points after wounding for the presence and intensity of lamellipodia formation using a videomicroscopy technique. It is well know that lamellipodia represent a kind of cell membrane protrusions generated during and necessary for effective cell migration (128), that has also been shown for airway ECs (195). The relative lamellipodia area was calculated by dividing the total lamellipodia area by the cell number within the examined wound edge.

3.2.3. Analysis of EC proliferation and viability

EC proliferation in response to different stimuli was analyzed directly (by means of 5-bromo-2-deoxy-uridine (BrdU) incorporation method) and indirectly (by automatic quantification of viable cells at different time points after stimulation).

For the BrdU assay, airway ECs were seeded at the density of 10^4 cells/ml on Lab-Tek II eight-chamber glass slides (Nalge Nunc International, Naperville, IL). When the cells reached confluence, they were incubated with various stimuli (LL-37, sLL-37, TGF-α) at concentrations as in the wound closure assay or medium alone for 24 or 48 h. Cell proliferation was assessed using BrdU labeling and detection kit II (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's protocol. Briefly, the cells were incubated with 10 μM BrdU for 1 h. After the aspiration of the BrdU labeling medium and washing, the cells were fixed in 70% ethanol at -20°C for 30 min. Then the cells were consequently exposed to mouse anti-BrdU, anti-mouse Ig-alkaline phosphatase, and, finally, to the color-substrate solution. The preparations were then examined in a light microscope. The proliferation index was calculated as the number of BrdU-positive cells divided by the total number of cells, multiplied by 100. At least 600 cells from each experimental group were counted. To analyze the involvement of selected signaling pathways, inhibitor studies were performed using the inhibitors described above. To assess the contribution of cell proliferation to the wound closure induced by stimuli, confluent NCI-H292 cell monolayers grown on Lab-Tek II glass slides were wounded and incubated with various stimuli as described above. At different time-points after injury (24 and 48 h), a BrdU assay was performed. Then the wound edges and post-wounding areas were analyzed for the presence of BrdU-positive cells.

Alternatively, cell proliferation was measured by determining the number of viable cells by incubation with WST-1 proliferation reagent (Roche). Briefly, PBECs or NCI-H292 cells were seeded into 96-well-plates, serum-starved for 24h and then stimulated for 24-120 h under different experimental conditions. After stimulation period, 10 μl of WST-1 was added into each well containing 100 μl of experimental medium. The stable tetrazolium salt WST-1 can be cleaved to a soluble formazan by a complex cellular mechanism that is dependent on the glycolytic production of NADPH in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. After incubation period (from 30 min to 90 min) with WST-1, the formazan dye formed was quantitated by measuring the absorbance at 450 nm using a Tecan Ultra 384 reader.

Different microbial molecular patterns were tested in the WST assay: (a) TLR2 agonists - PGN from *Bacillus subtilis* or *Staphylococcus aureus* 1-100 μg/ml (InvivoGen), FSL-1 (Pam2CGDPKHPKSF, InvivoGen) and MALP-2 (InvivoGen) both at 1 or 10 μg/ml, synthetic lipopeptide Pam3CSK4 1-20 μg/ml (InvivoGen), LTA from

Steptococcus pyogenes, 5-100 μg/ml (Sigma Aldrich), heat-inactivated *S. aureus* (10-100 bacteria per EC); (b) TLR3 (MyD88-independent) agonist – a synthetic analog of double-stranded (ds) RNA polyinosine-polycytidylic acid (poly(I:C)) 25-100 μg/ml (InvivoGen); (c) TLR4 agonist - LPS (10-20 μg/ml); (d) TLR5 agonist – flagellin *Salmonella typhimurium,* 0.1 or 1 μg/ml (InvivoGen); (e) TLR9 agonist - CpG-containing synthetic oligonucleotides (ODN2006 type B; 0.1 or 1 μg/ml); (g) NOD2 agonist MDP (MurNAc-L-Ala-D-isoGln) 10-20 μg/ml (Bachem). Cytokines IL-1β, TNF-α, and IP-10 were also tested (10 ng/ml, all from R&D Systems). TGF-α (20 μg/ml; Sigma Aldrich) was used as a positive control.

3.2.4. Cytotoxicity and apoptosis assays

The cytotoxic effect of different concentrations of LL-37 and sLL-37 (0.5-50 μg/ml) on NCI-H292 and PBECs was assessed by colorimetric quantification of the lactate dehydrogenase (LDH) in cell supernatants using the Cytotoxicity Detection Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. Briefly, after 24 h of stimulation, cell-free supernatant was collected and incubated with the substrate mixture from the kit. LDH activity was determined in a coupled enzymatic reaction, in which tetrazolium salt is reduced to formazan. The content of formazan dye was then quantitated by measuring the absorbance at 490 nm using ELISA reader.

The effect of stimuli on EC apoptosis was determined by the annexin binding apoptosis assay (Vybrant apoptosis assay, Molecular Probes). This assay is based on the detection of the externalized phosphatidylserine in apoptotic cells after specific interaction with fluorochrome-conjugated annexin. After stimulation, ECs were harvested and washed in cold PBS. Then 10 μl of Alexa Fluor 488-conjugated annexin V and 1μl of 100μg/ml of the propidium iodide working solution were added to each 100 μL of cell suspension. After incubation at room temperature for 15 min, annexin-binding buffer was added and the stained cells were analyzed by FACS. The assay allows to discriminate between apoptotic cells stained with green-fluorescent Alexa Fluor 488 annexin and necrotic cells, whose nuclei are stained with propidium iodide giving a red fluorescence, and live cells that show little or no fluorescence.

3.3. Analysis of gene and protein expression

3.3.1. Real-time PCR

Total RNA from NCI-H292 or PBECs was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Then 1.5 μg of total RNA

preparation was reverse transcribed with a cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) using oligo(dT)18. Generated cDNA was diluted 1/5 and 5 µl was used as template for amplification by means of quantitative real-time PCR with 15 µl/sample of an ABsolute QPCR SYBR Green PCR Mix (ABgene, Epsom, Surrey, UK) using ICycler (Bio-Rad, Munich, Germany). The following primers (TIB Molbiol, Berlin, Germany) were used (Table 1):

Gene	Sequence of sense primer	Sequence of anti-sense primer	AT (°C)
β-actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG	60
LL-37	CCACCATGGGCCTGGTGATGCCTCT GGCCATC	TGTACACTAGGACTCTGTCCTGGGT ACAAG	65
FPRL1	TGGTTGCCCTTCTGGGCACC	CTCTCGGAAGTCTTGGCCCAC	65
P2X7	CCCCGGCCACAACTACACCACGAGA AAC	CCGAGTAGGAGAGGGTTGAGCCGA TG	67
TLR2	GAGAGTGGGAAATATGGACACCTT	GCAGTTCCAAACATTCCACG	60
TLR4	ACCTGGAGTGGGAGGACAGT	AGATAGATGTTGCTTCCTGCCAA	60
CD14	CTGCAACTTCTCCGAACCTC	CCAGTAGCTGAGCAGGAACC	60
MD-2	CAACAATATCATTCTCCTTCAAGGG	GCATTTCTTCTGGGCTCCC	60
IL-6	CCAGAGCTGTGCAGATGAGTACA	CCTGCAGCTTCGTCAGCA	60
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCT	58
IP-10	TGAAATTATTCCTGCAAGCCAA	CAGACATCTCTTCTCACCCTTCTTT	60
ICAM-1	ACGTACCTCTATAACCGCCAGC	ATATGGGAAGGCCGAGGAAGAG	60
COX-2	CCTCCTGTGCCTGATGATTGC	TGGCCCTCGCTTATGATCTG	64
MUC1	GCCAGTAGCACTCACCATAGCTCG	CTGACAGACAGCCAAGGCAATGAG	50
erbB2	GGTGCCGTGGAGAACCCCGAG	TCACACTGGCACGTCCAGACC	55

Table 1. Characteristics of PCR primers (sequences, annealing temperatures (AT))**.**

Quantitative PCR results were obtained using the ΔΔCt (cycle threshold) method and expressed as mRNA content relative to β-actin mRNA. For qualitative analysis, PCR products were subjected to electrophoresis on a 1.5% agarose gel, and DNA was visualized by ethidium bromide staining.

5.4.2. Cytokine ELISA

Cytokine levels in culture supernatants were determined using a commercially available DuoSet ELISA Development kits for IL-6, IL-8, TNF-α according to the manufacturer's instructions (R&D Systems). In brief, 96-well microplates were pre-covered with capture antibody and incubated overnight. Then after washing and incubation with a blocking reagent, 100 µl per well of sample or standard were added and incubated overnight at 4°C. After washing and subsequent incubation with detection

antibodies during 2 h and Streptavidin-POD Conjugate (Roche Diagnostics) during 20 min at room temperature, 100 µl of TMB+Substrate-Chromogen (Dako Deutschland GmbH, Hamburg, Germany) was added. The reaction was stopped by adding 3N H_2SO_4, and the absorbance was measured using ELISA reader at 450 nm or 490 nm.

5.4.3. Western blot

Immunoblotting was used to analyze phosphorylation of EGFR. NCI-H292 cells were stimulated for the indicated time periods with different substances. The cell pellet was lysed with radioimmunoprecipitation (RIPA) buffer containing 10 mM TRIS-HCl (pH 7.4), 0.5 % (v/v) NP-40, 0.5 % (v/v) deoxycholate, 150 mM NaCl, 10 mM NaF and supplemented with proteinase inhibitor cocktail (Complete EDTA-free, Roche Molecular Biochemicals) and then centrifuged at 14,000 x g for 15 min at 4°C . Then supernatants were collected, and protein content in each sample was measured using the BCA (bicinchoninic acid) Protein Asay Kit (Pierce, Perbio, Bonn). Equal amounts of total protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Tris-HCL precast gels (Bio-Rad) and then transferred onto nitrocellulose (Blotting Nitrocellulose Membrane 0.45 µM, Bio-Rad). Blots were incubated with 0.5-2 µg/ml of anti-phospho-EGFR antibodies (Upstate, Charlottesville, Virginia) in 5% milk in PBS overnight at 4°C with agitation. As a loading control, β-actin-antibody (ABcam, Cambridge, UK) was used. After incubation with secondary enhanced chemiluminescence peroxidase labelled anti-mouse antibody (Amersham Biosciences, Uppsala, Sweden; 1:2000 dilution in 5% milk solution in sterile PBS) during 45-60 min at room temperature, the membranes were incubated with SuperSignal West (Pico – for β-actin; Femto – for EGFR) Maximum Sensitivity Substrate (Pierce) for 60 seconds and then exposed to x-ray film (Eastman Kodak, Rochester, N.Y.).

3.4. Analysis of immune cell responses

3.4.1. Analysis of DC activation

We analyzed modulation of dendritic cell (DC) activation by ECs (as described in detail above) and LL-37. Immature monocyte-derived DCs (1 x 10^6 cells/ml) were seeded in 6-well or 24-well plates in RPMI medium containing 10% FBS. DC maturation was induced by addition of live or heat-inactivated *P. aeruginosa* (strain PAO1; 10 bacteria per DC; at 1 h after incubation live bacteria were washed out and the medium was replaced with medium containing 100 µg/ml of gentamicin), or microbial molecular patterns such as LPS (0.1 or 1 µg/ml), LTA (10 µg/ml), or flagellin (0.1 or 1 µg/ml).

To test the effect of LL-37 on DC maturation, the cells were incubated with 5 or 20 μg/ml of LL-37 alone or together with microbial stimuli. In some experiments, DCs were pre-incubated with LL-37 for 2 h. Then the cells were washed in medium without LL-37 and then stimulated with TLR ligands. Alternatively, LL-37 and LPS were co-incubated in 37°C during 30 min allowing them to form a complex, and then the mixture was added to DC. These different modes of DC stimulation were used to determine the character of possible LL-37 interaction with DCs, from one side, and TLR agonists, from another.

After 24 h of incubation, the cell culture supernatants were removed and analysed for cytokines by ELISA as described above. The cells were harvested, washed once with PBS and used for FACS analysis. DCs were incubated for 30 min at room temperature with following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD14, phycoerythrin (PE)-conjugated anti-CD83, PE-Cy5-conjugated anti-CD80, and allophycocyanin (APC)-conjugated anti-HLA-DR (all BD Pharmingen, Heidelberg, Germany) or with their corresponding isotype controls (BD Pharmingen) in PBS supplemented with 1% FBS. FITC-conjugated anti-CCR7 was purchased from R&D Systems. Then the cells were washed twice with PBS containing 1% FBS, resuspended in CellFix (BD Pharmingen) and analyzed using a FACSort flow-cytometer (BD Biosciences). DC survival was analyzed using WST-1-based cell viability assay.

3.4.2. Analysis of T cell proliferation

Differently stimulated DCs were harvested, washed once with sterile PBS and then used for co-incubation with T cells. Allogeneic naïve $CD4^+$ T cells were isolated from blood of healthy volunteer donors as earlier described. To analyze T-cell proliferation, the carboxy-fluoroscein diacetate, succinimidyl ester (CFSE) assay was performed according to the manufacturer's instructions (Invitrogen) with some minor modifications. Briefly, allogeneic naïve $CD4^+$ T from peripheral blood of a healthy donor were incubated with 5 μM of CFSE for 10 min at 37° C. After subsequent washing CFSE-labelled T cells (1×10^6) were co-cultured with differently treated DCs (1×10^5) during 5 days in the presence of 1 μg/ml anti-CD3 monoclonal antibody (clone UCHT1; R&D Systems). After co-incubation period, the cells were harvested and assessed for their CFSE dilution using FACS analysis. Given that each division cycle is associated with a two-fold decrease in CFSE fluorescence intensity, the percentages of the daughter cells under each CFSE peak, corresponding to the number of divisions, were determined. As a negative control, CFSE-labelled T cells cultured in the presence of anti-CD3 but without DCs have been analyzed.

3.5. Statistical analysis

For all experiments, at least duplicate or triplicate determinations were made for each experimental condition. The results of replicates were averaged and expressed as one data point. In indicated cases, the results of representative experiments were shown. All data are expressed as mean plus or minus standard deviation (SD). Comparisons between experimental groups were performed using Student's t test. Results were considered statistically significant for *P* values less than 0.05.

4. RESULTS

4.1. Antimicrobial peptide LL-37 regulates airway epithelial homeostasis

4.1.1. Effect on wound closure

To evaluate the effect of LL-37 on bronchial epithelial wound closure *in vitro,* confluent monolayers of NCI-H292 and PBECs were mechanically damaged and then exposed to LL-37, TGF-α, or medium.

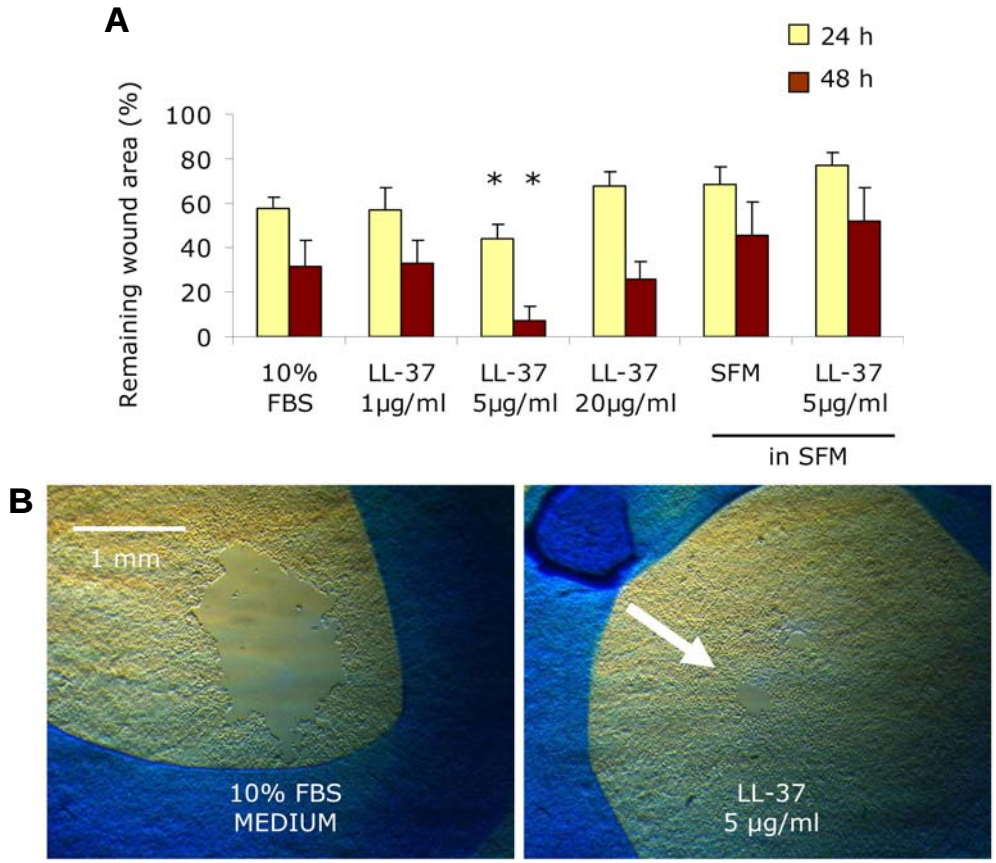


Figure 8. Effect of LL-37 on airway epithelial wound closure. Mechanically wounded NCI-H292 cells were incubated with different concentrations of LL-37 (A); the remaining wound area (percentage of residual wound area compared with t = 0 h) was measured after 24 and 48 h. * $P < 0.05$ as compared with the medium group (n=15 per group). (B) NCI-H292 wound areas 72 h after damage; 10x; initial wound area in both groups was similar; SFM = serum free medium.

Treatment with 5 µg/ml of LL-37 resulted in an accelerated wound closure in NCI-H292 cell monolayers (Figure 8). The stimulating effect of LL-37 on wound closure was dependent on the concentration of the peptide and required the presence of serum (Figure 8). In NCI-H292 airway epithelial cell line, LL-37 stimulated wound closure only at 5 µg/ml, but not at higher or lower concentrations (Figure 8A).

The biological properties of cell lines differ significantly from those of primary cells. Therefore, the effect of LL-37 on PBECs was also tested.

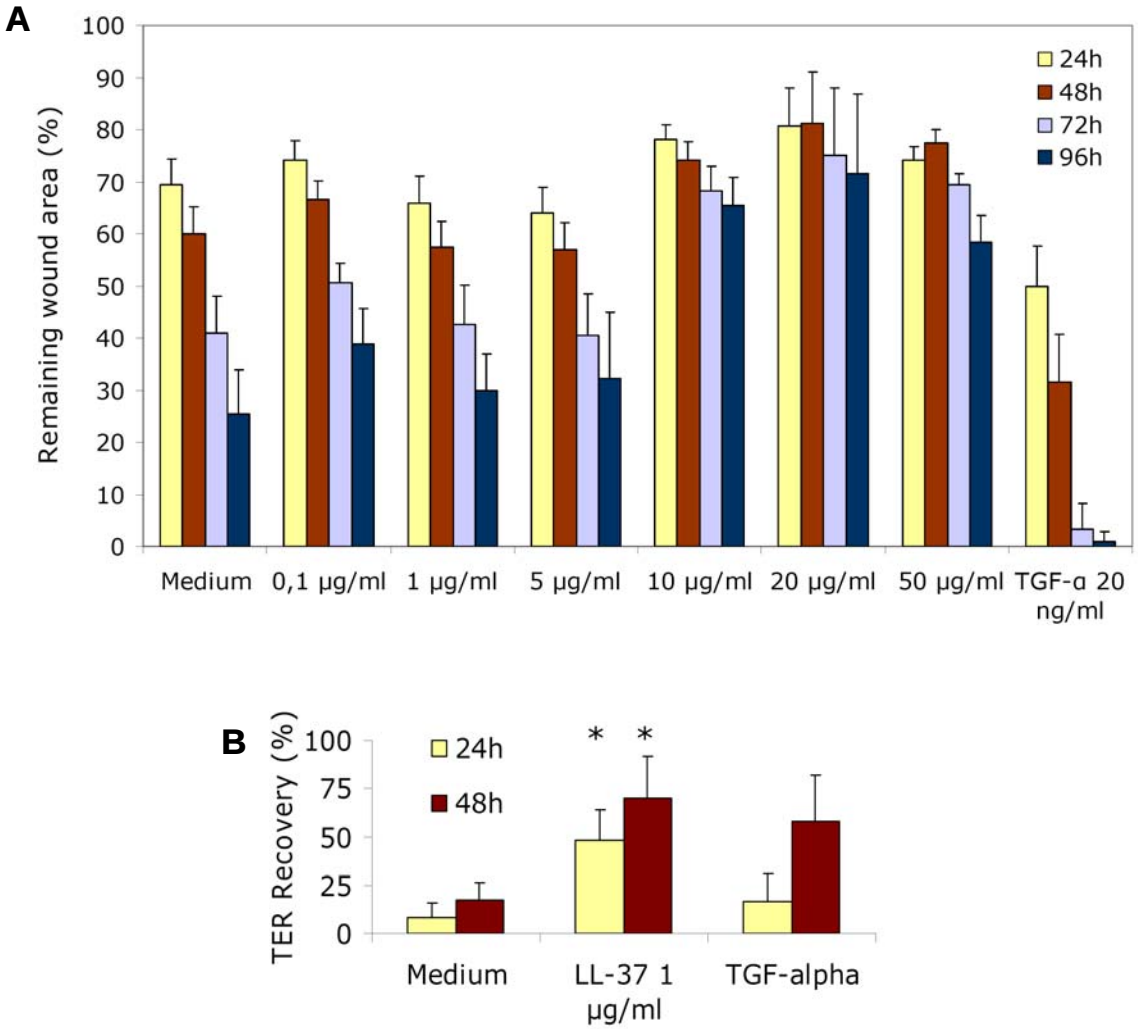


Figure 9. Effect of LL-37 on wound repair of PBECs. (A) Different concentrations of LL-37 were applied to the mechanically wounded monolayers of non-differentiated PBECs; the remaining wound area was measured during 96 h after damage (n=5 per group). Since there was no stimulatory effect of LL-37 on wound closure, significance of differences between the groups is not indicated. (B) Differentiated PBECs in ALI culture were wounded and the wound closure was determined by measuring transepithelial resistance. * $P < 0.05$ as compared with the medium group (n=3 per group).

Taken together, these data show that LL-37 increase the speed of wound closure in NCI-H292 epithelial cell line as well as in differentiated PBECs in a concentration-dependent manner.

4.1.2. Effect on EC migration

To determine whether the effect of LL-37 on wound closure is due to accelerated migration, the wound edges were examined for the presence of lamellipodia, cell membrane protrusions induced during cell migration. We have found that the intensity of lamellipodia formation was significantly increased in the LL-37 treated group at 24 h and 48 h after wounding (Figure 10). This suggests that LL-37 promotes epithelial wound closure by increasing migration of ECs into the wound area.

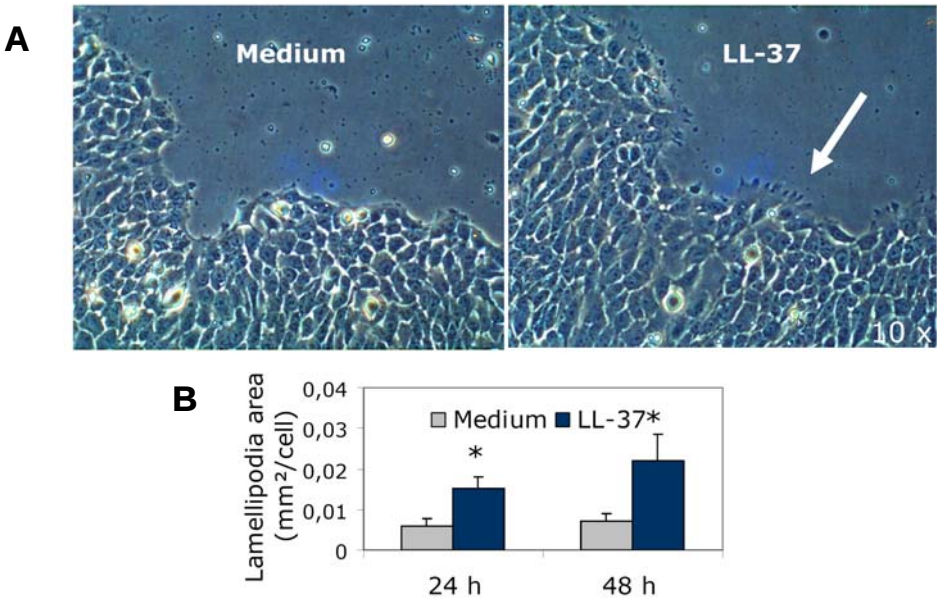


Figure 10. LL-37 increases the formation of lamellipodia (arrow) at the wound edge of NCI-H292 cells treated with 5 μg/ml LL-37 (A), mean lamellipodia area at 24 h and 48 h after wounding (B) * $P < 0.05$ ($n = 5$ per group).

Since LL-37 increased cell migration in a wound closure assay, we hypothesized that LL-37 might be chemotactic for airway ECs. We used LL-37 in a modified Boyden's chamber assay and found that LL-37 stimulated NCI-H292 migration in a concentration-dependent manner (Figure 11). There was no stimulation of migration in cells treated with sLL-37 (Figure 11). In contrast to wound closure, the effect of LL-37 on NCI-H292 cell migration in the chemotaxis assay was not modified by the presence of serum (data not shown). Because LL-37 induced chemotaxis in serum-independent manner and also at concentrations that were not effective in wound closure assay, it seems likely that induction of epithelial chemotaxis is not directly involved in the wound repair process enhanced by the peptide.

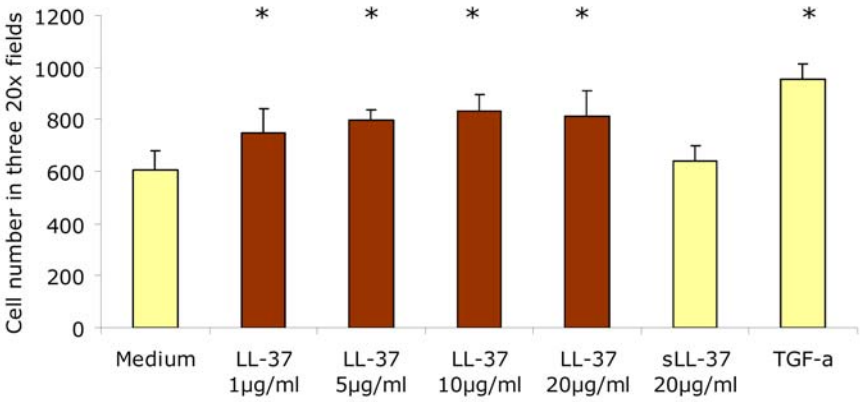


Figure 11. Effect of LL-37 on airway EC chemotaxis was analyzed by Boyden chamber assay**.** LL-37, TGF-α, or sLL-37 were placed into each of the bottom wells. NCI-H292 cell migration was quantified as the number of migrated cells on the lower surface of the filter in a three random fields using a light microscope at × 20 magnification. **P* < 0.05 as compared with the cells in the medium group (n = 4 per group).

4.1.3. Effect on EC proliferation

Cell proliferation is an important component of epithelial repair. To determine whether LL-37 stimulates bronchial EC proliferation, we measured BrdU incorporation in NCI-H292 cells 48 h after stimulation with LL-37, sLL-37, TGF-α, or medium alone. Similar to chemotaxis assay, LL-37 increased NCI-H292 labeling with BrdU in a concentration-dependent manner (Figure 12), and treatment with sLL-37 at the equivalent concentrations had no significant effect.

The stimulatory effect of LL-37 on EC proliferation did not require the presence of serum (data not shown), and occurred dose-dependently at concentrations lower and higher than 5 μg/ml. Of interest, the high concentration of LL-37 (50 μg/ml), which was toxic to airway ECs and induced a rapid detachment of cells during the BrdU assay, still displayed increased BrdU incorporation (Figure 12B). These observations tempted us to speculate that cell proliferation is not involved in the effect of LL-37 on epithelial wound closure. To ensure this issue, wounded monolayers of NCI-H292 cells were incubated with BrdU and the incorporation was detected. We found no increase of stained cells at the wound edge compared to the control group or a region remote from the wound edge (data not shown). Altogether, these findings show that LL-37 induces EC proliferation, but it is likely not a predominant mechanism responsible for its effect on wound closure.

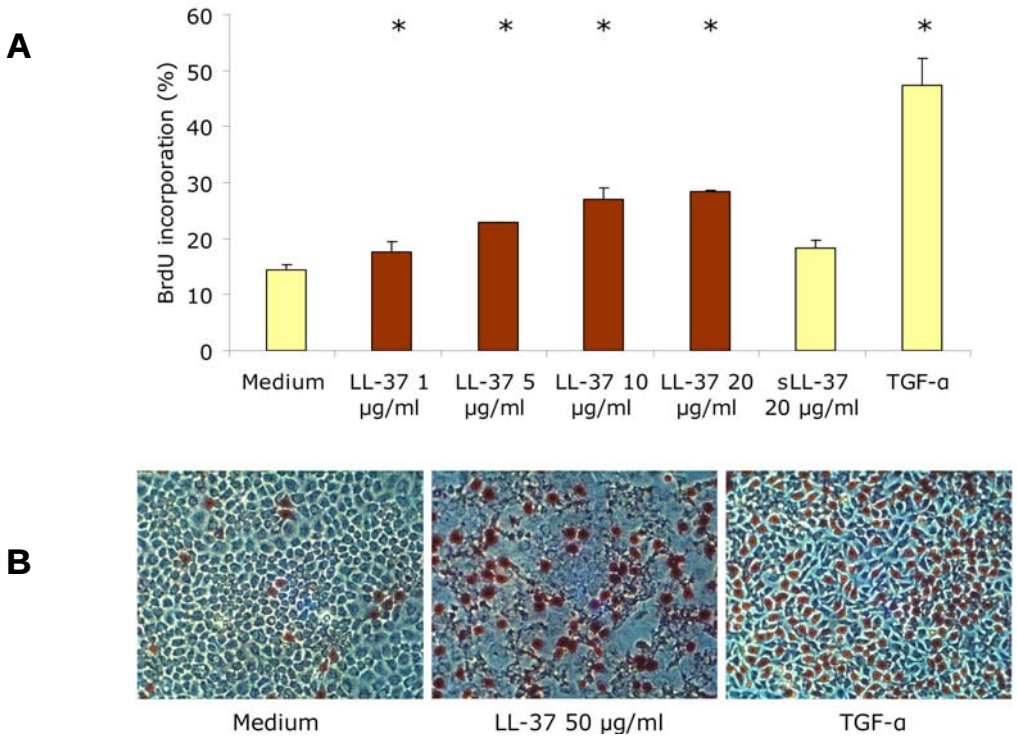


Figure 12. Effect of LL-37 on airway EC proliferation. NCI-H292 cells were incubated with BrdU in the presence of LL-37 or under control conditions (medium, sLL-37, TGF-α) for 48 h. (A) The numbers of BrdU positive cells were determined 48 h after incubation. Data are expressed as a percentage of the BrdU positive cells (BrdU incorporation index). * $P < 0.05$ as compared to the medium group ($n = 4$ per group). (B) BrdU incorporation in the nuclei of NCI-H292 cells after 24 h of incubation under indicated conditions; 10x.

4.1.4. Effect on EC viability

It is known that cationic peptides may be cytotoxic because of their ability to interact with negatively charged membranes resulting in increase of their permeability. To determine cytotoxic effects of LL-37, we performed an LDH release assay with NCI-H292 and PBECs. The presence of LDH outside the cells serves as a marker of cell membrane damage, therefore, we analyzed whether LL-37 causes necrotic changes in airway ECs. We found that LL-37 induced significant release of LDH from PBECs (Figure 13A) and NCI-H292 cells (Figure 13B) at high concentrations (≥ 20 µg/ml), consistently with our earlier observation that, at high concentrations, LL-37 does not increase and even inhibits epithelial wound closure (Figures 8A and 9A).

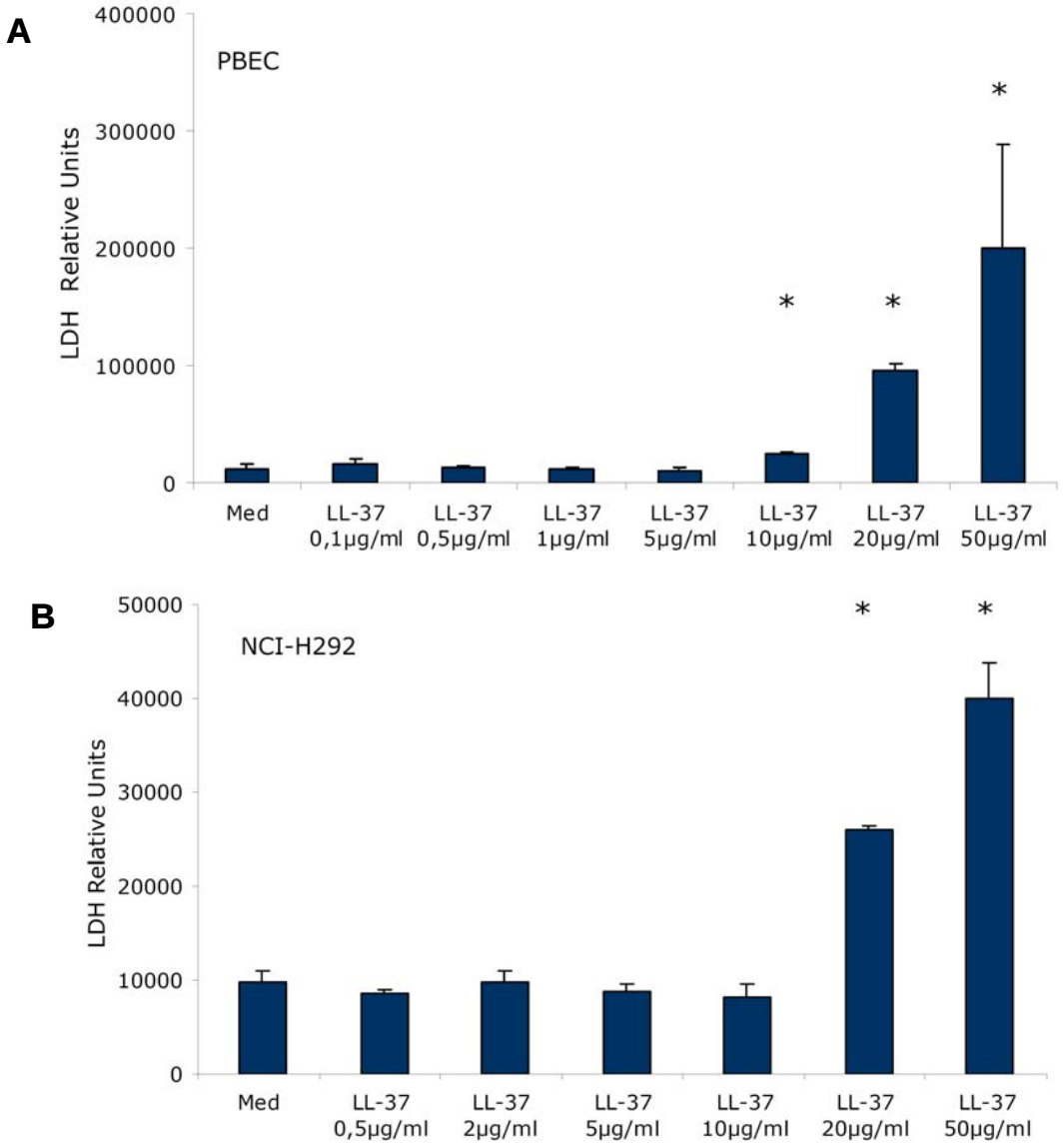


Figure 13. Cytotoxic effects of LL-37 on airway ECs. LDH release in response to the indicated concentrations of LL-37 from (A) PBEC and (B) NCI-H292 cells was measured. * $P < 0.05$ as compared to the medium group ($n = 4$ per group).

To examine whether LL-37 also induces EC apoptosis we performed an annexin binding assay, which is based on the detection of the externalized phosphatidylserine in apoptotic cells after specific interaction with fluorochrome-conjugated annexin. Co-incubation with propidium iodide, a dye that stains the nuclei of necrotic cells, permits a specific discrimination between necrotic and apoptotic cells. No significant effect of LL-37 on apoptosis of NCI-H292 cells was found in this assay as compared to the induction of necrotic cell death (Figure 14).

Taken together, these data suggest that high concentrations of LL-37 (≥ 20 µg/ml) are toxic to airway ECs.

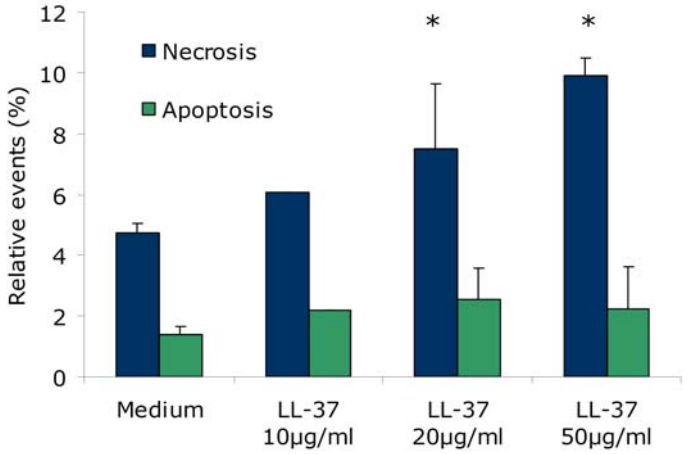


Figure 14. Effect of LL-37 on airway EC death. LL-37 did not induce apoptosis of NCI-H292 in an annexin-binding assay, however, resulted in dose-dependent increase of necrotic cell death. * $P < 0.05$ as compared to the medium group ($n = 4$ per group).

4.1.5. Signaling pathways

In order to determine whether the effect of LL-37 is dependent on the peptide structure, but not on its cationicity, we used the scrambled version of LL-37 (sLL-37), in which the amino acid sequence is changed but the number of residues and charge do not differ from the original peptide, and the all-D-form of LL-37 (D-LL-37). We found that sLL-37 (Figure 15) and also D-LL-37 (data not shown) did not accelerate airway epithelial wound closure at any concentration regardless the presence of serum. Thus, the effect of LL-37 on epithelial wound closure is likely dependent on the structure of the peptide.

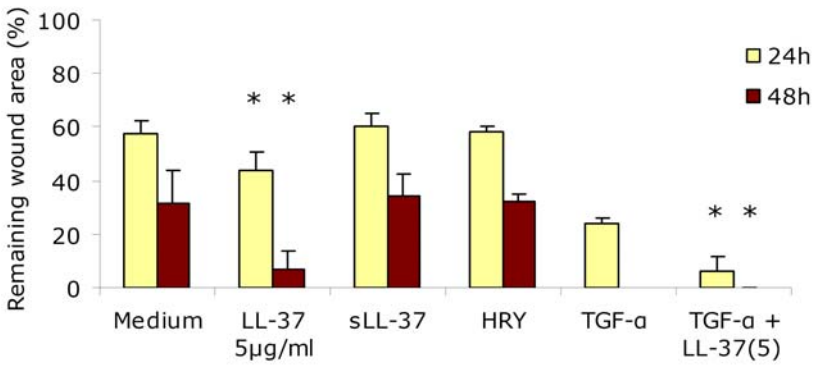


Figure 15. Analysis of upstream signaling pathways involved in airway epithelial wound closure induced by LL-37. Wounded NCI-H292 monolayers were (A) incubated with 5 µg/ml LL-37 or scrambled LL-37 (sLL-37), HRY-peptide, TGF-α, or TGF-α + LL-37. The remaining wound area was determined 24 and 48 h after wounding; inhibition index was calculated as described in the Materials and Methods section. * $P < 0.05$ as compared to the medium group ($n = 5$ per group).

Since the effect of LL-37 appeared to be dependent on the specific structure of the peptide, the involvement of a specific receptor is possible. Several receptors have been proposed to interact with LL-37 and mediate its effects on the host cells. One of them, formyl peptide receptor-like 1 (FPRL1) is a chemoattractant receptor mostly expressed on PMNs and involved in recognition of N-formyl peptides of bacterial origin (120). It has recently been demonstrated that LL-37 attracts neutrophils and other blood cells acting as a ligand for FPRL-1 (40). Since cell migration was suggested to be responsible for effect of LL-37 on wound closure (Figure 10) and LL-37 was found to be chemotactic for NCI-H292 cells (Figure 11), we thought that LL-37 may modify EC function via this receptor. To test this possibility, we applied a synthetic HRYLPM-COOH peptide, recently described as a full and selective activator of FPRL-1 (9), in a wound closure assay. However, we did not find the stimulatory effect of this peptide on NCI-H292 wound closure (Figure 15). Consistently, we detected very weak expression of FPRL-1 mRNA in airway ECs (Figure 16).

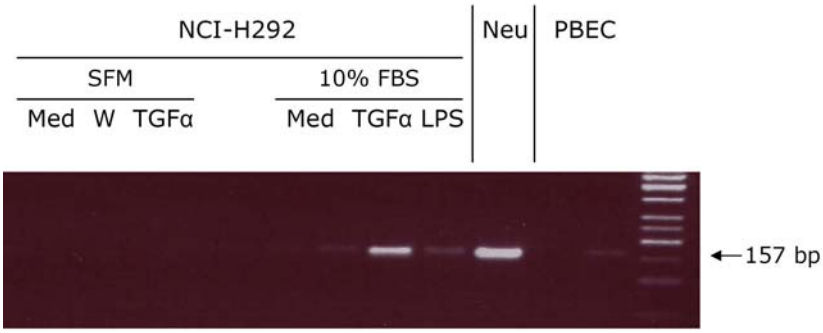


Figure 16. FPRL1 mRNA expression in airway ECs (NCI-H292 cells and PBECs) was determined by RT-PCR, PCR products were subjected to electrophoresis on a 1.5% agarose gel, and DNA was visualized by ethidium bromide staining; in NCI-H292 cells expression of the gene was analyzed in both serum-free (SFM – serum free medium (Med)) and 10% FBS-containing Med; without stimulation, or 24 h after wounding (W) or stimulation with TGF-α 20 ng/ml or LPS 10 μg/ml; neutrophil (Neu) cDNA was used as a positive control.

Another candidate receptor is a purinergic receptor P2X7, which has recently been implicated in the regulation of monocyte (45) and neutrophil (123) functions by LL-37. By real-time PCR we determined P2X7 expression in both NCI-H292 and PBECs (data not shown). Then we used a specific inhibitor of this receptor KN-62 and found no effect of this inhibitor on LL-37-stimulated wound closure (Figure 17A) suggesting that P2X7 receptor is also not involved in this process.

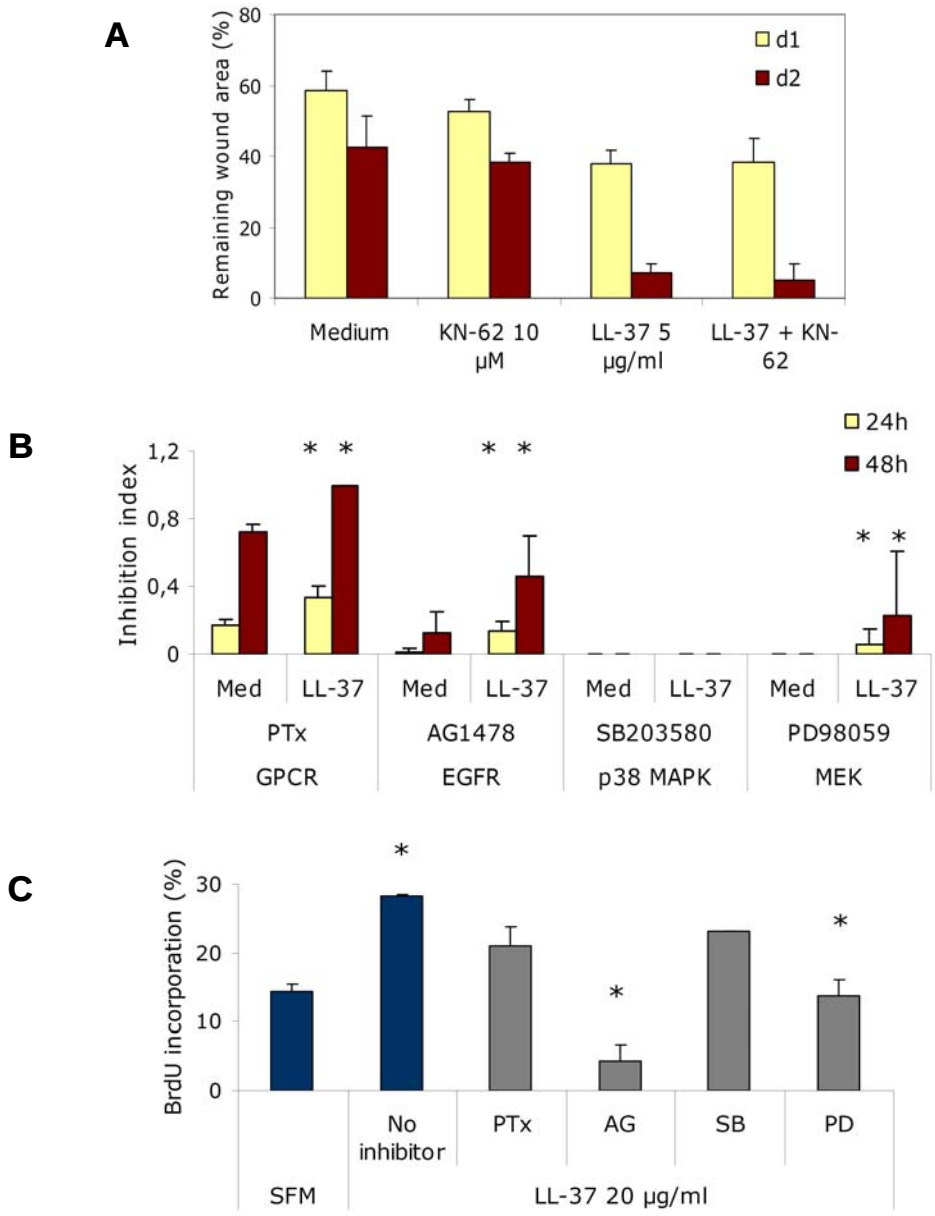


Figure 17. LL-37 signaling pathways involved in airway epithelial wound closure and proliferation. Wounded (A, B) or intact (C) NCI-H292 monolayers were pre-treated with inhibitors 30 min before addition of LL-37 (A, B - 5 µg/ml). (A) The remaining wound area was determined at d1 and d2 after wounding; inhibition index was calculated as described in Methods. * P<0.05 as compared to the medium group (n=5 per group). (B) The percentage of BrdU positive cells (BrdU incorporation, %) was determined 48h after incubation. * $P < 0.05$ as compared to the medium group (LL-37) or to the LL-37 group (inhibitor groups) (n = 4 per group).

In a recent study LL-37 induced responses in airway ECs by transactivation of the EGFR (174). Since GPCR signaling is known to be involved in EGFR transactivation (9,38), we tested whether the effect of LL-37 on airway epithelial repair can be reduced by specific inhibitor of GPCRs *Pertussis toxin* (PTx). We found that PTx inhibited

wound closure (Figure 17B) and, to a less extent, EC proliferation (Figure 17C) induced by LL-37. To determine the involvement of EGFR, we exposed wounded monolayers to specific EGFR inhibitor AG1478. Such a pre-treatment significantly decreased the effect of LL-37 on both wound closure (Figure 17B) and EC proliferation (Figure 17C).

The molecular crosstalk between LL-37 and EGFR signaling in airway ECs can be further supported by observations that addition of LL-37 substantially increased effect of TGF-α on wound closure (Figure 15), and that application of this EGFR ligand induced expression of FPRL1 in NCI-H292 cells (Figure 16). Expression of EGFR in tested ECs was confirmed by detection of ErbB2 transcripts using real-time PCR (data not shown).

To gain insight into possible down-stream signaling events, inhibitors of MEK and p38 MAPK were used. Inhibition of MEK, but not p38 MAPK, effectively reduced LL-37-stimulated wound closure (Figure 17A) and EC proliferation (Figure 17B).

4.2. Epithelial innate immune recognition modulates airway epithelial homeostasis

4.2.1. Effect on epithelial repair

Invasion and colonization of epithelial surfaces by pathogenic bacteria is usually associated with damage to epithelial barrier (167), we hypothesized that recognition of microbial molecular patterns by ECs may directly influence the homeostatic processes to facilitate the optimal tissue repair.

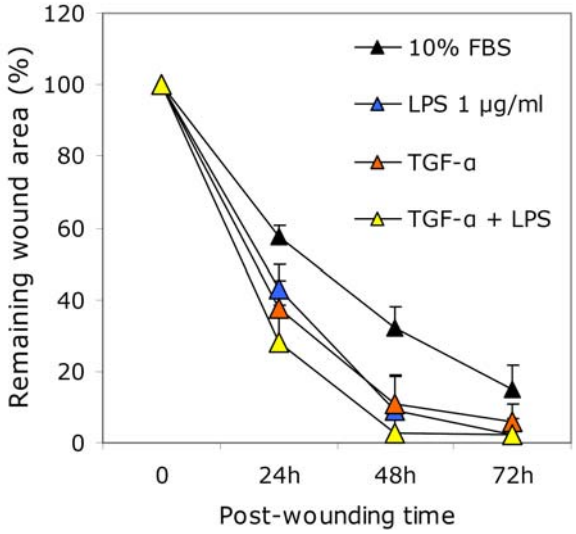


Figure 18. Effect of LPS on epithelial wound closure. Mechanically wounded NCI-H292 monolayers were stimulated with LPS (Sigma), TGF-α (20 ng/ml), LPS + TGF-α, or 10% FBS-medium alone; remaining wound area was measured during 72 h. The difference between values in stimulated groups and 10% FBS group was statistically significant ($P<0.05$; $n=5$).

To evaluate this hypothesis we first exposed the mechanically wounded NCI-H292 epithelial monolayers to LPS (*E. coli*, Sigma), a major suprastructure of gram-negative bacteria, and found that endotoxin significantly increases the speed of wound closure in the presence of serum (Figure 18). LPS stimulated wound closure as effectively as a positive control TGF-α. Interestingly, addition of LPS to TGF-α further increased TGF-α-induced wound closure (Figure 18).

The finding that LPS directly stimulates epithelial repair mechanism served for as a "proof-of-concept" for our further studies, in which different microbial factors were tested for their possible role in epithelial growth and repair. These studies revealed that peptidoglycan (PGN), a cell wall component of gram-positive bacteria, substantially increased epithelial wound closure (Figure 19).

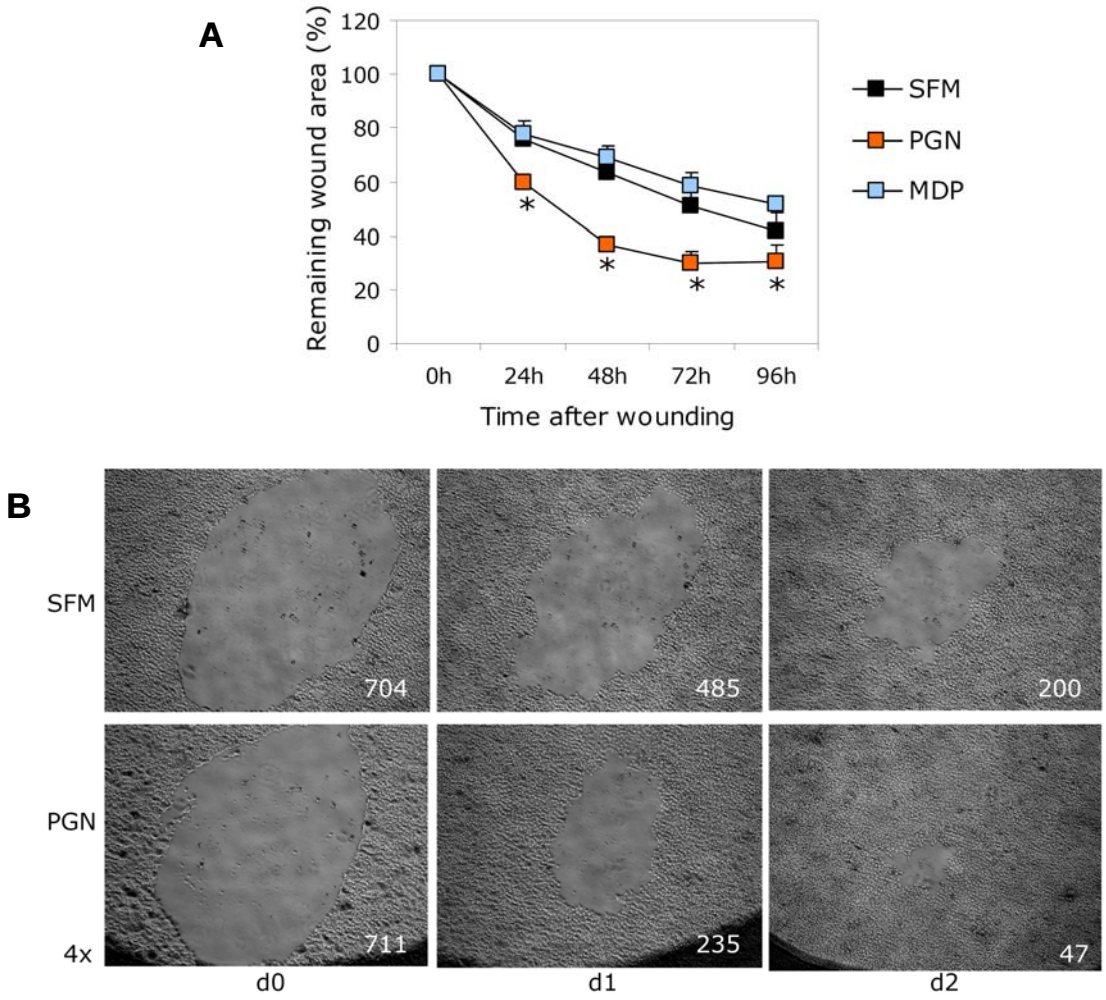


Figure 19. PGN directly stimulates airway epithelial wound closure. (A) Addition of PGN (*S.aureus*) but not MDP (both at 10 μg/ml) to wounded NCI-H292 monolayers increased wound closure compared to SFM alone ($P < 0.001$; n=5). (B) Representative graph of wound closure in PGN and SFM groups (wound area measurement data in pixels are indicated).

Notably, muramyl dipeptide (MDP), a product of PGN degradation that signals via NOD2, did not induce the similar effect (Figure 19A). This suggests the likely involvement of TLR2 in the observed phenomenon. Then we tested *S. aureus*, gram positive bacteria that express PGN and other TLR2-activating patterns, and found that it accelerates epithelial wound closure similarly to PGN (Figure 20).

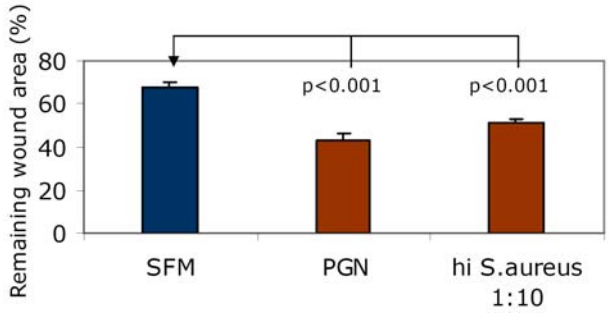


Figure 20. *S. aureus* increases airway epithelial wound closure. Heat-inactivated (hi) *S. aureus* (1:10 dilution of the stock preparation OD_{600} 1.0) and PGN (*S. aureus*; 10 µg/ml) were added to wounded NCI-H292 epithelial monolayers under serum-free conditions; the remaining wound area was measured 24 h after stimulation and were compared with SFM group; n=5.

To ensure that PGN-mediated effect is based on increased cell migration, we performed the analogous assay, but now very small linear wounds (width ~1 mm) were created, and cell migration into the wound areas was analyzed during the first 24 hrs.

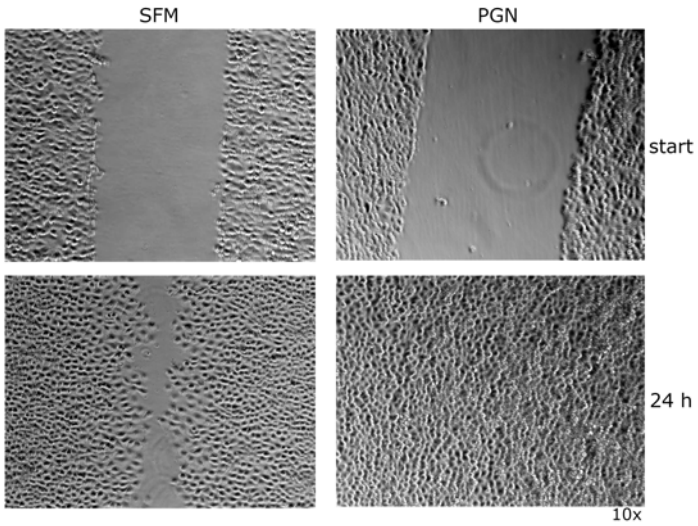


Figure 20. PGN increases EC migration during wound closure. Small wounds (width ~1 mm) were created in NCI-H292 cell monolayers after 18 h of serum starvation, then PGN (*S. aureus*, 10 µg/ml) or SFM alone were added and the wound areas were photographed; images were taken immediately (upper panel) and 24 h (lower panel) after wounding.

We have found that PGN substantially accelerated NCI-H292 cell migration in this assay, resulting in complete closure of the wound, whereas in control group cell migration was clearly less effective (Figure 20).

It was important to determine whether induction of epithelial repair by microbial patterns is restricted to cancer cells or represents a general phenomenon that occurs in any epithelia as a defense response to microbial invasion. To clarify this issue, we analyzed the effect of different microbial factors on the repair of mechanically wounded differentiated primary airway epithelium. We found that under serum-free conditions repair of primary airway epithelium developed with a very low speed, and 1 day after injury the transepithelial resistance (TER) was lower compared to the start value (Figure 21). Addition of PGN and LTA at concentrations 20 and 100 µg/ml, respectively, prevented the decrease in TER during the first 12 hrs and supported recover of epithelial integrity during the second day of repair. Although without reaching statistical significance, Fsl-1, a synthetic ligand of TLR2, and *S. aureus* also provided protective effect for PBECs in terms of increased repair.

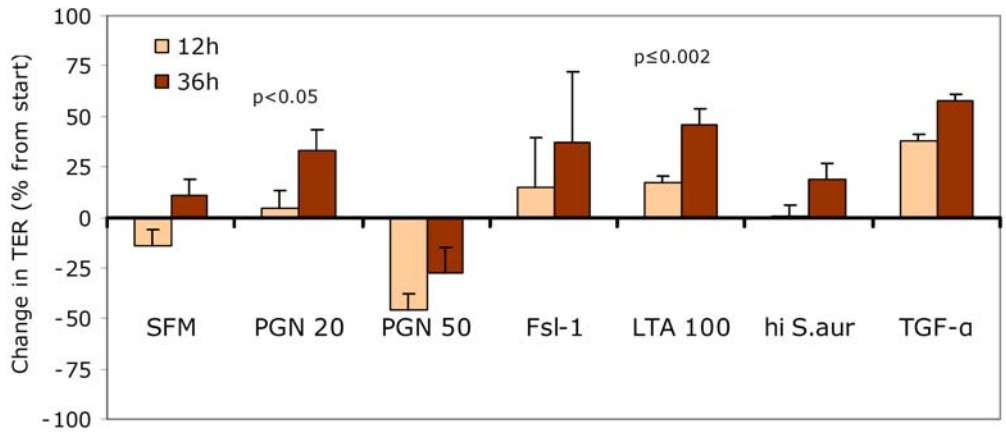


Figure 21. TLR2 ligands increase survival and repair of differentiated primary bronchial epithelial cells (PBEC). Different TLR2 stimuli (PGN 20 µg/ml and 50 µg/ml; Fsl-1 2 µg/ml; LTA 100 µg/ml; heat-inactivated (hi) *S. aureus* 1:10 dilution of the stock preparation OD_{600} 1.0), TGF-α (20 ng/ml; positive control), or medium (SFM) alone were added to the mechanically wounded differentiated (starting TER > 500 Ω) PBECs from the apical and basolateral sides; transepithelial resistance (TER) was measured at indicated time-points after stimulation and the change in TER is displayed.

4.2.2. Effect on epithelial growth and survival

To test whether microbial factors contribute to epithelial growth, we used the WST assay that allows quantification of metabolically active, i.e. alive, cells in culture. Increase of cell number with time can be interpreted as result of cell proliferation, while decrease of cellularity is a marker of cell death. We found that LPS (*E. coli*, Sigma) increased epithelial cellularity in a time-dependent manner (Figure 22A). Then we analyzed the effect of other microbial factors on epithelial growth (Figures 22 B and C).

As shown in Figures 22 B and C, TLR2 ligands PGN and LTA dose-dependently increased NCI-H292 cell number. This effect rather reflects increased cell proliferation than survival, since cell numbers in the control group did not change during the incubation period (Figure 22 B). Concentrations of PGN and LTA lower than 10 μg/ml and 50 μg/ml, respectively, were not effective, consistently with previous observations showing that relative high concentrations of these patterns are necessary to activate ECs (105). PGN and LTA induced proliferative response in ECs in a dose-dependent manner, suggesting that the effect is likely receptor-mediated. PGN and LTA are classical ligands for TLR2 (193), but there are some data supporting TLR-independent effect of PGN (179) and LTA (105). Therefore, we also applied synthetic lipoprotein Pam_3CSK4, which activates selectively TLR2, and found that it also induced strong proliferative effect in tested ECs (Figure 22 B). Notably, heat-inactivated *S. aureus* also substantially increased NCI-H292 cell number in a WST-based proliferation assay (Figure 22 C).

Interestingly, factors that signal via MyD88-dependent pathway induce a similar effect on airway epithelial growth in our model. In addition to LPS and TLR2 ligands, stimulatory ODN, a TLR9 agonist, and inflammatory cytokine IL-1β, both signaling via MyD88, also increased EC number (Figure 22 D). In contrast, Poly(I:C), a ligand for TLR3 that recruits MyD88-independent pathway, decreased EC survival (Figure 22 D).

Next, we examined the effects of microbial factors on survival and proliferation of PBECs grown as submersed nondifferentiated monolayers. As shown in Figure 23, serum deprivation during 24 h resulted in a considerable decrease of cell number, reflecting a decreased survival without serum. However, addition of TLR2 stimuli to the serum-free medium significantly increased survival of PBECs, and the highest concentration of PGN tested (50 μg/ml) completely prevented cell death. Inactivated *S. aureus*, applied at relatively high dose showed a similar protective effect. Synthetic TLR2 agonist Fsl-1 used in this set of experiments also prevented decrease of epithelial cellularity (Figure 23), again supporting the involvement of TLR2. The protective effect on differentiated PBECs survival during wound closure was shown in Figure 21.

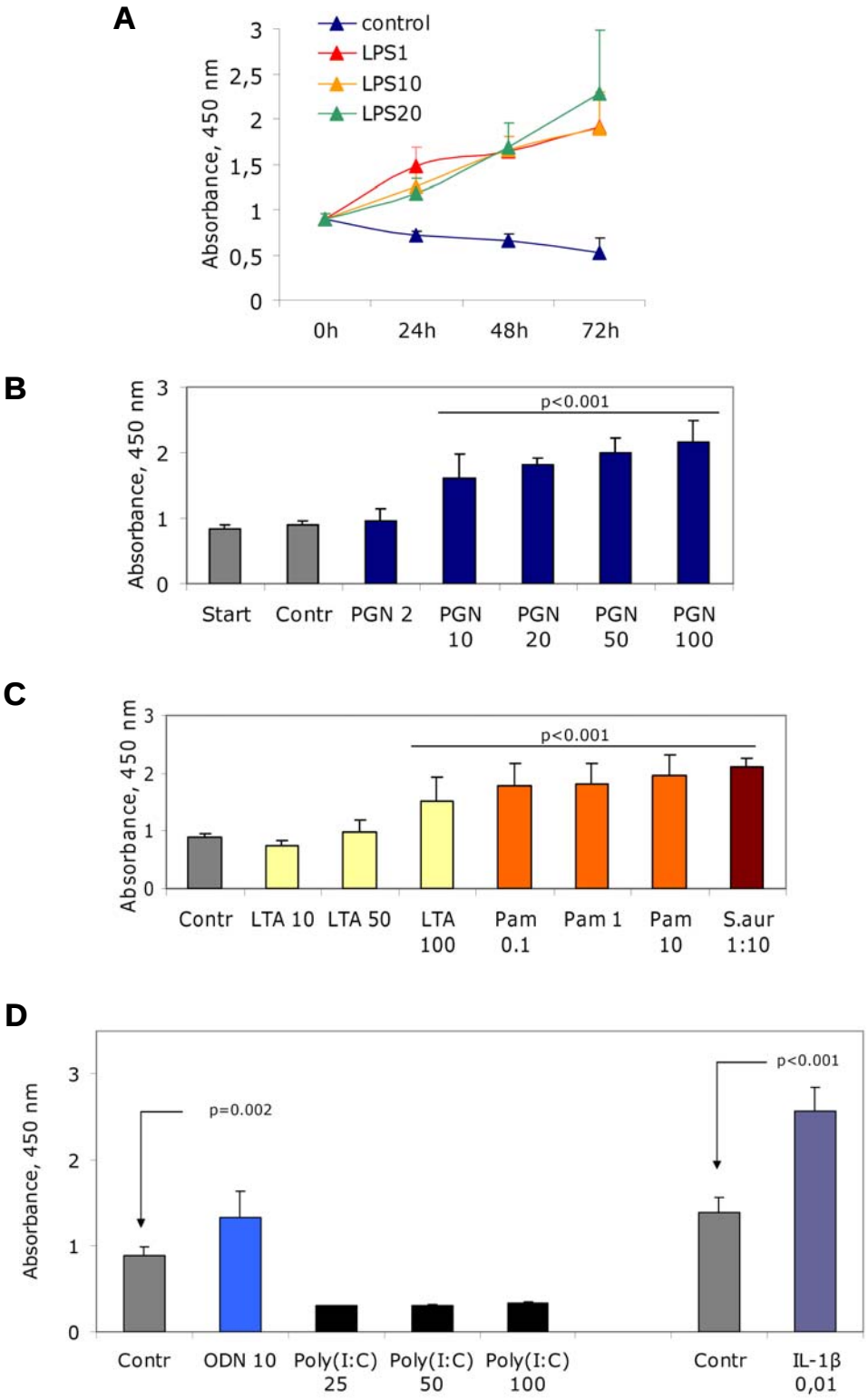


Figure 22. Effect of microbial factors on airway epithelial growth. Confluent NCI-H292 cell monolayers were serum-starved for 18 h and then stimulated with (A) LPS (*E. coli*, Sigma); (B) PGN (*S. aureus*), (C) LTA, PAM_3CSK4 (Pam), (D) ODN, Poly(I:C), IL-1β at indicated concentrations (in μg/ml); heat-inactivated *S. aureus* 1:10 dilution as described above; or medium alone (in A – SFM + 100 ng/ml LBP) during 24 h; in B – the initial cellularity before stimulation (start) is shown; then absorbance corresponding to live cell number was measured at 450 nm after 30 min of incubation with WST-1; n=8. P indicates significance of difference with control.

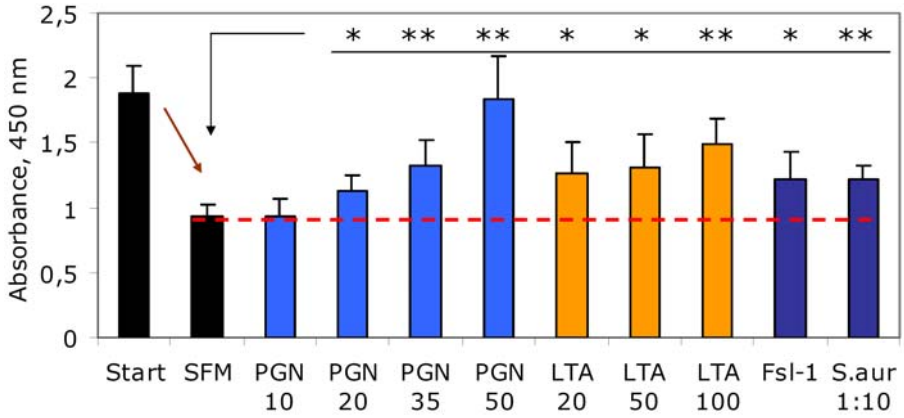


Figure 23. Effect of TLR2 agonists on survival of PBECs. Confluent PBEC monolayers were serum-starved for 18 h and then stimulated with different TLR2 stimuli (PGN, LTA, Fsl-1 (1 µg/ml) at indicated concentrations (in µg/ml); heat-inactivated *S. aureus* (2 x 1:10 dilution as described above); or serum-free medium (SFM) alone; after 24 h of incubation the number of metabolically active cells was determined after 30 min incubation with WST-1 reagent and subsequent measuring absorbance at 450 nm; * $P < 0.005$; ** $P < 0.001$; n=8.

4.2.3. Signaling pathways

First, it was essential to confirm that TLRs are responsible for activating the pathways involved in epithelial homeostasis. To test this, TLR2 siRNAs were transfected into the ECs which were then wounded and stimulated with PGN. The transfection procedure did not have any nonspecific inhibitory effect on ECs since control siRNA did not delay PGN-induced wound closure, however, the both TLR2 siRNAs effectively suppressed the effect of PGN (Figure 24).

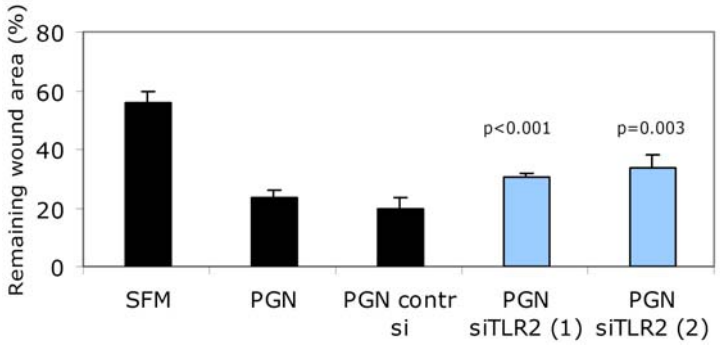


Figure 24. Involvement of TLR2 in PGN-induced airway epithelial repair. NCI-H292 cells were transfected with 5 nM of two different siRNAs for TLR2, control siRNA; 48 h after the medium was changed to serum-free medium (SFM); after 16 h, monolayers were mechanically wounded and then were stimulated with 10 µg/ml PGN (*S. aureus*) or remained unstimulated; remaining wound areas (related to start; %) were measured 24 h after wounding; n=5; P values (statistic significance of the difference with the PGN group) are shown for siTLR2 groups.

Second, it was important to determine whether epithelial TLR signaling is linked to the pathways involved in epithelial repair processes directly or the observed effect occurs due to secondary mediators (e.g. cytokines) induced in ECs as a result of TLR signaling. To exclude the latter possibility, we used neutralizing antibodies against IL-1β and TNF-α, the inflammatory cytokines known to increase EC proliferation and migration (10). We have found that mixture of these antibodies did not result in a significant inhibition of PGN-stimulated epithelial wound closure (Figure 25), suggesting that IL-1β and TNF-α unlikely mediate the PGN-induced effect. Another pro-inflammatory cytokine IL-6, which is produced by airway ECs, did not stimulate epithelial wound closure (data not shown).

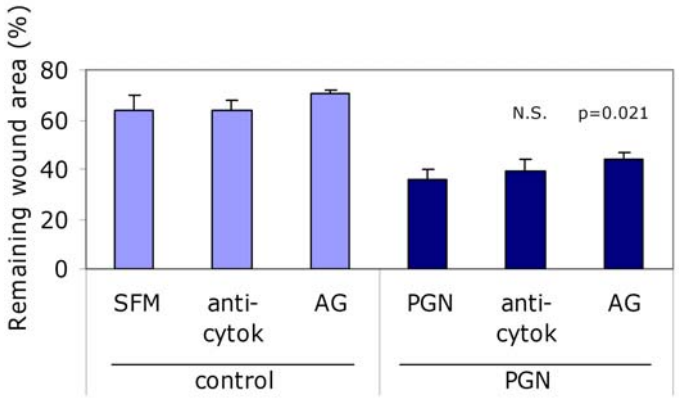


Figure 25. Effect of PGN on airway epithelial wound closure is not mediated by pro-inflammatory cytokines IL-1β and TNF-α. Confluent NCI-H292 monolayers were serum-starved for 18 h and then were incubated with a mixture neutralizing antibodies against IL-1β and TNF-α (each at 1 μg/ml) or inhibitor of EGFR AG1478 (1 μM) during 1 h; then monolayers were wounded and treated with 10 μg/ml PGN or remained unstimulated; 24h after, remaining wound areas were measured; n=5; P value - comparison with PGN; N.S. – nonsignificant difference.

Then we analyzed the molecular pathway that is recruited by TLR signaling and mediate epithelial repair. EGFR is known to play a central role in airway EC migration, survival and proliferation (97). Since signaling via EGFR is dependent on its tyrosine kinase activity, we pre-incubated NCI-H292 epithelial monolayers with a specific inhibitor EGFR kinase AG1478 before wounding and stimulation with PGN. We have found that such a protocol resulted in a significant decrease of the effect induced by PGN (Figure 25). Because epithelial restitution depends on cell migration during the repair process, we optimized the protocol of inhibition by addition of AG1478 before and after the wounding in order to provide constant inhibition of EGFR tyrosine kinase activity.

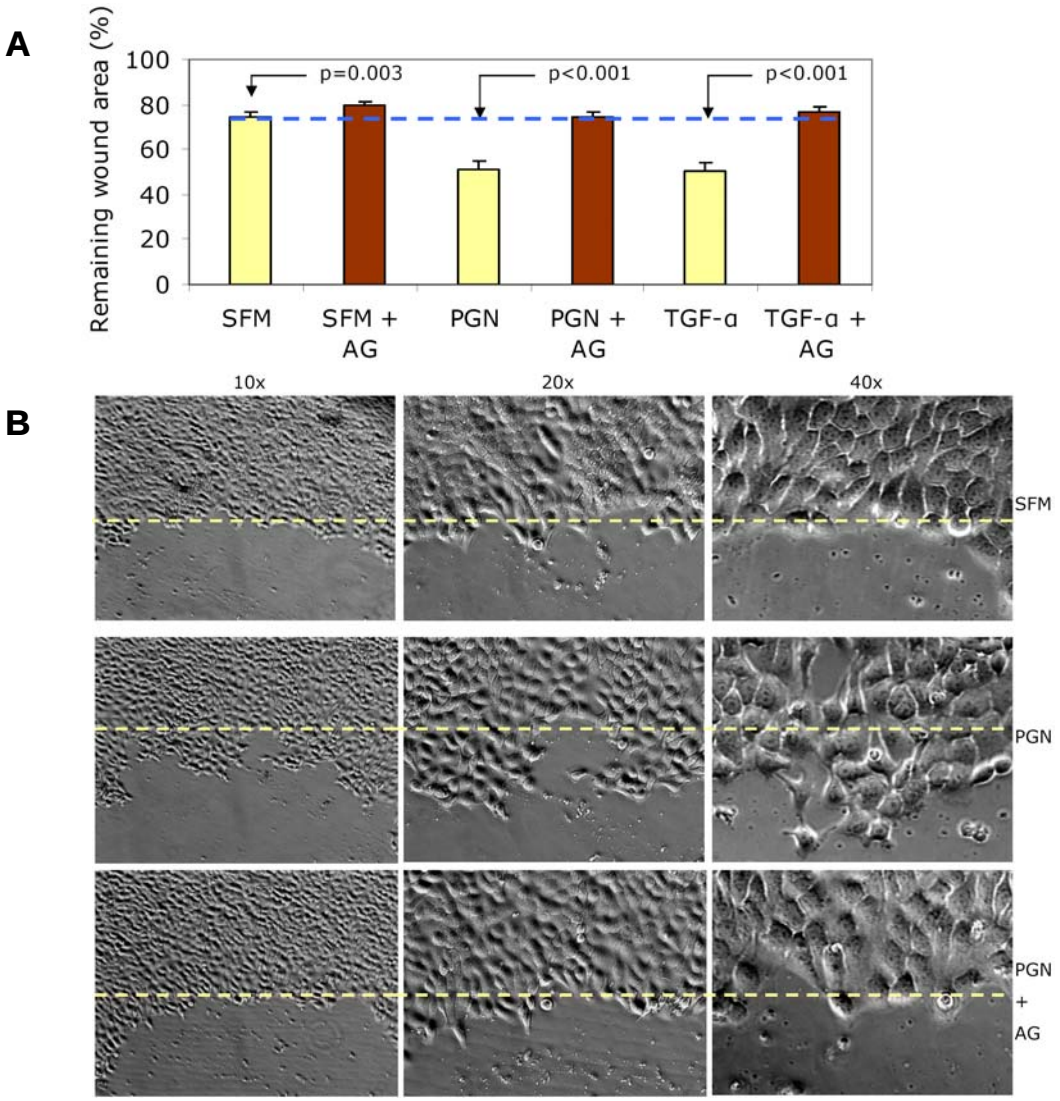


Figure 26. Effect of PGN on airway epithelial wound repair is EGFR-dependent. After 18 h of serum starvation, confluent monolayers of NCI-H292 cells were mechanically wounded and treated with 10 μg/ml PGN (*S. aureus*) or remained unstimulated; some groups were incubated with inhibitor of EGFR tyrosine kinase AG1478 (1 μM) during 1 h before wounding and then after wounding before addition of stimuli, or remained untreated; (A) remaining wound areas were measured 24 h after wounding; n=5; (B) representative micrographs showing the migration of cells from the wound edges 24 h after wounding.

We have found that such a protocol resulted in a weak but statistically significant delay of the wound closure process in the control group (Figure 26A), consistent with the requirement for continuous EGFR activation for epithelial repair. Importantly, AG1478 completely blocked the stimulatory effect of PGN on epithelial wound closure, similar to inhibition of the effect of TGF-α, which is a direct ligand of EGFR (Figure 26A). These finding suggests that EGFR activity is necessary for epithelial repair induced by TLR signaling. In order to ensure, that PGN-induced EC migration during wound closure is

dependent on EGFR activation, we analyzed the wound edges 24 h after injury. As shown in Figure 26B, EGFR kinase inhibitor effectively reduced EC velocity, suggesting that TLR-initiated EC migration is mediated via EGFR pathway.

To determine whether TLR signaling may result in EGFR activation, we analyzed phosphorylation of this receptor after stimulation of ECs with a TLR4 ligand LPS able to induce both MyD88-dependent and –independent signaling, and a TLR3 ligand Poly(I:C) signaling via exclusively MyD88-independent pathway. As shown in Figure 27A, LPS preparation used in this study induced EGFR phosphorylation at concentration effective to increase epithelial wound closure, however, Poly(I:C) did not show a similar effect.

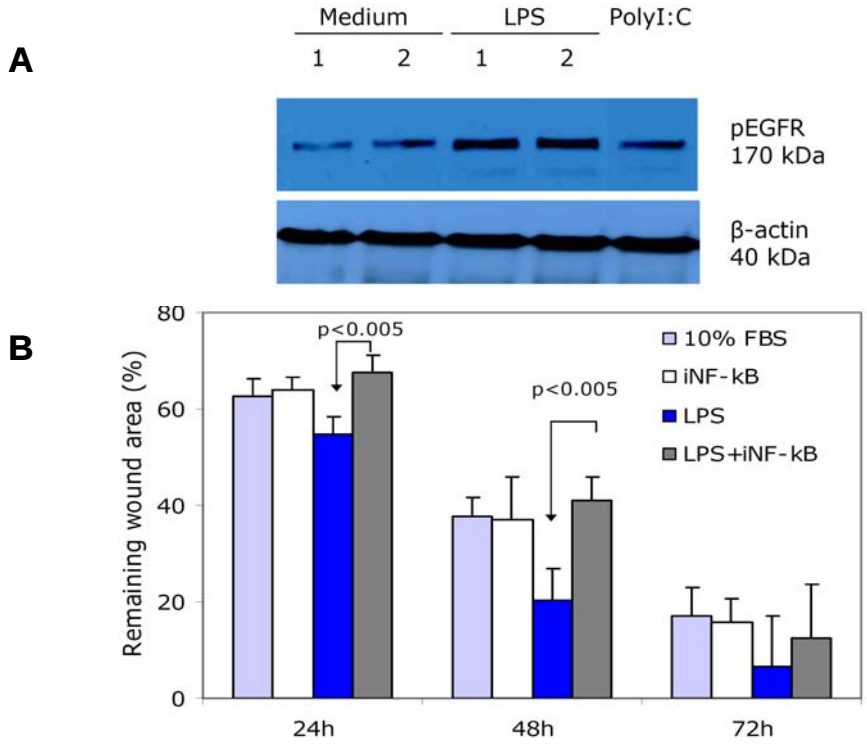


Figure 27. LPS signaling in ECs involves EGFR and NF-kB. (A) NCI-H292 cells were stimulated with LPS (*E.coli,*10 μg/ml, Sigma), or Poly(I:C) (25 μg/ml) in SFM containing 25 ng/ml LPB for 30 min; Western blot analysis for phosphorylated EGFR and beta-actin was performed; **(B)** Wounded NCI-H292 cell monolayers were stimulated with LPS (*E.coli,*10 μg/ml, Sigma) in the presence or absence of inhibitor of NF-kB activation (iNF-kB, 10 nM) in 10% FBS-containing medium; remaining wound areas were measured during 72 h after wounding; n=5; P describes statistical significance of difference between the LPS and LPS + iNF-kB groups.

Using PCR and ELISA, we determined that LPS induced MyD88-dependent (IL-6, IL-8 production; ICAM-1 and COX-2 gene transcription) as well as MyD88-independent (IP-10 and MUC1 mRNA up-regulation) responses in NCI-H292 cells (data not shown).

However, MyD88-associated signaling is likely required for the regulation of epithelial homeostatic processes, since MyD88-independent stimuli Poly(I:C) neither increased cell number (Figure 22D) nor induced phosphorylation of EGFR in airway ECs (Figure 27A). Similarly, chemokine IP-10, which is induced via TRIF-dependent MyD88-independent pathway, even decreased epithelial survival (data not shown). Since early activation of NF-kB is a hallmark of MyD88-dependent pathway (130), we tested the effect of the specific inhibitor of NF-kB activation on LPS-induced epithelial wound closure and found that this effect is blocked when NF-kB is inactivated. These data suggest that TLR-MyD88-NF-kB axis is involved in regulation of epithelial homeostasis.

4.3. Interaction between airway EC and DC *in vitro*.
Regulation of EC and DC innate immune activation by LL-37

4.3.1. Airway epithelial barrier prevents DC activation

To mimic the *in vivo* relationships between the airway epithelium and IEDCs, we developed an *in vitro* model of EC – DC interaction described in detail in Methods (Figure 7). PBECs were differentiated as ALI cultures on the semipermeable membrane (upper chamber) of the transwell culture system. When the cells have reached complete differentiation (as determined by the measuring TER), immature DCs were added to the lower chamber of the transwell system. ECs and DCs were then co-incubated during 24 h. This process was called epithelial "education" of DC, since we proposed that soluble EC-derived could modulate the steady-state phenotype and function of DCs.

After co-incubation period, we stimulated educated DC indirectly (Figure 28A) by applying live *P. aeruginosa* or various microbial factors via the apical epithelial surface. We started with this model of DC activation, because the contact of luminal microbes with apical membranes of ECs represents a prevalent mode of host-pathogen interactions in the airways. After 24 h of activation, DCs were analyzed for expression of maturation markers in comparison to similarly activated but not educated DCs. We have found that *P. aeruginosa* (Figure 28B) or flagellin (Figure 28C) did not induce activation of educated DCs when applied from the apical epithelial side, whereas they caused substantial maturation of non-educated DCs directly exposed to these stimuli. Similar results were obtained when DCs were stimulated with other microbial factors including LPS and LTA (Figure 28D). This result suggests that epithelial education plays a preventive role, either inhibiting the steady-state maturation capacity or inducing modification of DC phenotype less sensitive to microbial stimulation.

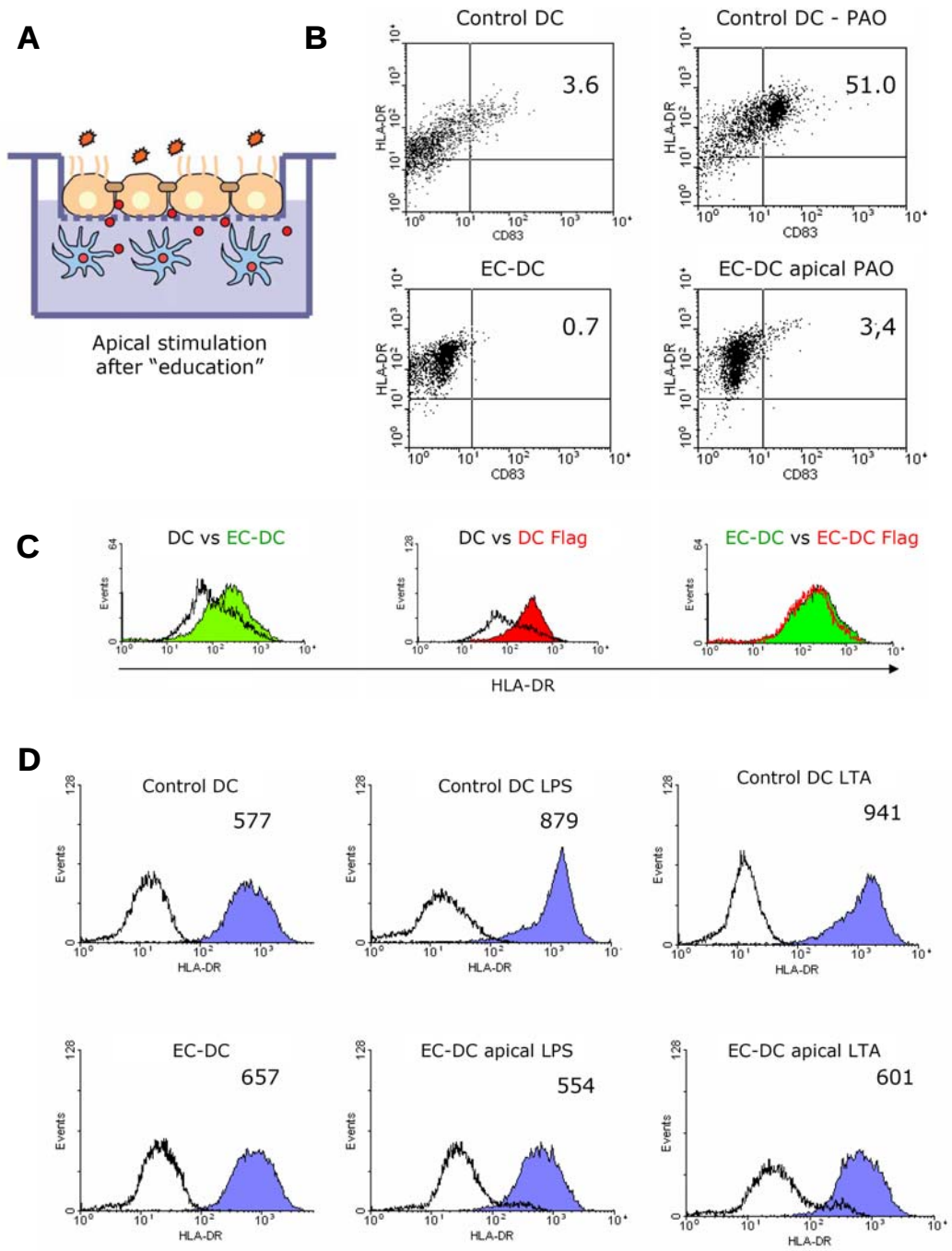


Figure 28. Airway epithelial barrier prevents DC activation. Differentiated PBECs were cultured in upper chamber and immature DCs were applied into the lower chamber (A); DC were co-incubated with ECs during 24 h ("education"); then (B) live *P. aeruginosa* (strain PAO1, 10 bacteria / 1 DC) or (C) flagellin (0.1 μg/ml) or (D) LPS (0.1 μg/ml) or LTA (2 μg/ml) were added from the apical side of ECs (as shown in A); 24 h after, DCs were evaluated for expression of CD83 (B) and HLA-DR (B-D) or their co-expression (percentages of cells co-expressing high levels of markers are indicated in B) by FACS analysis comparatively with similarly stimulated non-educated DCs (in Figure B – upper panel); DC – non-educated DC (in C - without color), EC-DC – educated DC (in C – green histogram), flagellin (in C – red); in D - white histograms – isotype control; blue histograms – experimental sample; mean fluorescence intensity is indicated.

The inhibition of background DC maturation is unlikely since incubation with ECs did not reduce but even increased HLA-DR expression compared to non-educated DCs (Figure 28C), indicating that some epithelial factors induce steady-state semi-mature phenotype of DCs. Other maturation markers did not increase after co-culture (CD83 – Figure 28B; CD80 – data not shown), arguing against possible contamination of culture medium with microbial factors that could activate DCs.

Absence of DC activation after stimulation in this model may also be explained by the barrier function of ECs that mechanically separated DCs from stimuli, providing a mechanistic reason why airway IEDCs are not persistently activated by luminal microbes. Apical application of *P. aeruginosa* stimulated PBECs to secrete inflammatory cytokines IL-6 and IL-8, and, in addition, decreased epithelial integrity (data not shown). Therefore, it is possible that DCs located in the bottom chamber were exposed to high levels of pro-inflammatory cytokines and bacteria that crossed the barrier. Taking these considerations into account, it was hard to believe that the absence of DC activation in this model is simply because of the absence of a direct contact with factors that can induce their maturation. Therefore, insufficient activation of DC in this model of interaction with ECs may be explained by both epithelial education and epithelial barrier function. To evaluate the individual input of these factors we performed further experiments.

4.3.2. Airway epithelium regulates DC activation

Next, we examined whether DCs educated by ECs differ from non-educated DCs during direct exposure to maturation stimuli. For this, we added microbial factors from the basolateral side of PBECs, enabling DCs interact with stimuli directly (Figure 29A). A feature of this model is possibility of simultaneous stimulation of basolateral epithelial TLRs known to be more sensitive to some microbial patterns, including flagellin (60). In such a model, we aimed at mimicking the *in vivo* situation of bacterial penetration of the airway epithelial barrier when both DCs and ECs are exposed to microbial factors, but there is no longer preventive impact of epithelial barrier on DC activation.

As shown in Figure 29B, direct stimulation of educated DCs with flagellin in the presence of ECs did not result in increase of their maturation. This suggests that epithelial education might represent an independent factor regulating DC activation. However, it is unclear why direct interaction with flagellin and exposure to cytokines, generated by ECs in response to this factor, were not sufficient to overcome negative regulation provided by epithelial education. One possibility is that activation of ECs by microbial patterns results in concomitant induction of (a) factor(s) with such a regulatory activity.

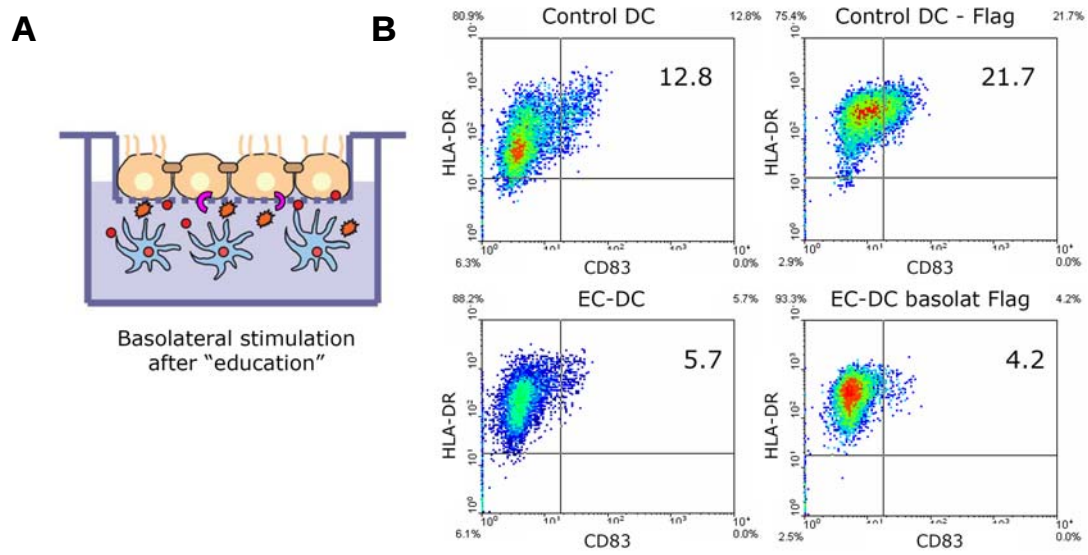


Figure 29. Airway epithelium regulates DC activation. (A) Differentiated PBECs and immature DCs were co-cultured during 24 h; then flagellin (Flag; 0.1 µg/ml) was added from the basolateral side of ECs; (B) 24 h after activation, DCs were evaluated for expression of HLA-DR and CD83 (% of cells co-expressing high levels of both markers are indicated) by FACS analysis comparatively with non-educated DC (control DC), EC-DC – (educated DC); in B - colors correspond to density of cells in corresponding areas (red>yellow>orange>green>blue).

To analyze the role of ECs during DC activation, we performed a model of direct DC activation (Figure 30).

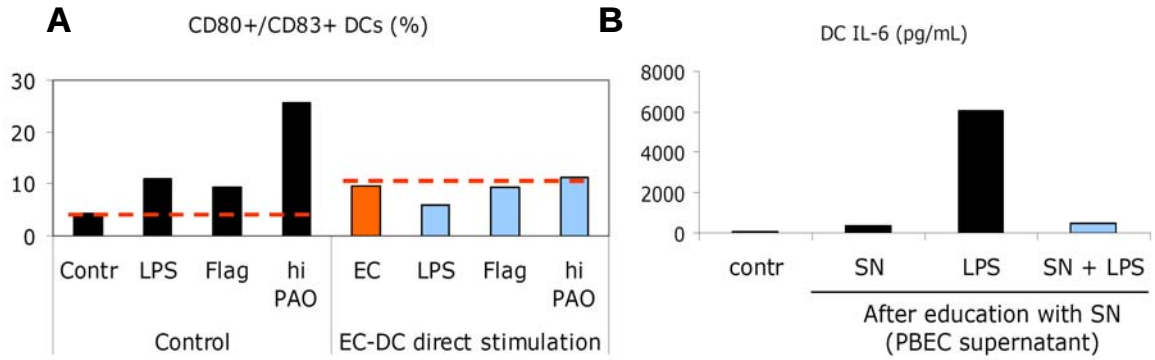


Figure 30. Epithelial education prevents DC activation. After 24 h of incubation with PBECs (A), DCs were separated from co-culture and directly stimulated with LPS, flagellin (both 0.1 µg/ml), or heat-inactivated *P. aeruginosa* (hi PAO, 1:10 dilution) for 24 h; then DCs were analyzed for expression of CD80 and CD83 by FACS (% of cells co-expressing high levels of both markers) during 24 h; EC-DC – educated DCs; alternatively, (B) DCs were stimulated for 24 h with LPS after 24 h pre-incubation with SN of unstimulated PBECs; then concentration of IL-6 was determined in DC supernatants using ELISA; the results of the representative experiments are shown.

In this model, after co-incubation with differentiated PBECs, DCs were separated from the transwell system and stimulated directly with microbial factors in the absence of ECs. We found that direct stimulation of DCs that were pre-incubated with PBECs did not result in their activation as measured by surface expression of maturation markers CD80 and CD83 (Figure 30A). This suggests that epithelial education plays a major role in prevention of DC activation by bacteria and bacteria-associated molecular patterns. The effect is likely mediated by soluble factors that are produced by ECs constitutively, because pre-incubation of DCs with supernatants of unstimulated differentiated PBECs produced the similar effect as a co-culture of DC with PBECs (Figure 30B). Although decrease of DC reactivity to microbial factors can be observed as a result of earlier exposure to epithelial microenvironment, we found that even non-educated DCs are not able to reach full maturation, when they are applied beneath PBECs, previously damaged and exposed to bacteria (model 2 depicted in Figure 7; data not shown). Taken together, ECs control DC activation by virtue of complex regulatory mechanisms.

4.3.3. DCs alter epithelial barrier function

To investigate the influence of DC on epithelial barrier function, we co-cultured DCs and differentiated PBECs as described above and measured transepithelial resistance at different time-points during the course of co-incubation (Figure 31).

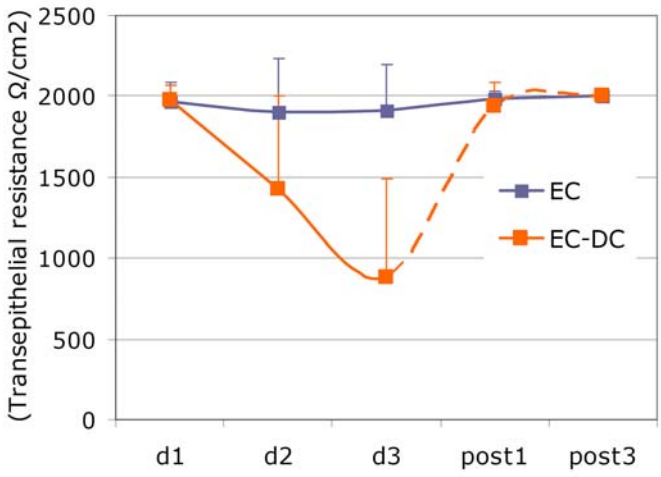


Figure 31. Effect of DCs on epithelial integrity. PBECs were co-cultured with $1x10^6$/ml DCs (orange line) during three days or cultured alone (blue line); transepithelial resistance was measured every day (in a co-culture group: during three days of co-culture (d1-d3) and during 3 days after co-culture (solid line; post1 - post3), i.e. after removal of DC from the culture system (interrupted line)); n=5.

As shown in Figure 31, the presence of DCs in the culture system dramatically decreased transepithelial resistance in co-cultured ECs in a time-dependent manner. This effect was reversible as demonstrated by complete recovery of epithelial integrity within 24 h after removal of DCs from the culture.

4.3.4. LL-37 modulates innate immune activation of ECs and DCs

Given that LL-37 is present in the airway epithelial cells (14) and also, as shown recently, in monocyte-derived DC (2), we supposed that this peptide may regulate innate immune processes in these cell types and mediate their interaction.

4.3.4.1. Effect on ECs

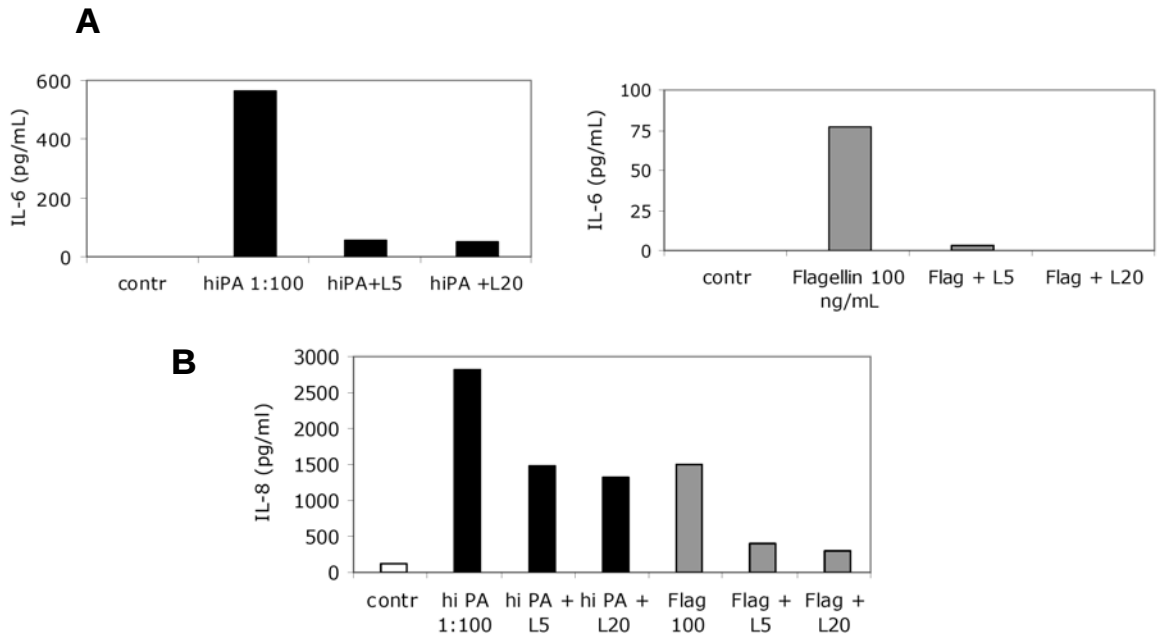


Figure 32. LL-37 regulates production of inflammatory cytokines by airway ECs in response to microbial patterns. Confluent NCI-H292 cells were exposed to heat-inactivated *P. aeruginosa* (1:100 dilution from standard stock solution) or flagellin (0.1 μg/ml); in some groups 5 μg/ml of LL-37 was added to cells prior stimulation; 24 after incubation, cell supernatants were collected and analyzed for IL-6 (A) and IL-8 (B) concentration by ELISA; the representative results of repeated experiments are shown.

As shown in Figure 32, physiologically relevant concentration of LL-37 (5 μg/ml) effectively reduced pro-inflammatory cytokine IL-6 and IL-8 production by NCI-H292 cells in response to inactivated *P. aeruginosa* and flagellin. However, we did not obtain a similar inhibition in PBECs and other EC lines like 16HBE cells differentiated as ALI

cultures (data not shown). This correlated with low level of CD14 mRNA in these cells compared to NCI-H292 cells which highly expressed this transcript as detected by real-time PCR (Figure 33).

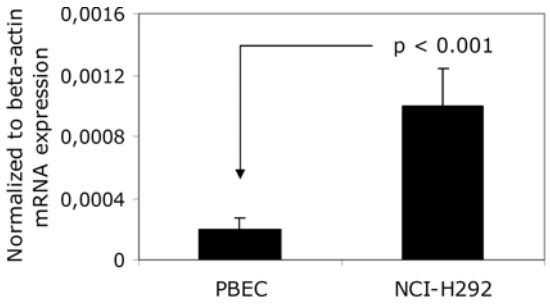


Figure 33. Expression of CD14 mRNA in airway ECs. Real-time PCR analysis of PBECs and NCI-H292 cells for expression of CD14 mRNA was performed; n=3.

Since flagellin is a microbial pattern associated with *P. aeruginosa*, inhibition of EC responses to these factors by LL-37 could be related to a similar mechanism, which may be CD14-dependent (data not shown). Increased expression of CD14 by the epithelial cancer cell line might, therefore, explain its higher responsiveness to LPS.

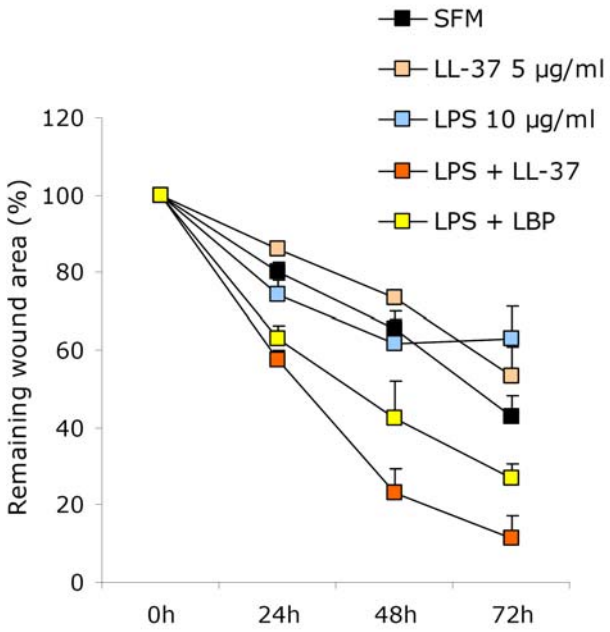


Figure 34. LL-37 and LPS synergistically increase epithelial wound closure of NCI-H292 cells under serum-free conditions. Confluent NCI-H292 cell monolayers were mechanically damaged and then exposed to LPS, LL-37, LPS + LL-37, or LPS + LBP (25 ng/ml), or serum-free medium (SFM) alone; remaining wound area was measured every day after wounding; n=5; the representative results of repeated experiments are shown.

It is well established that LL-37 is able to interact with LPS electrostatically and neutralize it (103). However, nothing is known about the modulation of LPS responses by LL-37 in ECs. We have shown that LL-37 stimulates airway epithelial wound closure and that microbial factors including LPS are involved in regulation of epithelial growth and repair processes. Therefore, it was interesting to investigate whether LL-37 regulates epithelial repair responses induced by LPS. We have found that LPS and LL-37 alone did not induce NCI-H292 wound closure under serum-free condition, however after addition of LL-37 to the serum-free medium, ECs responded to LPS by increased wound closure (Figure 34). Notably, this effect was similar to addition of LBP to the medium.

To our knowledge, this is first observation of facilitation of an LPS effect by LL-37, since this peptide is known to bind and neutralize endotoxin, as mentioned above. Then we analyzed whether inflammatory response to LPS is regulated by LL-37 in a similar manner. As expected, LL-37 decreased LPS-induced IL-6 production in the presence of serum (Figure 35). However, under serum-free conditions, release of the cytokine by NCI-H292 cells was substantially increased when LL-37 was applied together with LPS, consistently with the wound closure data. These results suggest that interaction between LL-37 and LPS may be more complicated than simple neutralization of LPS by LL-37 depending on the presence of serum.

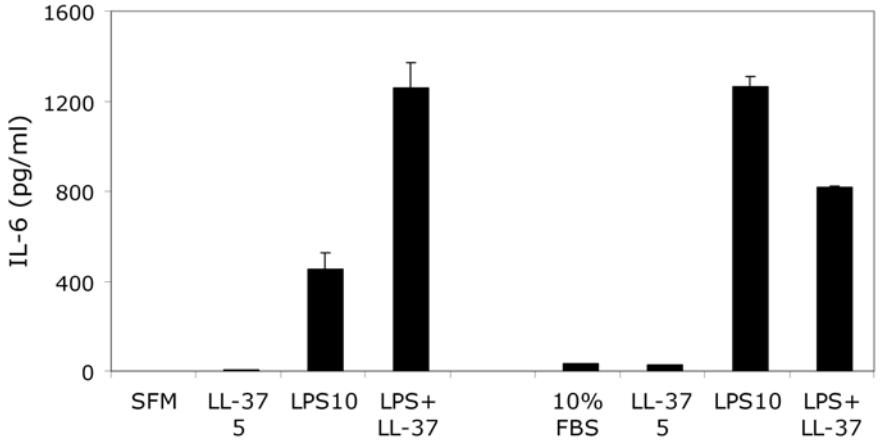


Figure 35. Regulation of airway epithelial inflammatory response to LPS by LL-37. NCI-H292 cells were stimulated with LL-37 (5 μg/ml), LPS (10 μg/ml), LL-37 + LPS; in 24 h, cell supernatants were collected and IL-6 concentrations were determined by ELISA.

4.3.4.2. Effect on DCs

Since LPS, a TLR4 ligand, is one of the maturation factors for DC, and LL-37 is able to regulate LPS responses, we were interested to analyze the effect of LL-37 on DC

maturation induced by LPS. Because in our experiments on CD14-expressing ECs LL-37 reduced the flagellin responses, we also included this stimulus in this assay.

As expected, LL-37 effectively decreased DC activation by LPS, as measured by expression of surface maturation markers CD80, CD83 (Figure 37A) and HLA-DR (data not shown), when LL-37 was applied 5-10 min before addition of LPS. Notably, LL-37 also prevented DC activation by flagellin (Figures 37A and 37B). This effect was not dose-dependent, since 5 µg/ml (Figure 37B) and 20 µg/ml (Figure 37A) of LL-37 were equally effective. LL-37 increased survival of DCs in a WST-1 assay (data not shown) indicating that inhibition of DC activation was not because of the cytotoxic effect.

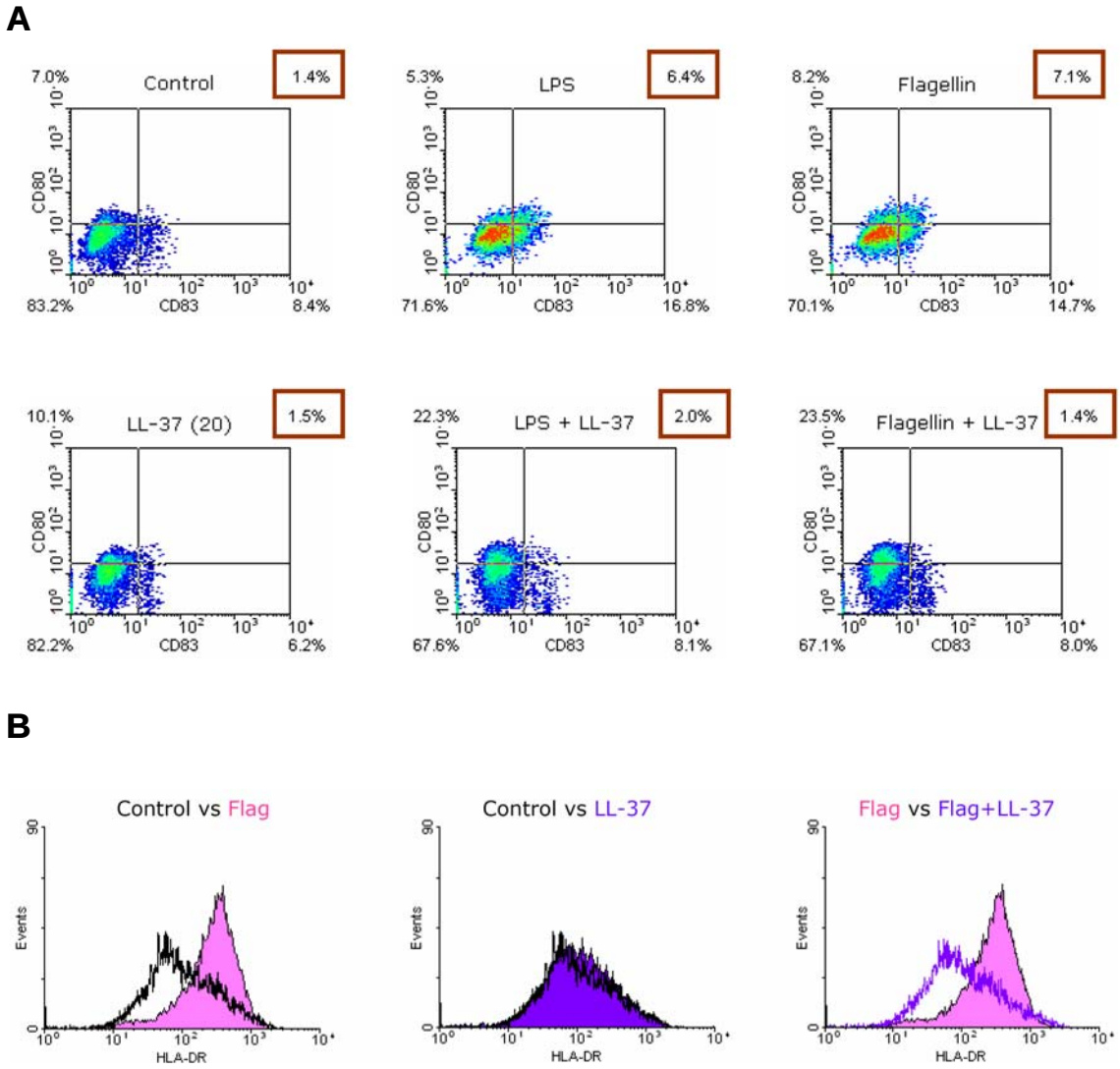
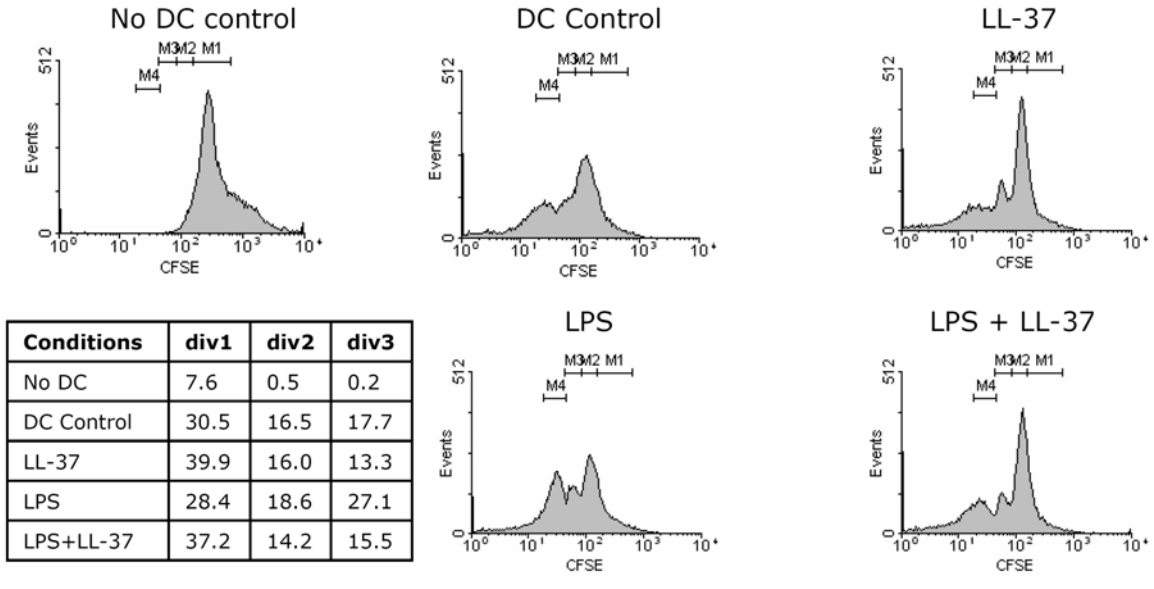


Figure 37. Effect of LL-37 on activation of DC by TLR ligands. Monocyte-derived DC were incubated with LPS (10 ng/ml) or flagellin (Flag; 100 ng/ml) in the presence or absence of LL-37 (A - 20 µg/ml; B – 5 µg/ml) in 10% FBS-containing medium; 24 h after incubation, the cells were analyzed for the expression of maturation markers (A) CD80 and CD83; and (B) HLA-DR by FACS; representative density plots (A) and histograms (B) are shown.

Maturation of DCs is necessary for T cell priming. Therefore, we asked whether LL-37-prevented DC maturation will result in a decreased induction of naïve T cell proliferation. To answer this question, we analyzed naïve CD4+ T cell proliferation in an allogeneic mixed lymphocyte reaction with differently stimulated (with and without LPS, with and without LL-37) DCs using CFSE assay, as described in Methods.



Conditions	div1	div2	div3
No DC	7.6	0.5	0.2
DC Control	30.5	16.5	17.7
LL-37	39.9	16.0	13.3
LPS	28.4	18.6	27.1
LPS+LL-37	37.2	14.2	15.5

Figure 38. LL-37 inhibits the LPS-increased ability of DCs to induce naïve CD4+ T-cell proliferation. DCs were cultured during 24 h with or without LPS (100 ng/ml) and with or without LL-37 (5 μg/ml). The CFSE-labeled allogeneic naïve CD4+ T-cells (1 x 10^6) isolated by negative magnetic sorting were co-cultured with differently treated DCs (1 x 10^5) or cultured without DCs (negative control) in the presence of 1 μg/ml anti-CD3 monoclonal antibody. After 5 days, the cells were harvested and assessed for their CFSE dilution by FACS analysis. The percentages of the daughter T cells under each CFSE peak, corresponding to the number of divisions (M1 – division 0, M2 – division 1, M3 – division 2, M4 – division 3), have been determined for each group and are presented in the inserted table.

As expected, five-day co-culture of naïve CD4+ T cells with LPS-stimulated DC resulted in a significant increase of the numbers of cells undergoing more than two divisions (Figure 38). Pre-treatment of DCs with LL-37 before stimulation with LPS blocked their ability to induce naïve CD4+ T-cell proliferation, returning the percentages of the cells undergoing more than one division to the control level. Therefore, incubation of DCs with LL-37 before or during stimulation with LPS decreases the capacity of DCs to induce naïve CD4+ T cell proliferation.

5. DISCUSSION

In the present study using human airway epithelium as a model we have identified several links between innate immunity and epithelial homeostasis, such as (a) regulation of epithelial repair and innate immune processes by the endogenous AMP LL-37; (b) involvement of innate immune signaling in regulation of epithelial growth and repair; and (c) bidirectional interaction between airway ECs and DCs. This information helps to understand host defense as a network of mechanisms regulating immunity and tissue homeostasis. Although these mechanisms are distinct in their nature and primary functions, they do extensively cooperate upon tissue injury and microbial challenge.

Airway epithelium represents a physiological tissue barrier with two sides and two functions. First, it protects the respiratory compartment of the lung from pathogens by mechanical and chemical defense factors such as mucus, antimicrobial substances, or beating of cilia. Second, intact airway epithelium protects from developing inflammation in response to microbes present in the airway lumen primarily by preventing the contact of bacteria with subepithelial inflammatory cells (macrophages and DCs) which are very sensitive to any kind of danger. Thus, the function of the airway epithelial barrier, as hypothesized and investigated in this work, is to protect from pathogens without development of inflammation. Accordingly, we found that ECs possess an autonomous program to maintain the integrity of barrier during damage, induced by microbes.

5.1. Involvement of an antimicrobial factor in epithelial homeostasis

Consistently with the concept stated above, we have found that human cathelicidin antimicrobial peptide LL-37, known to be expressed in host defense cells including airway ECs and to exert a potent antimicrobial activity against many respiratory pathogens (14), is able to stimulate repair of the damaged airway epithelium. At physiologically relevant concentration, LL-37 increased airway epithelial wound closure, EC migration and proliferation. In normal human bronchial epithelium, the peptide induced effect only when the cells were fully differentiated.

The biological functions of LL-37 have been extensively studied during the last decade. As result, many non-antimicrobial activities have been identified. The peptide was implicated in chemotaxis of inflammatory and immune cells including neutrophils, monocytes, lymphocytes (40), eosinophils (175) and mast cells (126). LL-37 has also been shown to regulate tissue homeostasis: it stimulated regeneration of skin wounds (73), induced angiogenesis (100), increased growth of breast (72) and lung (von Haussen J and Bals R, submitted) cancer cells.

Of note, receptor-mediated mechanism, involving FPRL1 (40,100,123) and/or P2X7 (45), has been identified for some of non-antimicrobial effects of LL-37, suggesting that the mode(s) of targeting of microbial and host cells by the peptide may be principally different. Although it remains uncertain whether any specific receptor interacts with the peptide to initiate repair-associated signaling, the results of our study do not indicate that this is not possible. Stimulation of epithelial wound closure by LL-37 was dependent on the peptide structure, since scrambled and D-form versions of LL-37 did not induce the similar effect. However, our experiments can not support the involvement of FPRL1 and P2X7 in airway epithelial wound repair stimulated by LL-37.

The effect of LL-37 on epithelial wound closure and proliferation was dependent on the activity of EGFR tyrosine kinase and MEK. Interestingly, transactivation of EGFR with subsequent activation of downstream MEK has been previously implicated in LL-37-induced release of pro-inflammatory cytokine IL-8 from airway ECs (174). In a more recent study, such a mechanism was demonstrated for increased keratinocyte migration by LL-37 (176). However, the upstream signaling events leading to EGFR activation remain elusive. One possibility could be that electrostatic interaction of this cationic peptide with negatively charged components of cell membranes results in a receptor-independent activation of membrane-associated signaling cascades. Indeed, EGFR has been shown to localize in membrane-associated lipid raft microdomains (115). A precise mode of interaction of the peptide with cell membranes and its biological consequences represent an intriguing issue for future studies.

EC migration and proliferation, both dependent on similar signaling mechanism involving EGFR and MEK, are affected by LL-37 differently. Consistently, wound closure was enhanced predominantly via induction of cell migration, as determined by increased lamellipodia formation and no significant change in numbers of proliferating cells at the wound edges of injured epithelial monolayers after treatment with LL-37. This observation is in agreement with other studies, which demonstrated that airway epithelial wound closure depends mostly on intensity of cell migration into the wound area (48,194). The effect of LL-37 on wound closure was observed exclusively under serum-containing conditions and could not be enhanced by increase of the concentration of the peptide (> 5 μg/ml). By contrast, EC proliferation was amplified by LL-37 in the absence of serum in a dose-dependent manner. Therefore, the quality of the effect *in vivo* may be determined by the particular tissue context regulating amount of the released peptide.

Interestingly, high concentrations of LL-37 (> 10 μg/ml) were toxic for airway ECs. Such concentrations of the peptide might be generated during inflammatory conditions that are characterized by recruitment of neutrophils into the mucosa. Neutrophils are an important source of various antimicrobial substances, including cathelicidins, and, when accumulated in tissue, they are able to release them in relatively high amounts (48,197). Indeed, increased levels of LL-37 in tissue fluids have been detected in patients with inflammatory lung diseases, like pneumonia (48,147) and cystic fibrosis (32). In such pathological settings, LL-37 may cause damage to epithelium instead of protective effect induced by low concentrations of the peptide. Persistent release of increased amounts of LL-37 may potentially cause prolonged EGFR activation. In the light of recent data, implicating EGFR activation in epithelial repair and airway remodeling in asthma (138), LL-37 can be considered to play a role in the pathogenesis of this disease. Therefore, the roles of LL-37 in tissue homeostasis in health and disease are likely very different.

5.2. Innate immune recognition is coupled to epithelial homeostasis

Another important finding of the present study is regulation of airway epithelial homeostasis by microbial factors. As extensively discussed in Introduction, tissue injury is often caused by infection. Airway epithelium is continuously exposed to a plenty of different microbes and other environmental factors, and, therefore, epithelial damage is not a rare event. On the other hand, ECs possess an intrinsic compensatory mechanism allowing repair of structural defects within relatively short time (48). It happens due to the temporal overexpression and activation of various signaling pathways necessary for cell migration and proliferation, the components of tissue repair. In epithelial tissue, these pathways include EGFR and the autocrine membrane-associated ligands, IGFR, KGFR and other factors as discussed above.

Pathogens possess a capacity to cause damage to host tissue barriers (110) and, in particular, to the epithelial barrier (167). Because many pathogen-associated molecular patterns can be recognized by ECs (79), including airway ECs (63,119), we proposed that recruitment of signaling networks necessary for initiation of tissue repair process may be a consequence of such recognition. We have found that a TLR4 ligand LPS, a TLR9 ligand CpG, and molecular factors known to signal via TLR2 (*S. aureus*, PGN, LTA, and synthetic lipopeptides) are able to induce various “nonimmune” responses in airway ECs, including stimulation of wound closure, EC migration, proliferation and survival. Both normal airway ECs and cancer cells responded to these factors directly, i.e. independently on the presence of inflammatory cells of haemotopoietic origin in the system.

To the best of our knowledge, this is the first experimental evidence for a direct monocyte/macrophage-independent induction of epithelial growth and repair by TLR ligands. According to the prevalent opinion, ECs require help from myeloid cells in form of cytokines in order to respond to TLR ligands (63,139). In our study, airway ECs did not need that help. Moreover, neutralization of inflammatory cytokines IL-1β and TNF-α, which can potentially serve as TLR-inducible autocrine mediators of EC migration and proliferation (10,63), did not inhibit responses initiated by TLR agonists. It suggests that some pathway(s) involved in epithelial homeostasis can be directly recruited after innate immune recognition by ECs.

We determined the involvement of EGFR in TLR-initiated epithelial repair by complete blockade of PGN-induced wound closure in the presence of specific inhibitor of EGFR tyrosine kinase AG1478. In addition, LPS induced phosphorylation of EGFR in NCI-H292 cells. As discussed above, EGFR is known to play a central role in EC migration, survival and proliferation. In the lungs, EGFR activation is directly linked to cancer development (133). Although the molecular mechanism linking TLR and EGFR signaling was not a focus of our study, existence of such a link represents a novel phenomenon and intriguing issue for future investigations.

Notably, the effect on epithelial homeostatic processes was surprisingly restricted to molecular patterns signaling via MyD88-dependent pathway. All TLRs suspected to be involved in observed responses (TLR1-2-6 in response to PGN, LTA, lipopeptides, and *S. aureus*; TLR4 in response to LPS; TLR9 in response to CpG ODN), and IL-1R (in response to IL-1β) utilize the MyD88 adaptor protein for their downstream signaling (3). By contrast, TLR3 ligand Poly(I:C) known to signal via exclusively MyD88-independent pathway, and IP-10, a chemokine induced by TRIF-mediated MyD88-independent signaling, did not induce but even suppressed epithelial growth and survival. Moreover, all stimuli that increased epithelial growth or repair in our study are known to be dependent on the early activation of NF-κB transcription factor during their intracellular signaling (130). Consistently, epithelial wound closure stimulated by LPS was dependent on NF-κB, since specific inhibitor of activation of this transcription factor completely blocked the effect of LPS. Therefore, the TLR-MyD88-NF-κB axis is likely involved in the regulation of epithelial homeostatic processes.

That is in contrast to a very recent study of Chien et al. showing that TLR3-associated MyD88-independent downstream signaling in epithelial cancer cells promotes their survival via RalB GTPase-mediated activation of the IκB family kinase TBK1 (33).

However, the authors did not examine whether direct stimulation of TLR3 in cancer ECs results in a similar outcome. Thus, compared to our work, this study does not provide a direct evidence for the role of epithelial TLRs in regulation of EC behavior. Secondly, the authors did not find a similar effect in primary nonmalignant ECs. In our work, TLR2 ligands increased survival of normal PBECs. Interestingly, the same cells were used. Indeed, as stated before, we also found that TLR3 signaling does not induce but even decrease EC survival. Therefore, the aberrant response in cancer cell lines observed by Chien et al. may probably be explained by specific features of cancer cells that, as also found in our study, may be very different from normal cells in terms of sensitivity to TLR stimulation. Despite some disagreements with our data, the study of Chien et al. provides an important independent evidence for involvement of epithelial innate immune signaling in epithelial homeostasis, hypothesized in our work.

An interesting question is which factors may determine the higher responsiveness of cancer ECs to TLR stimulation. We have found that NCI-H292 carcinoma cells, which displayed a robust response to TLR agonists, express significantly higher levels of CD14 mRNA as compared to the normal airway ECs. Membrane-associated CD14 is implicated in recognition of LPS and several TLR2 ligands (22), and, therefore, overexpression of this receptor may explain the higher sensitivity of these cancer cells to microbial factors tested in our study. Another possible explanation is increased expression of EGFR in lung epithelial cancer cells (133) and in NCI-H292 cells (173), in particular. It was discussed above, that EGFR was found to play a role in epithelial repair induced by TLR agonists. Taking into account these suggestions, introduction of TLR agonists as adjuvants into cancer vaccines, currently considered as a promising approach to increase anti-tumor immunity, needs to be critically re-evaluated.

Notably, relatively high concentrations of TLR ligands tested were necessary to induce the observed effects. ECs are known to be less sensitive to TLR stimulation (79), and previous studies have also detected response of airway ECs using high concentration of TLR2 ligands (105). It is very possible that such concentrations of microbial products are present in the airways during massive infection associated with epithelial damage.

Regulation of epithelial responses by LPS represents another interesting issue. It is widely appreciated, that ECs are generally less sensitive to endotoxin than inflammatory cells (79) because the molecules necessary for LPS recognition are expressed at lower levels (CD14 and MD2 (89)) or unusually located (e.g. presence of TLR4 inside the cell instead of classical cell surface expression (65)). In line with this, macrophages has been

shown to be necessary for lung inflammatory responses to endotoxin *in vivo* (99). However, others were able to demonstrate direct activation of airway ECs by LPS *in vitro* (125) and *in vivo* (161). In one recent study, stimulation of NCI-H292 cells with LPS resulted in increased expression of MUC5AC by transactivation of EGFR (155). In our study, LPS stimulated wound closure and phosphorylation of EGFR in airway ECs.

Interestingly, LL-37 that is known to bind LPS and neutralize its sepsis-related biological activities (35,103,122), unexpectedly substituted LBP, enabling LPS to induce wound closure in the absence of serum. Notably, LL-37 or LPS alone did not increase wound closure under the serum-free conditions. Moreover, addition of LL-37 to serum-free medium substantially enhanced LPS-induced secretion of IL-6 from airway ECs. It should be stressed that our early experiments have been done with Sigma preparations of LPS. Although our subsequent studies have determined that ultra pure preparations of LPS provided by other companies are less effective in induction of epithelial responses, LL-37 did not show synergistic relationships with TLR2 ligands suspected to contaminate LPS provided by Sigma. Therefore, we believe that this kind of co-operation between LL-37 and LPS found in wound closure and IL-6 release assays does really exist. Theoretically, LL-37 may increase LPS responses in serum-free conditions by several mechanisms. First, as a membrane-active peptide, it may deliver LPS into EC, where intracellular TLR4 is present (65). Indeed, it has been demonstrated that cationic LL-37 targets extracellular DNA plasmid to the nuclear compartment of mammalian cells via lipid rafts and proteoglycan-dependent endocytosis (146). A similar mechanism may also be utilized in case with LPS, which contains anionic lipid A and interacts with LL-37 electrostatically. Second, LL-37 can interact with CD14 (121), which is involved in LPS internalization (96). These mechanisms are currently under investigation.

Taken together, we can conclude that (1) TLR ligands can in principle be directly recognized by airway ECs; (2) TLR-MyD88-NF-κB pathway is involved in autonomous regulation of epithelial repair, growth and survival; (3) recognition of danger-associated molecules by ECs recruits cellular pathways necessary for tissue repair, including EGFR; (4) homeostatic responses to microbial factors can be modulated by endogenous defense molecules, like antimicrobial peptide LL-37; (4) epithelial cancer cells are particularly sensitive to certain TLR stimuli, and can respond to them with increased growth and survival. Innate immune mechanisms are linked to tissue homeostasis. The physiological sense of such a link may be simultaneous and coordinated protection from pathogens, prevention of tissue damage and regulation of inflammation at the epithelial level.

5.3. Control of immune activation by epithelial factors

The concept of co-operative relationships between epithelial homeostasis and innate immunity, developed in our work, premises that not only immune factors are involved in epithelial physiology, but also epithelial factors can influence immune processes. The latter issue was investigated in our studies of EC – DC interactions.

The role of DCs in mucosal immune system was a major research focus during the past decade. Being localized in peripheral tissues, mucosal DCs represent sentinel cell type able to sense Ags, capture and process them, migrate to regional lymph nodes in order to present these Ags to specific T cells, thereby priming their clonal expansion and effector function (17). Recent evidence suggests that local factors present within tissue microenvironment may substantially modify DC function. Given that ECs are the closest neighbors of mucosal DCs, influence of epithelial factors on DCs seems to play a special role. According to the most prevalent opinion, ECs tend to keep DCs in an immature (143) or semi-mature (84) state and promote their Th2-priming capacity (22,145). Although the existence of DCs within airway epithelium is now firmly established (78), the character of interaction between DCs and airway ECs under steady-state and following exposure to pathogens.

Using several *in vitro* models of interaction between polarized human bronchial epithelium and monocyte-derived DCs we have found that: (1) airway epithelial barrier prevents activation of subepithelial DCs by bacteria and bacterial products; (2) "education" of DCs with epithelial factors supported their semi-mature phenotype and reduced their responsiveness to maturation factors; (3) subepithelial DCs compromise epithelial barrier function.

The first observation derives from the studies using a co-culture model, in which DCs were first pre-incubated with ECs to generate an *"in vitro"* equivalent of airway IEDC and then live *P. aeruginosa* or microbial products, like flagellin, LPS or LTA, were applied from the apical surface of the ECs. This model is very similar to those developed recently by Rimoldi et al. (143) and Butter et al. (30) with one important exception, namely, that the semipermeable membrane of the transwell co-culture system used in our experiments had a very small pore size (0.4 μm). Therefore, DCs were not allowed to migrate through the membrane between the chambers and only soluble factors produced by the cells were able to diffuse. Using this model, which makes a direct interaction between DCs and maturation stimuli unlikely, educated (pre-incubated with epithelial factors) DCs could not be activated.

There are two possible explanations for this observation: (1) desensitization of DCs to stimulation as result of epithelial education; or (2) absence of a direct contact with stimuli, that is probably necessary for appropriate DC maturation. In order to discriminate between these possibilities, we developed a second co-culture model, in which educated DCs were directly exposed to maturation stimuli in the presence or absence of ECs. This model mimics *in vivo* situation, when bacteria invades and penetrates epithelial barrier and then can be recognized by tissue-resident (i.e. educated) Ag-presenting cells. We found that following epithelial education, DCs were not able to mature even when the direct contact with stimuli was achieved. The similar effect was observed when ECs were not present during the activation process, indicating that the major regulatory role of ECs occurs during the education process. This observation is in agreement with study of Rimoldi et al., in which the authors demonstrated that pathogenic bacteria *Salmonella typhimurium* could not induce maturation of DCs educated by intestinal ECs (143). The factors responsible for modulation of DC responsiveness are rather soluble and likely released by ECs constitutively, because pre-incubation of DCs with supernatants collected from unstimulated PBECs resulted in a similar outcome.

A number of factors produced by ECs are supposed to modulate DC activation. For example, in the mentioned above work of Rimoldi and coauthors, TSLP produced by colon ECs was found to be responsible for regulation of DC maturation (143). In the lung epithelium, GM-CSF (50) and PGE2 (91), constitutively produced by airway ECs, might be very possible candidates. Given that production of these mediators is increased in allergic airway diseases, including asthma and allergic rhinitis, they may promote Th2-priming capacity of lung mucosal DCs (21,50). An interesting unresolved question is why inflammatory cytokines produced by ECs after stimulation with pathogenic bacteria and bacteria themselves are not effective to overcome the steady-state inhibitory effect of epithelial environment. It would be interesting to know whether recognition of microbes by ECs induces production of regulatory factors that control the amplitude of mucosal inflammatory responses that in healthy tissue should likely be minimal. These factors might be also enough to control activation of non-educated DCs, as demonstrated in one of our experiments.

Interestingly, epithelial education resulted in unusual steady-state DC phenotype. In repeated experiments, surface expression of maturation marker CD83 was found to be decreased, but HLA-DR was upregulated. Similar observation has been made by Jahnsen et al., who also observed selective HLA-DR upregulation in airway IEDCs (50,84). In

their study, DCs were able to achieve full phenotypic maturation only after leaving the epithelium, namely, following migration to the lymph node and, especially, upon cognate interaction with Ag-specific T cells (84). Therefore, the epithelial environment is likely not favorable to DC maturation and controls DC activation by virtue of complex regulatory mechanisms.

However, the results of our experiments should not be overinterpreted. There are some technical limitations of the models used in our experiments. Since we focused mainly on the effect of soluble epithelial factors on DCs, the possibility of a direct cell-to-cell interaction during education process was excluded by selecting a transwell membrane with a small pore size. Secondly, we did not include in our co-culture experiments other mucosal cells, like mast cells and fibroblasts that can also function as sentinels of pathogens and probably modify some DC functions. Indeed, in one ongoing study, we observed that direct co-culture of DC with fibroblasts modulated sensitization of DCs to some TLR ligands (R. Shaykhiev, unpublished data). However, in the present work we focused only on interaction between ECs and DCs.

Finally, we have found that interaction between airway ECs and DC is complex and bidirectional. Subepithelially located DCs dramatically decreased epithelial barrier function resulting in an increased epithelial permeability. This observation might have a remarkable clinical relevance. It is well established that airway mucosa in asthma is characterized by elevated DC number (83) and dysregulation of epithelial barrier (77). These changes are associated with severity of allergic airway inflammation and increased susceptibility to infection. Different factors may cause this effect. Penetration of airway epithelial layers by DC is possible, as demonstrated in patients with allergic rhinitis (172) and in *in vivo* model of airway infection (84). However, according to the current opinion, penetration of epithelium by dendritic processes is mediated by temporal formation of tight junctions with ECs (142) and, therefore, does not impair epithelial barrier function. Alternatively, soluble factors released by DCs may be responsible for this effect. Whatever the exact mechanism, our *in vitro* studies provide for the first time mechanistic link between DCs and integrity of airway epithelial barrier.

Taken together, EC-DC interaction studies indicate that ECs are able to control DC activation by prevention of a direct contact with bacteria and / or due to the regulatory properties of soluble factors released by ECs. DCs “educated” within airway epithelial microenvironment are less sensitive to stimulation with microbial factors. On the other hand, accumulation of DCs beneath ECs may impair airway epithelial barrier function.

Based on these considerations and taking into account that LL-37 is present in the airway ECs (14) and also in monocyte-derived DC (2), we supposed that this peptide may be involved in regulation of innate immune activation of these cell types and mediate their interaction. The influence of LL-37 on various host defense cells, including DCs (39) and ECs (174), has recently been investigated by different groups. However, the most of these studies were performed using LL-37 at relatively high concentrations (≥ 10 μg/ml), which probably do not represent physiological situation and, moreover, may play a detrimental role during mucosal inflammation. We tested the effects of relatively low concentrations of LL-37 and found that the peptide at 5 μg/ml modulated innate immune activation of airway ECs and DCs by TLR ligands.

In airway ECs, LL-37 decreased release of cytokines IL-6 and IL-8 in response to flagellin and inactivated *P. aeruginosa*, suggesting that, at physiological concentrations, the peptide is able to regulate epithelial inflammatory responses independently on its antimicrobial activity. Although it is known that LL-37 can reduce responses to LPS (116,121), interaction between LL-37 and flagellin has not been addressed previously. In DCs, as expected, application of LL-37 inhibited expression of maturation markers in response to LPS and, consequently, decreased naïve CD4+ T cell proliferation in the allogeneic mixed lymphocyte reaction. Of note, similar to its effect on ECs, LL-37 down-regulated maturation of DC by flagellin. That is in contrast to already mentioned studies, in which high concentrations of LL-37 caused release of IL-8 from airway ECs (174) or activated some DC functions (18,39). At low concentrations, tested in our studies, LL-37 was not able to induce inflammatory responses in host defense cells, but rather controlled them.

Therefore, it seems likely that constitutive release of the peptide by ECs contributes to airway epithelial barrier function by following mechanisms: (1) inactivation of pathogenic microorganisms; (2) control of inflammatory response to microbes and their products; and (3) regulation of tissue repair in an autocrine manner. In addition, LL-37 might represent a regulator of airway EC – DC crosstalk.

5.4. General conclusion

Taken together, these findings let us to conclude that epithelial homeostasis and mucosal innate immunity are closely connected, and their co-operative regulation might play a key role in the maintenance of tissue integrity and control of immune balance (Figure 39).

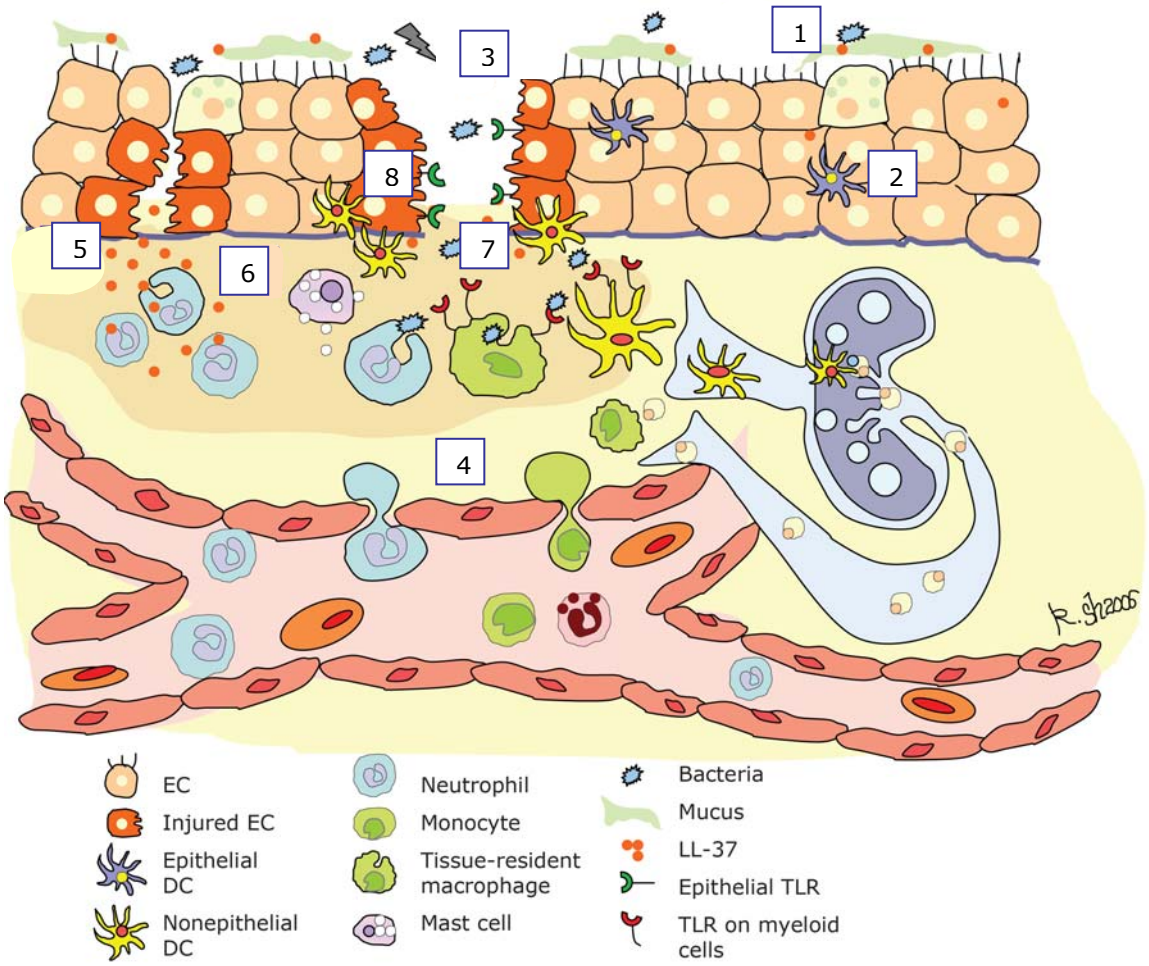


Figure 39. A model of co-operative regulation of innate immunity and epithelial homeostasis. Defense mechanisms that control microbial pathogenesis and tissue integrity in healthy (1-2) and damaged (3-7) airway mucosa differ significantly. Constitutive epithelial factors exert protective effects. Among them, antimicrobial peptide LL-37, which is released by intact airway ECs at low concetrations, binds and inactivates microbes, maintains epithelial integrity and prevents inflammatory responses of ECs and DCs to microbial factors (1); healthy ECs keep epithelial DCs in a state less capable to be activated (2). Upon damage (3), injured ECs provide signals to recruit and activate inflammatory cells (4); recruited neutrophils release high levels of cytotoxic mediators, including LL-37 that at such high concentrations further increases epithelial damage (5) and amplifies inflammatory response by acting on neutrophils, monocytes, mast cells and DCs (6). Secondary tissue damage (5) may also be caused by DCs accumulated beneath the epithelium (7). Epithelial TLRs initiate epithelial repair and growth processes after recognition of microbe-associated molecular patterns (8).

Further studies are undoubtedly necessary to reveal physiological relevance of these data in order to translate the knowledge about "epithelial-immune" co-operation into clinically meaningful information. This might be important for further progress in the

development of novel therapeutic approaches to chronic diseases (asthma, interstitial lung diseases, inflammatory bowel disease, cancer and others), which are associated with concomitant immune dysregulation and epithelial tissue injury.

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CURRICULUM VITAE

Renat Rashidovich Shaykhiev

Date of Birth: October 19, 1977

Place of Birth: Kazan, Tatarstan, Russia

Education

1984 – 1989 – School No 74, Kazan, Russia

1989 – 1992 – School No 16, Nukus, Uzbekistan

1992 – 1994 – School No 50, Kazan, Russia (gold medal)

1994 – 2000 – Kazan State Medical University (honours, best graduate)

2000 – 2002 – Residency in allergy and immunology, Kazan State Medical University

Work

1995 – 2000 – founder and leader, New Biological Club (NB Club), Kazan

2000 – 2002 – resident physician

2002 – 2003 – consultant physician, Immunology, Kazan Nongovernmental Clinic

2003 – present – Dr. med. program, AG PD Dr. Dr. Bals, University of Marburg

Memberships and awards

2001 – present – JMA European Academy of Allergy and Clinical Immunology

since 2006 – Inflammation Research Association

since 2006 – Society for Leukocyte Biology

2005 – Speaker and grant; North American Cystic Fibrosis Conference (Baltimore)

2005 – EAACI Travel grant (Rotterdam)

2001 – FOCIS travel grant (Boston)

2001 – Pavlov Scholarship, Honour Pin (Bronze), Int Academy of Science/ICSD

2000 – World Allergy Organization Travel Award (Sydney)

2000 – Best Graduate of the Kazan State Medical University

1998 – 2000 – Scholarship of the President and Government of Russia

1998 and 1999 – Scholarship of the Vernadsky Foundation (Moscow)

1998 and 1999 – Khabibullin Prize of the Tatarstan Academy of Sciences (Kazan)

1997 – Young Investigator Award of the Major of Kazan

Publications

- Shaykhiev R, Beisswenger C, Kandler K, Senske J, Puchner A, Damm T, Behr J, Bals R. Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. Am J Physiol Lung Cell Mol Physiol. 2005; 289(5):L842-8.
- Kandler K, Shaykhiev R, Kleemann P, Klescz F, Lohoff M, Vogelmeier C, Bals R. The anti-microbial peptide LL-37 inhibits the activation of dendritic cells by TLR ligands. Int Immunol. 2006 Oct 13; [Epub ahead of print].
- von Haussen J, Koczulla R, Shaykhiev R, Herr C, Pinkenburg O, Reimer D, Wiewrodt R, Aigner A, Czubayko F, Bals R. The host defense peptide LL-37/hCAP-18 is an autocrine growth factor for lung cancer cells (submitted).

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- Shaykhiev R, Bals R. Interaction between epithelial cells and leukocytes (review, in preparation)
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Presentations (related to this work)

- SFB/TR 22 Symposium Regulation of immune responses in the lung (Tutzing, Germany, 2006)
- Otztal Lung Research Seminar (Obergurgl, Austria, 2006)
- SFB/TR 22 Symposium Regulation of immune responses in the lung (Willingen, Germany, 2006)
- BMFZ Group Presentation (Marburg, Germany, 2005)
- North American Cystic Fibrosis Conference (Baltimore, USA, 2005)
- Meeting of the Clinical Research Group "Chronic Airway Diseases" (Marburg, Germany, 2005)
- Otztal Lung Research Seminar (Obergurgl, Austria, 2005)
- Summer School of the European Academy of Allergy and Clinical Immunology on Asthma (Rotterdam, The Netherlands, 2005)
- Annual Meeting of the Cell Biology Section of the German Society for Pneumology (Bonn, Germany, 2005)
- Otztal Lung Research Seminar (Kleinwalsertal, Austria, 2004)
- International Symposium on Inflammatory Diseases of Barrier Organs (Kiel, Germany, 2004)

Academic teachers

Kazan	**Marburg**
Akhtyamova	Bals
Akhmerov	Bauer
Burnasheva Z	Fehrenbach
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Kiyasov	Gemsa
Nefedova	Lohoff
Orlov	Renz
Ryazanov	Vogelmeier
Sabitova	
Tsybulkina	
Yunusov	

Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel **„Co-operative regulation of epithelial homeostasis and immunity“** im Universitätsklinikum Gießen und Marburg, Standort Marburg, Innere Medizin, Schwerpunkt Pneumologie unter der Leitung von Herrn PD Dr. Dr. Robert Bals mit der Unterstützung durch die unten genannten Personen ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- und ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

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Renat Shaykhiev

Marburg

ABBREVIATIONS

ALI	Air-liquid interface
AMP	Antimicrobial peptide
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BrdU	Bromodeoxyuridine
CD	Cluster of differentiation (antigens)
CF	Cystic fibrosis
CFSE	Carboxyfluorescein succinimidyl ester
COPD	Chronic obstructive pulmonary disease
DC	Dendritic cell
EC	Epithelial cell
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FPRL1	Formyl peptide receptor-like 1
GM-CSF	Granulocyte macrophage colony-stimulating factor
hBD	Human beta-defensin
hCAP18	Human cathelicidin antimicrobial protein 18
HLA	Human leukocyte antigen
HNP	Human neutrophil peptide (common name for alpha-defensins)
IEDC	Intraepithelial dendritic cell
IFN	Interferon
IL	Interleukin
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH
LPS	Lipopolysaccharide (also known as endotoxin)
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MD-2	Myeloid differentiation protein-2
MEK	Mitogen-activated protein kinase/extracellular signal to regulated kinase

MHC	Major Histocompatibility Complex
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear Factor kappa B
NOD	Nucleotide oligomerization domain
PAMP	Pathogen-associated molecular pattern
PBEC	Primary bronchial epithelial cell
PBS	Phosphate-buffered saline
PGN	Peptidoglycan
PRR	Pattern recognition receptor
RT-PCR	Reverse transcription-polymerase chain reaction
siRNA	Small interfering RNA
TCR	T cell receptor
TER	Transepithelial resistance
Th	T helper lymphocyte
TJ	Tight junction
TNF	Tumor necrosis factor
TRIF	Toll/IL-1 receptor (TIR)- domain-containing adaptor inducing IFN-β
WST	Tetrazolium salt