NOD2/CARD15 Mediates Induction of the Antimicrobial Peptide Human Beta-defensin-2*

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Production of inducible antimicrobial peptides offers a first and rapid defense response of epithelial cells against invading microbes. Human beta-defensin-2 (hBD-2) is an antimicrobial peptide induced in various epithelia upon extracellular as well as intracellular bacterial challenge. Nucleotide-binding oligomerization domain protein 2 (NOD2/CARD15) is a cytosolic protein involved in intracellular recognition of microbes by sensing peptidoglycan fragments (e.g. muramyl dipeptide). We used luciferase as a reporter gene for a 2.3-kb hBD-2 promoter to test the hypothesis that NOD2 mediates the induction of hBD-2. Activation of NOD2 in NOD2-overexpressing human embryonic kidney 293 cells through its ligand muramyl dipeptide (MDP) induced hBD-2 expression. In contrast, overexpression of NOD2 containing the 3020insC frameshift mutation, the most frequent NOD2 variant associated with Crohn disease, resulted in defective induction of hBD-2 through MDP. Luciferase gene reporter analyses and site-directed mutagenesis experiments demonstrated that functional binding sites for NF-κB and AP-1 in the hBD-2 promoter are required for NOD2-mediated induction of hBD-2 through MDP. Moreover, the NF-κB inhibitor Helenalin as well as a super-repressor form of the NF-κB inhibitor IκB strongly inhibited NOD2-mediated hBD-2 promoter activation. Expression of NOD2 was detected in primary keratinocytes, and stimulation of these cells with MDP induced hBD-2 peptide release. In contrast, small interference RNA-mediated down-regulation of NOD2 expression in primary keratinocytes resulted in a defective induction of hBD-2 upon MDP treatment. Together, these data suggest that NOD2 serves as an intracellular pattern recognition receptor to enhance host defense by inducing the production of antimicrobial peptides such as hBD-2.

Human epithelia are in permanent contact with various potential pathogenic microorganisms. To overcome these microbial threats, epithelia have developed a chemical defense system based on the production of various antimicrobial proteins (1–4). Human beta-defensin-2 (hBD-2) was the first inducible human antimicrobial protein discovered and was originally isolated from lesional psoriatic skin (5). hBD-2 belongs to the beta-defensin family, a group of small (4–5 kDa), cationic antibiotic peptides first discovered in cattle (6). hBD-2 exhibits a broad spectrum of antimicrobial activity, and its capacity to kill bacteria in vivo has been demonstrated in a mouse gene therapy study with hBD-2-transfected tumor cells. Following a bacterial infection, mice with hBD-2-bearing tumors bore fewer viable bacteria than controls (7). In addition to its capacity to serve as an antibiotic peptide, hBD-2 may promote adaptive immune responses by recruiting dendritic and T cells to the site of microbial invasion through interaction with CCR6 (8). Furthermore hBD-2 was found to be a specific chemotactrant for tumor necrosis factor-α (TNF-α)-treated human neutrophils (9). hBD-2 is expressed in many epithelia (e.g. skin, respiratory tract, digestive tract, and genitourinary tract), and expression can be induced by cytokines such as interleukin 1 (IL-1) (10–14), TNF-α (5, 10, 15–17), IL-22 (18), and IL-17 (19) as well as by microbial stimuli (5, 12, 17, 20–27).

The putative intracellular peptidoglycan receptor NOD2 (CARD15) is a member of the Apaf-1/CARD superfamily and is composed of an N-terminal caspase recruitment domain (CARD), a centrally located nucleotide-binding oligomerization domain (NOD) and 10 C-terminal-located leucine-rich repeats (LRRs) (28). NOD2 was found to be expressed in antigen-presenting cells such as monocytes/macrophages, but more recent studies have also revealed abundant presence of NOD2 in epithelial Paneth cells of the small intestine as well as in other epithelial cells (28–32). The LRR region of NOD2 has been implicated in the recognition of intracellular pathogen components, similar to the homologous LRRs of plant disease resistance proteins (33) and Toll-like receptors (34). NOD2 has been shown to recognize peptidoglycan fragments (e.g. muramyl dipeptide, MDP) through its LRR region leading to proinflammatory responses through activation of NF-κB (35–37).

In this study we have shown an association between activation of NOD2 and the induction of hBD-2. Our findings suggest that activation of NOD2 in epithelial cells may strengthen epithelial innate immunity through the induction of antimicrobial peptides.

EXPERIMENTAL PROCEDURES

Culture of Epithelial Cells—HEK293 cells and primary keratinocytes were cultured in 75-cm² flasks (BD Biosciences) in a humidified atmosphere with 5% CO₂. Standard medium for feeding of HEK293 cells consisted of Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Foreskin-derived primary keratinocytes were isolated from neonatal foreskins as described (38) and cultured in Epilife medium (Sigma). For stimulation experiments cells were seeded in 12-well tissue culture plates (4 cm²/well; BD Biosciences) and used at 60–80% confluency.

RNA Isolation and cDNA Synthesis—After treatment cells were washed twice with PBS and harvested using TRIzol reagent (Invitrogen) according to the supplier’s protocol. RNA quality and quantity were determined by gel electrophoresis and photometry. Subsequently 1 μg
of total RNA was reverse transcribed to cDNA with oligo(dT) primers and 200 units of Superscript II (Invitrogen) according to the manufacturer’s protocol.

**Real-time Reverse Transcription PCR**—Real-time reverse transcription-PCR analyses were performed in a fluorescence temperature cycler (LightCycler; Roche Diagnostics) as described previously (12). This technique continuously monitors the cycle-by-cycle accumulation of fluorescently labeled PCR product. Briefly, cDNA corresponding to 10 ng of RNA served as a template in a 10-μl reaction containing 4 mM MgCl₂, 0.5 μM of each primer, and 1× LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics). Samples were loaded into capillary tubes and incubated in the fluorescence thermocycler (LightCycler) for an initial denaturation at 95 °C for 10 min followed by 45 cycles, each cycle consisting of 95 °C for 15 s, 60 °C (touchdown of −1 °C/cycle from 66 to 60 °C) for 5 s and 72 °C for 10 s. At the end of each run melting curve profiles were produced by cooling the sample to 65 °C for 15 s and then heating slowly at 0.20 °C/s up to 95 °C with continuous measurement of fluorescence to confirm amplification of specific transcripts. Cycle-to-cycle fluorescence emission readings were monitored and analyzed using LightCycler software (Roche Diagnostics). The specificity of the amplification products was further verified by subjecting the amplification products to electrophoresis on a 2% agarose gel. The fragments were visualized by ethidium bromide staining, and the specificity of PCR products was verified by sequencing of the fragments, Fig. 3

**Western Blot**—HEK293 cells were harvested in lysis buffer (1% Triton X-100 in PBS + protease inhibitor mixture; Roche Diagnostics) and lysed by sonication. After centrifugation (10,000 × g, 15 min), the resulting supernatant was mixed with 4× NuPAGE LDS sample buffer (Invitrogen), and 30 μg of this cell extract was separated onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen). To analyze NOD2 protein expression in primary keratinocytes, 2 × 10⁵ cells were immunoprecipitated with 1 μl of NOD2-specific rabbit antiserum (Cayman Chemical, Ann Arbor, MI) (28) using the immunoprecipitation kit (Protein G; Roche Diagnostics) according to the manufacturer’s instructions. Immunoprecipitated proteins were resolved in NuPAGE LDS sample buffer (Invitrogen) and separated onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen). Proteins were transferred to a Protran-nitrocellulose membrane (Schleicher & Schuell BioScience), blocked for 1 h in blocking buffer (5% (w/v) nonfat powdered milk in PBS + 0.05% Tween), and then incubated for 18 h at 4 °C in 3% (w/v) nonfat powdered milk in PBS + 0.05% Tween containing 1:4000 NOD2-specific rabbit antiserum (Cayman, MI) (28). The membrane was washed with PBS + 0.05% Tween six times for 5 min each and then incubated for 1 h in 3% (w/v) nonfat powdered milk in PBS + 0.05% Tween containing 1:20,000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (Dianova, Hamburg, Germany). After another six washes, the membrane was incubated for 5 min with chemiluminescent peroxidase substrate (Sigma) and visualized using a Diana III cooled CCD camera imaging system (Raytest, Straubenhardt, Germany).

**hBD-2 Reporter Plasmid Construction**—A 2338-bp-containing fragment of the hBD-2 promoter was subcloned into the promoterless pGL3-basic firefly luciferase vector (Promega, Madison, WI) to generate reporter plasmid hBD-2–2338-luc as previously described (23). Generation of mutations in the NF-κB and AP-1 binding sites of the hBD-2–2338-luc plasmid was described elsewhere (23). The resulting hBD-2–2338-luc plasmids containing mutated NF-κB and AP-1 binding sites were termed NF-κB-mut1–luc (containing one mutated NF-κB binding site at position −205 to −186, Fig. 3a), NF-κB-mut2–luc (containing one mutated NF-κB binding site at position −596 to −572, Fig. 3a), NF-κB-mut1 + 2–luc (containing two mutated NF-κB binding sites, Fig. 3a), AP1-mut–luc (containing a mutated AP-1 binding site, Fig. 3a), and NF-κB-mut1 + 2 + AP-1–luc (containing two mutated NF-κB binding sites and a mutated AP-1 binding site, Fig. 3a).

**Transfection and Determination of hBD-2 Promoter and NF-κB Activation**—HEK293 cells were transfected in 12-well plates (BD Biosciences) at 60–80% confluence. Luciferase-reporter plasmids for hBD-2 were generated as described above. To analyze activation of the transcription factor NF-κB the firefly luciferase reporter plasmid pNF-κB-Luc (Clontech, CA) was used. HEK293 cells were transfected for 24 h with 75 ng of the indicated reporter plasmids, 30 ng of the NOD2 expression plasmid, and 10 ng of an internal control Renilla luciferase expression plasmid (pRL-TK; Promega) using 1 μl of transfection reagent FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions. For stimulation with MDP, 1 μg of MDP (Bachem, Heidelberg, Germany) was added at the same time. 24 h after transfection cells were harvested with 500 μl of passive lysis buffer (Promega), and firefly luciferase activity from hBD-2-pGL3 reporter vector and Renilla lucif-
erase activity were measured by the dual luciferase assay system (Promega) on a TD-20/20 luminometer (Turner Designs). Promoter activity was reported as the ratio between firefly and Renilla luciferase activities in each sample. In some experiments HEK293 cells were transfected for 16 h with 0.5 μg of a plasmid (CMV-1kB-DN) allowing overexpression of a mutated 1kB variant (kindly provided by Dagmar Kulms, Münster, Germany) (39).

Small Interfering RNA (siRNA)—The following duplexed RNA oligonucleotides (Stealth RNAi) were synthesized by Invitrogen: NOD2-A, 5′-UAUUUGUUAACGGCCGAAUAACAGACG-3′; NOD2-B, 5′-UGCGGAGAACCAUACCCUACUC-3′. As a control, we used Stealth RNAi negative control duplexes (Invitrogen). Primary keratinocytes were cultured in 12-well plates and used at 50–70% density the day of transfection. Cells were transfected with Lipofectamine 2000 (Invitrogen); 1 μl of siRNA stock (20 μM) and 1 μl of Lipofectamine 2000 were each diluted with 250 μl of Epilife medium without supplements. After 5 min at room temperature, they were combined and incubated for 20 min. The reaction mixtures were overlaid on the cell culture for 5 h. The medium was then changed to fresh Epilife medium. After 20 h cells were treated with the NOD2 ligand muramyl dipeptide MDP-LD (or simply MDP, purchased from Bachem) or with the biologically inactive MDP expression vector (lane 1), NOD2 3020lnsc expression vector (lane 2), or empty vector (lane 3). Note the lower molecular mass of the mutated NOD2 protein (3020lnsc: lane 2), which is the result of the generation of a stop codon. The truncated NOD2 protein contains 1,007 amino acids instead of the 1,040 amino acids of the wild type protein (40, 57, 58). b, HEK293 cells were transfected with NOD2 or NOD2 3020lnsc expression vectors with or without 1 μg of MDP. NF-κB activity was analyzed after 24 h using an NF-κB-luciferase reporter assay. Data are means ± S.D. of one representative experiment of three, each done in triplicate.

**MDP Activates the hBD-2 Promoter via NOD2**—To investigate the influence of NOD2 on hBD-2 expression, NOD2 expression plasmids and a luciferase gene reporter vector containing 2338 bp of the hBD-2 promoter were co-transfected in HEK293 cells. Transfection of HEK293 cells with the NOD2 expression plasmid as well as with NOD2 3020lnsc plasmid activated the hBD-2 promoter (Fig. 2). Cells overexpressing NOD2 responded to MDP treatment by enhanced activation of the hBD-2 promoter. Cells transfected with a mock vector showed no further activation of hBD-2 promoter upon MDP treatment. Cells transfected with the NOD2 3020lnsc plasmid also showed no activation of hBD-2 promoter upon MDP treatment (Fig. 2).

**The Proximal NF-κB Binding Site in the hBD-2 Promoter Is Required for NOD2-mediated hBD-2 Induction**—To elucidate the role of putative binding sites for the transcription factors NF-κB and AP-1 in the hBD-2 promoter region we used hBD-2 promoter luciferase expression constructs containing mutations of the two NF-κB sites (position −205 to −186 and −596 to −572) and the AP-1 site (position −127 to −121) (Fig. 3a). NOD2-overexpressing HEK293 cells were transfected with these constructs, treated with MDP, and analyzed for hBD-2 promoter activation. Mutation of the two NF-κB sites together with the AP-1 site abolished the NOD2-mediated hBD-2 promoter activation upon MDP treatment (Fig. 3b). Mutation of the two NF-κB sites as well as mutation of only the proximal NF-κB site (position −205 to −186) almost completely inhibited hBD-2 promoter activation (Fig. 3b). In contrast, mutation of the NF-κB site at position −596 to −572 had only a marginal blocking effect on hBD-2 promoter activation (Fig. 3b). Mutating the AP-1 site reduced hBD-2 promoter activation from 7- to 5-fold (Fig. 3b).
NOD2 Mediates hBD-2 Induction

**FIGURE 2.** NOD2, but not NOD2 3020insC, activates the hBD-2 promoter upon MDP stimulation. HEK293 cells were co-transfected with an hBD-2 promoter luciferase plasmid and with NOD2 or NOD2 3020insC expression vector with or without 1 μg of MDP. hBD-2 promoter activation was analyzed after 24 h by measuring luciferase activity. Data are means ± S.D. of one representative experiment of three, each done in triplicate.

Our results demonstrate that NOD2, upon activation through its ligand MDP, mediates the expression of the inducible antimicrobial peptide hBD-2. It is known that activation of NOD2 through its ligand MDP triggers activity of the transcription factor NF-κB (36, 44). Because the hBD-2 promoter contains putative binding sites for NF-κB, we analyzed the relevance of these NF-κB binding sites for NOD2-mediated hBD-2 induction through MDP. Indeed, mutation of the two proximal NF-κB sites in the hBD-2 promoter region almost completely inhibited the secretion of hBD-2 peptide from primary keratinocytes we treated these cells with the NOD2 inhibitor IκB (41). As a consequence, NF-κB release, nuclear translocation, and functional DNA binding are prevented. Although NOD2-overexpressing HEK293 cells transfected with the empty CMV vector were not impaired in their hBD-2 activation upon stimulation with MDP, cells transiently transfected with the IκB dominant negative mutant lost their capacity to activate the hBD-2 promoter upon MDP treatment (Fig. 4).

The relative importance of the NF-κB and AP-1 binding sites for NOD2-mediated hBD-2 promoter activation. a, the hBD-2 promoter constructs used are diagrammed. Nucleotide positions are marked relative to the hBD-2 transcription start. Two NF-κB sites and one AP-1 site in the hBD-2 promoter (−2338 to −1 bp), linked to the luciferase gene, were mutated in different combinations. b, HEK293 epithelial cells were co-transfected with wild type (−2338-luc) or mutated hBD-2 promoter luciferase plasmids and with the NOD2 expression vector together with 1 μg of MDP. Induction of hBD-2 promoter was determined by measuring luciferase activity. Data are means ± S.D. of triplicate samples. Experiments were repeated twice, and similar results were obtained.

**DISCUSSION**

Helenalin, a Specific Inhibitor of NF-κB, Blocks NOD2-mediated hBD-2 Induction—To further test the influence of NF-κB on NOD2-mediated hBD-2 induction we blocked NF-κB activity in HEK293 cells by the specific inhibitor Helenalin (42, 43). Fig. 5 shows that MDP-mediated induction of the hBD-2 promoter in NOD2-overexpressing HEK293 cells can be blocked by treatment of the cells with Helenalin.

**FIGURE 4.** A dominant negative mutant of IκB inhibits NOD2-mediated hBD-2 induction. HEK293 cells were transiently transfected with a plasmid allowing overexpression of the super-repressor form of the NF-κB inhibitor IκB (CMV-IκB-DN) or with the empty control vector (CMV) only. After 16 h cells were transfected with a NOD2 overexpression plasmid and an hBD-2 promoter luciferase plasmid with or without 1 μg of MDP. After 24 h cells were harvested, and hBD-2 promoter activation was determined by measuring luciferase activity. Data are means ± S.D. of one representative experiment of two, each done in triplicate.
NOD2 Mediates hBD-2 Induction

hBD-2 promoter activation in NOD2-overexpressing HEK293 cells upon treatment with MDP. The involvement of NF-κB in the NOD2-mediated hBD-2 induction was further confirmed through inhibition of NF-κB by the inhibitor Helenalin as well as by overexpression of a dominant negative mutant of IκB. Both approaches strongly inhibited MDP-mediated hBD-2 induction in NOD2-overexpressing HEK293 cells. Furthermore, mutating the proximal AP-1 binding site at position −127 to −121 also led to a decrease of hBD-2 promoter activation. These results indicate that NF-κB and, to a lesser degree, AP-1 play a critical role in the regulation of the NOD2-mediated hBD-2 induction. These results are consistent with previous reports demonstrating an important role of NF-κB for induction of hBD-2 in epithelial cells upon stimulation with bacteria or bacterial products (23, 25, 26, 45–47).

NOD2 expression was originally discovered in monocytes (28) and later also found in epithelial Paneth cells of the small intestine (29, 30) as well as in other epithelial cell lines (31, 32). When we screened different tissues for gene expression of NOD2 we detected distinct NOD2 expression in the skin. In concordance with this observation, gene and protein expression of NOD2 could be detected in primary keratinocytes, suggesting that NOD2 may play a role in cutaneous innate immunity. Keratinocytes are an important source of inducible antimicrobial peptides such as hBD-2 (5, 12, 21, 48). To investigate whether NOD2 activation results in hBD-2 release, we stimulated primary keratinocytes with the NOD2 ligand MDP. We used chemically synthesized MDP to activate NOD2, thus avoiding the risk of contamination by other bacterial products. Because NOD2 is involved in intracellular sensing of microbial products, we used a protein transfection reagent to deliver MDP into the keratinocytes. This resulted in an augmented release of hBD-2, indicating that activation of NOD2 mediates hBD-2 expression in primary keratinocytes. Because we could not completely exclude that other pathogen recognition molecules distinct from NOD2 could also sense MDP, we used siRNA to specifically down-regulate NOD2 expression. As expected, primary keratinocytes transfected with

FIGURE 5. Helenalin, a specific inhibitor of NF-κB, blocks NOD2-mediated hBD-2 induction. HEK293 cells were transiently transfected with a NOD2 overexpression plasmid and an hBD-2 promoter luciferase plasmid. Cells were treated with or without 1 μg of MDP in the presence or absence of the NF-κB inhibitor Helenalin (4 μM). After 24 h cells were harvested, and hBD-2 promoter activation was determined by measuring luciferase activity. Data are means ± S.D. of one representative experiment of three, each done in triplicate.

FIGURE 6. NOD2 expression in skin and primary keratinocytes. a, analysis of NOD2 gene expression by reverse transcription-PCR with cDNA derived from various tissues (MW, molecular weight size marker). b, NOD2 protein expression in primary keratinocytes was analyzed by immunoprecipitation and subsequent Western blot analysis using NOD2 antiserum.

FIGURE 7. MDP-induced hBD-2 secretion in primary keratinocytes. Primary keratinocytes were treated for 2.5 h with different concentrations of MDP using ProteoJuice transfection reagent. Subsequently, medium was replaced and cells further incubated for 7 h. Cell culture supernatants were analyzed for the presence of hBD-2 peptide using an ELISA. Secretion of hBD-2 peptide was significantly increased from control siRNA-transfected MDP-treated keratinocytes compared with MDP-LL-treated keratinocytes (p < 0.5, Student’s t-test). In contrast, hBD-2 peptide secretion was not significantly changed between MDP- and MDP-LL-treated NOD2 siRNA-transfected keratinocytes. Data present means ± S.D. of triplicate samples. A representative result from three independent experiments is shown.

FIGURE 8. NOD2 mediates hBD-2 peptide release in primary keratinocytes. Primary keratinocytes were transfected with a NOD2-specific siRNA or an unrelated control siRNA for 20 h. After transfection, cells were treated with 5 μg/ml MDP or MDP-LL together with ProteoJuice transfection reagent. After 2.5 h the medium was replaced and cells further incubated for 7 h. A, cell culture supernatants were analyzed for the presence of hBD-2 peptide using an ELISA. Secretion of hBD-2 peptide was significantly increased from control siRNA-transfected MDP-treated keratinocytes compared with MDP-LL-treated keratinocytes (p < 0.5, Student’s t-test). In contrast, hBD-2 peptide secretion was not significantly changed between MDP- and MDP-LL-treated NOD2 siRNA-transfected keratinocytes. Data present means ± S.D. of triplicate samples. A representative result from three independent experiments is shown. B, for analyzing the effect of NOD2 siRNA, relative changes in NOD2 gene expression were determined by real-time PCR. Transfection of the NOD2-specific siRNA resulted in an inhibition of NOD2 gene expression of ~80% compared with keratinocytes treated with an unrelated control siRNA.
NOD2 Mediates hBD-2 Induction

NOD2-specific siRNA showed a strong decrease of NOD2 gene expression. These cells failed to induce elevated amounts of hBD-2 upon MDP treatment, thus confirming that hBD-2 induction in keratinocytes through MDP is mediated by NOD2. Together these data indicate that NOD2 may serve as a pattern recognition receptor in cutaneous innate immunity. Once activated by peptidoglycan fragments such as MDP, NOD2 promotes the release of antimicrobial peptides like hBD-2 in keratinocytes and thereby strengthens the cutaneous innate defense system.

Keratinocytes are constantly exposed to bacteria or bacterial products. Therefore one would assume that in the epidermis the extraocular sensing of the cutaneous flora should be avoided to prevent an undesirable inflammation. Colonization of human skin by *Staphylococci* and *Streptococci* is a characteristic feature of several skin diseases and is often followed by entry of the pathogenic bacteria into keratinocytes (49–51). It has been suggested that intracellular pattern recognition receptors such as NOD2 function to sense the presence of potential pathogenic invasive bacteria (52, 53). Therefore one could speculate that in keratinocytes NOD2 functions to sense pathogenic invading bacteria. However, this hypothesis remains to be proven. A recent study proposed a model for the activation of cytosolic host defense molecules by noninvasive pathogenic bacteria. It has been reported that *Helicobacter pylori* was recognized by epithelial cells via NOD1, an intracellular pathogen-recognition molecule with specificity for Gram-negative peptidoglycan (54). Activation of NOD1 was mediated by the direct delivery of peptidoglycan fragments through a type IV bacterial protein secretion system (54). This study demonstrated that noninvasive bacteria can activate cytosolic host defense molecules such as the NODs. Bacterial secretion systems are widely distributed through different bacteria species and often associated with virulent strains (55). Therefore one could speculate that delivery of pathogen-associated molecules into epithelial cells through bacterial secretion systems may represent a general mechanism to activate cytosolic host defense molecules. Delivery of bacterial products into the cell could serve as an “alarm signal” for host innate immunity, providing a mechanism to discriminate between pathogenic and commensal bacteria (56). Recognition of intracellular bacterial products in epithelial cells always represents a dangerous situation that requires activation of the innate immune system. Our data suggest that part of this activation could be the NOD2-mediated augmented production of antimicrobial peptides.

Several studies have shown that mutations in the LRR region of NOD2 are associated with susceptibility to Crohn disease, a chronic inflammatory disorder of the intestinal tract (40, 57, 58). The molecular mechanisms by which mutations in the NOD2 gene cause Crohn disease are still emerging. Recently, we found a decreased gene expression of the Paneth cell-derived alpha-defensins HDS and HD6 in patients with ileal Crohn disease, especially in patients with a mutation in the LRRs of NOD2 (59). These findings in humans could be confirmed and further substantiated in a rodent model. Mice lacking the NOD2 gene showed diminished levels of mouse alpha defensins (so-called cryptdins) and presumably as a result were more susceptible to an oral but not systemic bacterial infection (60). These observations further support the hypothesis that decreased production of antimicrobial peptides may promote bacterial-mediated inflammation in Crohn disease (61). Our data revealed that HEK293 cells overexpressing the 3020insC NOD2 variant (the major NOD2 mutation associated with Crohn disease) failed to induce hBD-2 upon stimulation with MDP. This gives reason to hypothesize that hBD-2 expression might be dysregulated in patients with Crohn disease and could also offer an explanation for the recent findings regarding a lack of induction of the inducible beta-defensins hBD-2 and hBD-3 in Crohn disease as compared with ulcerative colitis (62). However, preliminary studies in colonic epithelia from patients with and without NOD2 mutations revealed no statistically significant differences in hBD-2 expression. Further studies have to explore whether NOD2-mediated epithelial expression of hBD-2 (and probably other antimicrobial peptides) might be dysregulated in individuals carrying mutations in the NOD2 gene.

Our data revealed a functional expression of NOD2 in primary keratinocytes and a connection between activation of NOD2 and increased hBD-2 production in primary keratinocytes. Therefore it would be interesting to investigate whether mutations or a dysregulation of NOD2 are associated with an increased susceptibility to cutaneous infections. In particular, cutaneous infections that are associated with decreased expression and induction of antimicrobial peptides (e.g. atopic dermatitis, Refs. 15, 63) would be a favorable model to investigate this interesting hypothesis.

In summary, our data indicate that NOD2 contributes to epithelial innate immunity by mediating the expression of the inducible antimicrobial peptide hBD-2. A better understanding of the signal transduction pathways leading to induction of antimicrobial proteins may result in therapeutic approaches that enhance the innate defense system.

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