The Journal of Immunology

RESEARCH ARTICLE | JULY 15 2004

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J Immunol (2004) 173 (2): 1219–1223.
https://doi.org/10.4049/jimmunol.173.2.1219

Microbial DNA Induces a Host Defense Reaction of Human Respiratory Epithelial Cells[1]

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Epithelial cells represent the initial site of bacterial colonization in the respiratory tract. TLR9 has been identified in B cells and CD 123$^+$ dendritic cells and found to be involved in the recognition of microbial DNA. It was the aim of the study to investigate the role of TLR9 in the host defense reactions of the respiratory epithelium. Respiratory epithelial cell lines (IHAEo$^-$, Calu-3) or fully differentiated primary human cells as air-liquid interface cultures were stimulated with bacterial DNA or synthetic oligonucleotides containing CpG motifs (CpG oligodeoxynucleotides). Expression of TLR9, cytokines, and human β-defensin 2 was determined by quantitative RT-PCR or by ELISA. We found that TLR9 is expressed by respiratory epithelial cell lines and fully differentiated primary epithelial cells at low levels. Stimulation of the above-mentioned cells with bacterial DNA or CpG oligodeoxynucleotide resulted in an inflammatory reaction characterized by a dose-dependent up-regulation of cytokines (IL-6, IL-8) and human β-defensin 2. Up-regulation of NF-κB in epithelial cells in response to the CpG motif containing DNA was inhibited by overexpression of a dominant negative form of MyD88. These results provide clear evidence that the human respiratory epithelium is capable of detecting microbial DNA by TLR9. The respiratory epithelium has an important function in triggering innate immune responses and therefore represents an interesting target for anti-inflammatory therapy. ***The Journal of Immunology*****, 2004, 173: 1219–1223.**

The respiratory tract is continuously exposed to the environment. The innate immune system involves a broad functional spectrum of host defense mechanisms against microbes (1, 2). Pattern recognition receptors expressed on cells of the innate immune system recognize conserved structural motifs of microorganisms called pathogen-associated molecular patterns (3). TLRs of vertebrates have been identified by their structural homology to *Drosophila* Toll molecules, which are receptors involved in development of host defense. Members of the TLR family are essential components in the activation of innate immunity (1–3). Ten TLRs (TLR1–10) have been identified in mammalian systems; the current paradigm is that individual TLRs have distinct ligands (3, 4).

Recent studies demonstrate that TLR9 is involved in the recognition of microbial DNA (4). DNA containing CpG motifs activates immune cells such as macrophages, lymphocytes, NK cells and dendritic cells to secrete a variety of immunomodulatory cytokines, including IL-6, IL-12, IL-18, TNF-α, and IFN-γ (5–8). Bacterial DNA and CpG motifs also stimulate epithelial cells (9). Vertebrate genomes show CpG suppression and are usually methylated, whereas the CpG motifs in bacterial DNA are unmethylated and occur more frequently (10). Bacterial DNA evokes epithelial IL-8 production by a mitogen-activated protein kinase-dependent pathway (9).

Airway epithelial cells form the boundaries to the outer world and are at the front of microbial colonization. These cells express a variety of pathogen-associated molecular patterns including TLR1–6 (1, 11). Activation of TLR2 has been linked to the induction of human β-defensin 2 (hBD-2)[3] (12), an antimicrobial peptide expressed by epithelial cells. It has been the aim of our study to test whether respiratory epithelial cells can detect bacterial DNA motifs and whether this reaction is mediated by TLR9. We show that TLR9 is expressed in airway epithelial cells and that bacterial DNA and CpG oligodeoxynucleotides (ODN) stimulate respiratory cells by activation of TLR9.

Materials and Methods

Media and reagents

IHAEo$^-$ cells were cultured in DMEM supplemented with 10% FCS, 100 IU/ml penicillin G, and 100 IU/ml streptomycin sulfate. Calu-3 were cultured in Ham's F12:DMEM (1: 1) supplemented with 10% FCS and antibiotics (see above). Human respiratory epithelial cells were isolated from large airways resected during surgery and cultivated in air-liquid interface cultures as described previously (13). The protocol was approved by the Institutional Review Board of the University of Marburg and informed consent was obtained from the patients. DNA from *Escherichia coli* and human DNA were purchased from Sigma-Aldrich (Taufkirchen, Germany; endotoxin content in 100 μg/ml $<$20 ng/ml, Acila, Moerfelden, Germany). Additionally, bacterial DNA from *Porphyromonas ginigivalis* was a kind gift from K. Heeg (Microbiology, Philipps-Universität Marburg, Marburg, Germany). This DNA was intensively tested for the absence of contaminating LPS. Phosphorothioate-modified oligonucleotides (ODNs) were custom synthesized by MWG Biotech (Munich, Germany). The following sequences were used (the bold letters indicate the CpG motifs or the GpC motifs of the control ODN): 2006, T**CG** T**CG** TTT TGT **CG**T TTT GT**C** **G**TT; and 2006GC, T**GC** T**GC** TTT TGT **GC**T TTT GT**G** **C**TT. IFN-γ was purchased from Roche (Mannheim, Germany).

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Received for publication May 20, 2003. Accepted for publication May 6, 2004.

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[1] This study was supported by grants from the Wilhelm-Sander-Stiftung and the Deutsche Forschungsgemeinschaft (Ba 1641/5-1, 6-1) to R.B. and from the Deutsche Forschungsgemeinschaft (Da 592/1-1) to A.D.

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[3] Abbreviations used in this paper: hBD-2, human β-defensin 2; ODN, oligodeoxynucleotide; Ct, cycle threshold.

0022-1767/04/$02.00

Stimulation of the cells and harvest of material

Cells were stimulated with the indicated substances for various times. Experiments were repeated at least twice and run in triplicates. Supernatants were removed and used for measurement of cytokines IL-6 and IL-8. Total RNA was isolated by using an RNeasy Mini kit (Qiagen, Hilden, Germany). RNA isolation included DNase digestion using an RNase-free DNase Set (Qiagen).

Determination of cytokine concentrations

Cytokine levels in culture supernatants were determined using a commercially available ELISA kit for IL-6 and IL-8 according to the manufacturer's instructions (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Real-time RT-PCR

A total of 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. cDNA was diluted 1/5 and 5 μl was used as template in 25 μl of TaqMan-PCR mix according to the manufacturer's protocol (PE Biosystems, Weiterstadt, Germany). GAPDH primer (sense, GAA GGT GAA GGT CGG AGT C; antisense, GAA GAT GGT GAT GGG ATT TC), TLR9 primer (sense, ACT TCA CCT TGG ATC TGT CAC G; antisense, GCT TAT TGC GGG ACA GGT CTA), and hBD-2 primer (sense TCA GCT CCT GGT GAA GCT C; antisense, GGG CAA AAG ACT GGA TGA CA) were purchased from TIB Molbiol (Berlin, Germany). Fluorogenic probes (6-carboxyfluorescein) were (X indicates TAMRA): GAPDH, 6-FAM-CAA GCT TCC CGT TCT CAG CC X; TLR9, 6-FAM-TGT TTG CCC AGC TCT CGC ACC X; and hBD-2, 6-FAM-CCA TCA GCC ATG AGG GTC TTG TAT CTC C X. Specificity of RT-PCR was controlled by omission of the template or the reverse transcription and negative results were obtained. Quantitative PCR results were obtained using the $\Delta\Delta$Ct (cycle threshold) method. PCR efficiencies for all three reactions were similar.

Fluorescence-activated cell sorting

A PE-labeled Ab directed to TLR9 and isotype control used were obtained from eBioscience (San Diego). Cells were washed in PBS/2% FCS and fixed in PBS/2% paraformaldehyde. After washing, cells were permeabilized with PBS/0.5% saponin/2% FCS for intracellular staining. One million cells were analyzed on a Partec PAS flow cytometer (DakoCytomation, Hamburg, Germany). Data were analyzed using WinMDI software.

Luciferase reporter plasmid transfection and luciferase assay

To determine the effect of CpG DNA on NF-κB levels, IHAEo$^-$ were transiently transfected with different plasmids. 5xNF-κB-luc expresses *Firefly* luciferase under the control of a promoter that contains five NF-κB binding sites. β-Actin *Renilla* contains a *Renilla* luciferase with a β-actin promoter (14). ΔMyD88 is a dominant negative form of MyD88, a death domain-containing adapter molecules essential for TLR signaling (15). ΔMyD88 and empty pcDNA3 were a kind gift from C. MGreene (Royal College of Surgeons in Ireland, Dublin, Ireland) and 5xNF-κB-luc was obtained from C. Kupatt (University of Munich, Munich, Germany). Cells were transfected using Effectene (Qiagen) according to the manufacturer's protocol. Twenty-four hours before transfection, the cells were split onto 24-well plates with 2.5×10^4 cells/well. For transfection, a total amount of 0.2 μg of plasmid DNA per well was used. The ratio of 5xNF-κB-luc: ΔMyD88 or pcDNA3 was 1:2. The ratio (w/v) of plasmid DNA:Effectene was 1:10. Transfection complexes remained on the cells for 6 h, then they were removed and the medium was changed to normal growth medium. At 24 h posttransfection, the cells were stimulated with bacterial or human DNA (100 μg/ml) for 24 h. Preparation of cell lysates and luciferase assays were performed as recommended by the manufacturer (Dual-Luciferase Reporter Assay System; Promega, Mannheim, Germany) using a Luminometer FB12 (Berthold Detection Systems, Pforzheim, Germany).

Statistical analysis

Values are displayed as mean $\pm$ SEM. Comparisons between groups were analyzed by *t* test (two-sided) or ANOVA for experiments with more than two subgroups. Post hoc range tests were performed with the *t* test (two-sided) with Bonferroni adjustment. Results were considered statistically significant for $p < 0.05$.

Results

TLR9 is expressed by human respiratory epithelial cells

We initially addressed the question whether airway epithelial cells express TLR9. We determined TLR9 mRNA levels in the respiratory epithelial cell lines IHAEo$^-$ and Calu-3 and primary cells by quantitative RT-PCR (Fig. 1*A*). The expression levels are significantly lower than in the human B cell lymphoma cell line Raji that was used as positive control. We detected low levels of TLR9 protein in airway epithelial cell lines and primary cells using FACS analysis (Fig. 1*B*). Next, we investigated whether expression of TLR9 is regulated in airway epithelial cells. We observed a small but significant increase in TLR9 mRNA levels after adding IFN-γ to the cell line IHAEo$^-$ (Fig. 2). Other stimuli such as LPS, TNF-α, or bacteria did not change mRNA levels of TLR9 (data not shown). These data show that TLR9 is expressed in airway epithelial cells.

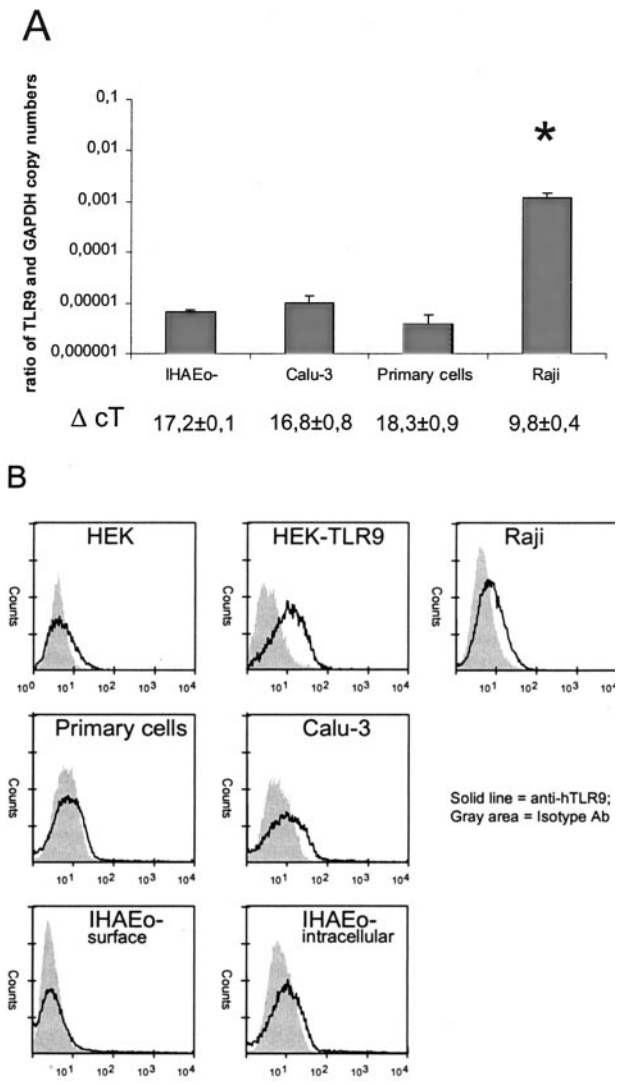

FIGURE 1. TLR9 is expressed in human respiratory epithelial cell lines and primary cells. *A*, TLR9 mRNA levels were quantified in the respiratory epithelial cell lines IHAEo$^-$ and Calu-3 and primary cells by quantitative RT-PCR. The ratio of TLR9 and GAPDH copy numbers was calculated from the ΔCt values using the formula $2^{-1 \times \Delta Ct}$. TLR9 mRNA levels in Raji are significantly (*) increased ($n = 4$). *B*, TLR9 protein is expressed in TLR9-transfected HEK293 cells, Raji, permeabilized IHAEo$^-$, Calu-3, and primary cells. Nonpermeabilized and nontransfected HEK293 cells showed no fluorescent shift as compared with the isotype control.

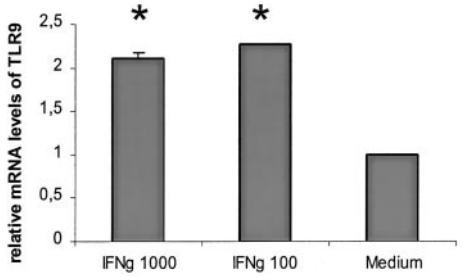

FIGURE 2. Effect of IFN-γ on TLR9 mRNA expression. IHAEo$^-$ were stimulated with the indicated concentrations of IFN-γ (U/ml) for 24 h. Small but significant differences to the control group of $p < 0.05$ are labeled with an ∗ ($n = 3$).

Stimulation of human respiratory epithelial cells with bacterial DNA or CpG ODN results in up-regulation of cytokines and hBD-2

Next, we characterized the effect of microbial DNA on human respiratory epithelial cells. We stimulated cell lines and well-differentiated primary cells with genomic DNA from different bacteria and synthetic CpG ODN. As controls we used human DNA and ODN containing an inverted CpG motif, respectively. As primary outcome parameters we determined levels of IL-8 and IL-6 by ELISA. Bacterial DNA and ODN 2006 caused dose-dependent up-regulation of IL-8 (Fig. 3, *A* and *B*) and IL-6 (data not shown) in IHAEo$^-$ as compared with the controls. In addition, primary respiratory epithelial cells as conventional submersed cultures were stimulated with DNA from *E. coli*, *P. ginigivalis*, and human source (Fig. 3*C*). In parallel experiments, we showed that LPS at concentrations of 50 ng/ml is necessary to elicit a response equivalent to the stimulation with bacterial DNA (data not shown).

Next, we analyzed the effects of the CpG motif containing DNA on polarized epithelial cells cultured in an air-liquid interface technique. It has been described earlier that responses of airway epithelia are correlated with differentiation status (16). Initially, we stimulated polarized Calu-3 with bacterial DNA from both the apical and basolateral side and found up-regulated IL-8 (data not shown) and IL-6 (Fig. 4*A*). Stimulation from the apical side of the epithelium showed a more prominent effect as compared with stimulation from the basolateral side. Furthermore, we stimulated differentiated primary airway epithelial cells grown as an air-liquid interface culture with ODN 2006 and ODN 2006GC from the apical or basolateral side. A significant up-regulation of IL-8 could be observed upon stimulation with ODN 2006 as compared with ODN 2006GC (Fig. 4*B*). Differences between apical and basolateral application were not significant (Fig. 4*B*).

Next, we determined whether host defense molecules such as antimicrobial peptides are regulated by microbial DNA and found that stimulation with DNA from *E. coli* induced an up-regulation of hBD-2 (Fig. 4*C*). This effect was more pronounced after application to the apical side of the culture.

Immunostimulatory effect of CpG DNA is inhibited by overexpression of a dominant negative form of MyD88

TLR9-mediated signaling is reported to involve MyD88 (17). To strengthen the evidence that TLR9 is involved in the recognition of bacterial DNA in epithelial cells, we expressed a dominant negative form of MyD88 (ΔMyD88) and investigated the stimulatory effect of CpG DNA. IHAEo$^-$ cells were transiently cotransfected with the plasmids 5xNF-κB-luc as a reporter for NF-κB activity, empty pcDNA3, and pcDNA containing the cDNA of ΔMyD88 in different combinations. *Renilla* luciferase activity under the control

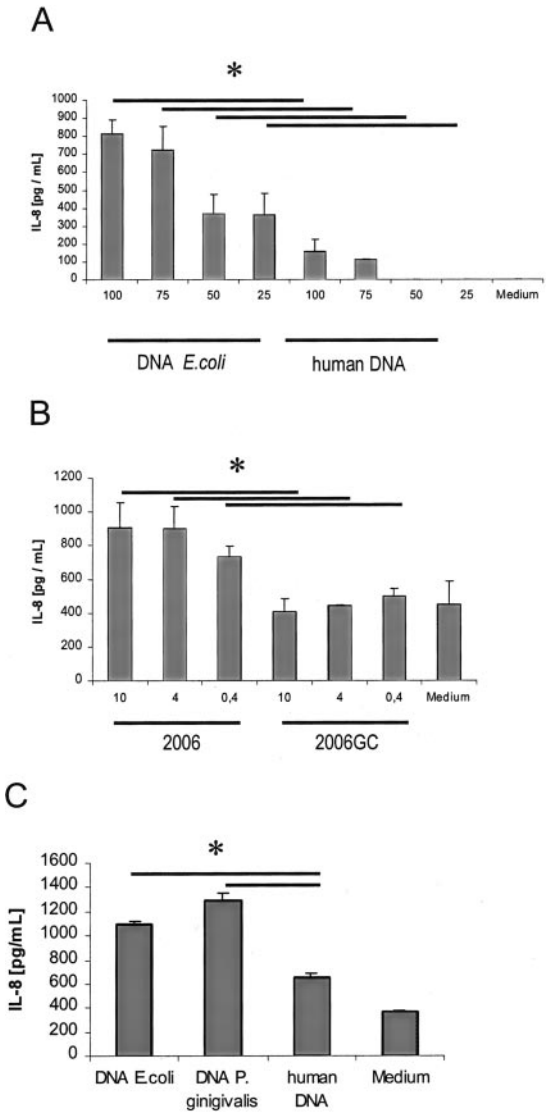

FIGURE 3. Effect of different CpG motif containing DNA on respiratory epithelial cells. Significant differences of $p < 0.05$ are labeled with an ∗. *A*, Effect of bacterial DNA on IL-8 secretion of IHAEo$^-$. Cells were stimulated with different concentrations (displayed in micrograms per milliliter) of DNA from *E. coli* for 24 h. Human DNA was used as control ($n = 4$). *B*, CpG ODN stimulate IHAEo$^-$ cells in a dose-dependent manner (concentrations are displayed in micrograms per milliliter) ($n = 4$). *C*, Effect of various DNAs on IL-8 secretion of primary respiratory epithelial cells. Primary respiratory epithelial cells as conventional submersed cultures were stimulated with DNA from *E. coli*, *P. ginigivalis*, and human source (100 μg/ml). All treatment groups are significantly different from the control ($n = 4$).

of a β-actin promoter was found to be constant in the experimental series (data not shown). First, IHAEo$^-$ cells were cotransfected with 5xNF-κB-luc and pcDNA3. Stimulation with bacterial DNA significantly increased NF-κB-dependent luciferase activity (Fig. 5). The stimulatory effect was significantly decreased when human instead of bacterial DNA was used for stimulation (Fig. 5). The level of luciferase activity in untransfected cells was 502 relative light units. When IHAEo$^-$ were cotransfected with 5xNF-κB-luc and ΔMyD88, the stimulatory effect of bacterial DNA relative to human DNA observed in the first experiment was abolished (Fig. 5).

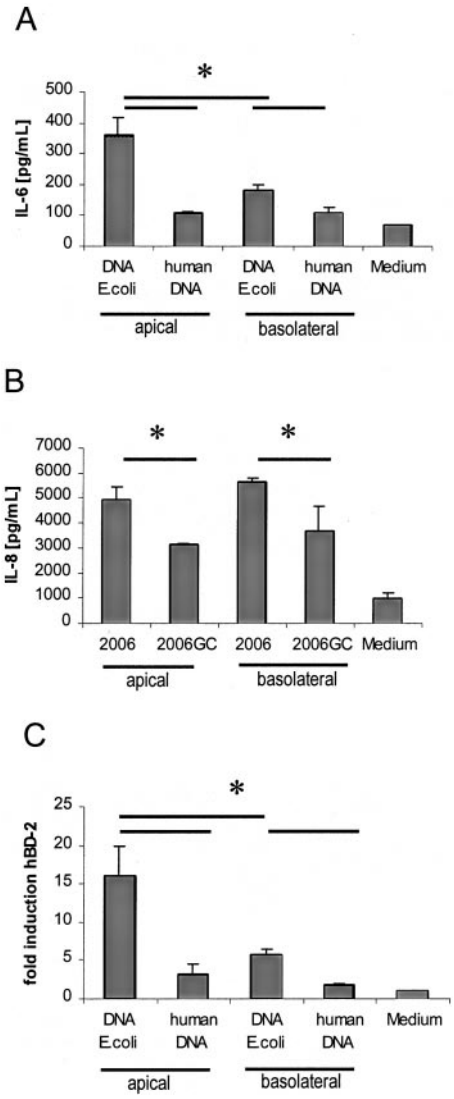

FIGURE 4. Effect of different CpG motif containing DNA on well-differentiated respiratory epithelial cells grown as air-liquid interface cultures. Significant differences of $p < 0.05$ are labeled with an $*$ ($n = 4$). *A*, IL-6 induction following stimulation from the apical or the basolateral side. We stimulated polarized Calu-3 as air-liquid interface cultures with bacterial DNA (100 μg/ml) for 24 h from the apical or basolateral side. Human DNA (100 μg/ml) was used as control. All treatment groups are significantly different from the control. *B*, IL-8 induction following stimulation from the apical or the basolateral side. We stimulated primary respiratory epithelial cells grown as differentiated air-liquid interface cultures with ODN 2006 and ODN 2006GC from the apical or basolateral side for 24 h. *C*, hBD-2 mRNA induction following stimulation from the apical or the basolateral side. We stimulated polarized Calu-3 as air-liquid interface cultures with bacterial DNA (100 μg/ml) for 24 h from the apical or basolateral side.

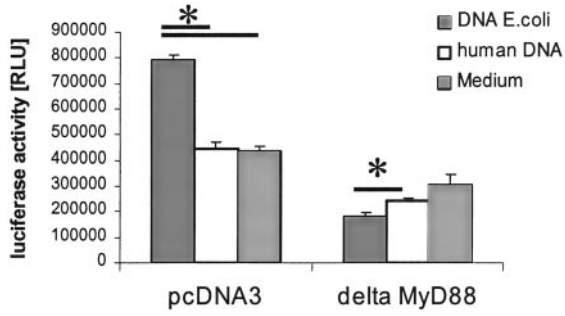

FIGURE 5. Overexpression of a dominant negative mutant of MyD88 suppresses the epithelial response to bacterial DNA. IHAEo$^-$ were cotransfected with 5xNF-κB-luc, empty pcDNA3, or pcDNA3 containing ΔMyD88. Cells were then stimulated with bacterial and human DNA (100 μg/ml). Significant differences of $p < 0.05$ are labeled with an $*$ ($n = 4$).

Discussion

The main finding of the present study is that the human respiratory epithelium is capable of detecting CpG motifs present in bacterial DNA. TLR9 is expressed in airway epithelia and is likely involved in this pattern recognition process. These data provide fundamental insights into the role of the human respiratory epithelium in pathogen recognition and host defense.

TLR9 has been reported to be essential for the recognition of CpG DNA in cells of the human immune system (17). Transfection of human TLR9 into nonresponsive cells reconstitutes CpG DNA responsiveness (4, 17–19). Notably, the cells used in many of the studies were of epithelial origin. CpG DNA effects on human immune cells such as dendritic cells and B lymphocytes include cytokine production, B cell proliferation, and dendritic cell maturation (8). These effects are absent in TLR9-deficient cells (4), suggesting that TLR9 is a critical element in recognition of CpG DNA. Bacterial DNA and immunostimulatory CpG ODN activate macrophages in vivo and in vitro to express activation markers, to translocate NF-κB, and to secrete proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12 (20–23).

In the present study, we describe for the first time that TLR9 is expressed in differentiated airway epithelial cells. TLR9 mRNA levels are relatively low as compared with the human B cell lymphoma cell line Raji and vary significantly between different cell lines or primary cells. In contrast to PBMC (19), epithelial expression of TLR9 seems not significantly regulated by IFN-γ. It has been shown earlier that airway epithelium expresses TLR1–6 (11) and can be activated by a variety of microbial components, such as LPS (11) or flagella (24, 25). In this study, we show that TLR9 mediates activation of epithelial cells by the CpG motif containing ODN or bacterial DNA. Several lines of evidence support this finding: 1) different types of DNA containing CpG motifs induce an up-regulation of various outcome measurements in a dose-dependent fashion. 2) Overexpression of a dominant negative form of MyD88 abrogates CpG DNA-stimulated up-regulation of NF-κB. One critical issue is the contamination of DNA preparations with other bacterial components, in particular with LPS. To rule out that contaminating LPS could cause the cellular reaction, we used synthetic ODN free of endotoxin. In addition, relatively high concentrations of LPS are necessary to stimulate airway epithelial cells (11). Therefore, it is unlikely that contaminating LPS in the DNA preparations is responsible for the observed effects. The level of bacterial DNA or CpG ODN necessary to elicit a response differs significantly between airway epithelial cells and classical immune cells such as B lymphocytes or dendritic cells. In parallel, the response of APCs to other pathogen-associated molecular patterns such as LPS is more sensitive as compared with epithelial cells. Epithelial cells are exposed to the outer body surface and constantly in contact with bacterial components. If equipped with a highly sensitive pattern recognition apparatus, epithelial cells would be a constant source of proinflammatory mediators. The concentrations of bacterial DNA used in the present study (25 μg/ml) approximately correspond to a bacterial density of 2.5×10^8 CFU/ml. This bacterial load can be found in severely infected airways such as in cystic fibrosis (26). It is hard to calculate concentrations of specific molecules at the airway surface. Based on

the very small depth of the airway surface fluid, a minute amount of lysed bacteria could be sufficient to reach high concentrations of cellular components.

In addition to up-regulation of the proinflammatory mediators IL-6 and IL-8, we observed a local host defense reaction by induction of the antimicrobial peptide hBD-2. Antimicrobial peptides are important effector molecules of the innate immune system produced by epithelial cells and other cell types. The expression of hBD-2 is partly controlled by NF-κB (27).

In conclusion, we show that the human respiratory epithelium is capable of detecting microbial DNA motifs and of inducing a host defense reaction. TLR9 is expressed in airway epithelial cells and likely mediates the recognition of bacterial DNA. These findings highlight the function of the respiratory epithelium in triggering an innate immune response.

Acknowledgments

We thank A. Püchner and T. Damm for excellent technical support.

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