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Regulation of *DMBT1* via NOD2 and TLR4 in Intestinal Epithelial Cells Modulates Bacterial Recognition and Invasion[1]

Philip Rosenstiel,[2]* Christian Sina,[2]* Caroline End,[†‡] Marcus Renner,[†] Stefan Lyer,[†] Andreas Till,* Stephan Hellmig,[§] Susanna Nikolaus,*[§] Ulrich R. Fölsch,[§] Burkhard Helmke,[¶] Frank Autschbach,[¶] Peter Schirmacher,[¶] Petra Kioschis,[‡] Mathias Hafner,[‡] Annemarie Poustka,[†] Jan Mollenhauer,[3†] and Stefan Schreiber[3,4]*

Mucosal epithelial cell layers are constantly exposed to a complex resident microflora. Deleted in malignant brain tumors 1 (DMBT1) belongs to the group of secreted scavenger receptor cysteine-rich proteins and is considered to be involved in host defense by pathogen binding. This report describes the regulation and function of DMBT1 in intestinal epithelial cells, which form the primary immunological barrier for invading pathogens. We report that intestinal epithelial cells up-regulate DMBT1 upon proinflammatory stimuli (e.g., TNF-α, LPS). We demonstrate that *DMBT1* is a target gene for the intracellular pathogen receptor NOD2 via NF-κB activation. DMBT1 is strongly up-regulated in the inflamed intestinal mucosa of Crohn's disease patients with wild-type, but not with mutant NOD2. We show that DMBT1 inhibits cytoinvasion of *Salmonella enterica* and LPS- and muramyl dipeptide-induced NF-κB activation and cytokine secretion in vitro. Thus, DMBT1 may play an important role in the first line of mucosal defense conferring immune exclusion of bacterial cell wall components. Dysregulated intestinal DMBT1 expression due to mutations in the *NOD2/CARD15* gene may be part of the complex pathophysiology of barrier dysfunction in Crohn's disease. *The Journal of Immunology,* 2007, 178: 8203–8211.

Deleted in malignant brain tumors 1 (*DMBT1*)[5] belongs to the superfamily of scavenger receptor cysteine-rich (SRCR) genes (1, 2). It encodes for a large secreted human glycoprotein comprising 14 SRCR domains, the number of which may vary due to genetic polymorphisms, two C1s/C1r-Uegf-Bmp1 domains, and a C-terminal zona pellucida domain, which mediates oligomerization. Lung gp340 (DMBT1^{gp340}), and salivary agglutinin (SAG; DMBT1^{SAG}) represent the respiratory and oral variants of DMBT1 (3–7). It has been demonstrated that DMBT1 is predominantly expressed in epithelial cells, by which it is secreted in a polarized manner (e.g., to the extracellular matrix (squamous epithelia) or to the lumen (monolayered epithelia and glands); Refs. 2, 4, and 6). An up-regulation of DMBT1 was detected in pulmonary epithelial cells upon inflammatory stimuli in vitro and in vivo (8, 9). DMBT1^{gp340} interacts with the defense collectins surfactant protein A and surfactant protein D and stimulates migration of alveolar macrophages, suggesting a role for DMBT1 in the cross-talk between epithelial cells and the underlying mucosal immune cells (10). DMBT1^{SAG} has been shown to bind and aggregate a broad spectrum of Gram-positive and Gram-negative bacteria including the cariogenic *Streptococcus mutans*, *Staphylococcus aureus*, *Escherichia coli*, and *Helicobacter pylori* (11). A recent study has demonstrated the peptide, VEVL XXXXW, as the minimal bacteria-binding motif of the SRCR domains of DMBT1 (12). To date, it is unclear whether the binding of bacteria to DMBT1 molecules inhibits bacterial invasion or promotes adhesion to biological surfaces (13–16).

Loss of DMBT1 expression is an early and frequent event (84% of the cases) in tumors originating from squamous epithelia. However in gastrointestinal tumors, a more complex pattern of deregulated DMBT1 expression has been described, where the down-regulation of DMBT1 in dedifferentiated tumors appears to be preceded by a massive up-regulation of the transcript during tumorigenesis (4, 6, 17–19). Albeit distinct functional mechanisms of DMBT1 in the initiation or progression of gastrointestinal tumors remain yet to be shown, several lines of evidence support a role in epithelial differentiation and cell fate decision, e.g., a tight regulation of DMBT1 expression along the crypt axis with a predominant staining of proliferating compartments has been demonstrated in immunohistological studies (6, 9, 20) and an interaction with trefoil factors (21), important mediators of intestinal epithelial cell (IEC) migration and differentiation (22, 23). The rabbit ortholog of DMBT1 has been shown to be a regulator for the induction of polarity and terminal differentiation in kidney epithelial cells (24–26). A recent study demonstrated that DMBT1 is

*Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Kiel, Germany; [†]Division of Molecular Genome Analysis, Deutsches Krebsforschungszentrum, Heidelberg, Germany; [‡]Institute of Molecular Biology and Cell Culture Technology, University of Applied Sciences Mannheim, Mannheim, Germany; [§]Department of General Internal Medicine, University Hospital Schleswig-Holstein, Kiel, Germany; and [¶]Institute of Pathology, University of Heidelberg, Heidelberg, Germany

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[2] P.R. and C.S. contributed equally to this work.

[3] J.M. and S.S. share senior authorship.

[4] Address correspondence and reprint requests to Dr. Stefan Schreiber, Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Campus Kiel, Schittenhelmstrache 12, Kiel, Germany. E-mail address: s.schreiber@mucosa.de

[5] Abbreviations used in this paper: DMBT1, deleted in malignant brain tumors 1; rhDMBT1, recombinant human DMBT1; SRCR, scavenger receptor cysteine-rich; CD, Crohn's disease; MDP, muramyl dipeptide; NOD2, nucleotide binding and oligomerization domain 2; PAMP, pathogen-associated molecular pattern; IEC, intestinal epithelial cell; pIEC, primary IEC; SNP, single-nucleotide polymorphism; SAG, salivary agglutinin; IBD, inflammatory bowel disease; LB, Luria-Bertani; siRNA, small interfering RNA; wt, wild type.

regulated by the MAPK/ERK pathway in gastric epithelial cells (20). Up to now, however, no intracellular signaling pathways have been identified that might be modulated by DMBT1.

The putative dual role of DMBT1 for the integrity of the epithelial function prompted us to investigate its role in intestinal inflammation. We found that *DMBT1* gene expression is up-regulated in epithelial cell lines (HT-29, SW620, SW948, HeLa S3) and primary IECs (pIEC) by TNF-α and LPS. This up-regulation is dependent on NF-κB activation and NF-κB-sensitive elements in the *DMBT1* promoter. Recombinant DMBT1 inhibits TLR4-mediated cellular responses to LPS, i.e., NF-κB activation and cytokine secretion, and the cytoinvasion of *Salmonella enterica* into IECs. We further demonstrate that DMBT1 is part of the defense program activated by NOD2, an intracellular receptor for the bacterial wall component muramyl dipeptide (MDP). A frameshift mutation, *L1007fsinsC*, in the bacteria-sensing domain (leucine-rich region) of NOD2, which leads to a partial truncation of the leucine-rich region in the protein, and several other single-nucleotide polymorphisms (SNP) are associated with the development of Crohn's disease (CD), a human chronic relapsing-remitting inflammatory bowel disease (27–29). DMBT1 is strongly up-regulated in inflammatory bowel disease (IBD) and the *NOD2* frameshift mutation found in CD patients is associated with a significantly lower DMBT1 expression in the affected mucosa of the patients. Thus, DMBT1-mediated protection and its anti-inflammatory effects could be important for mucosal homoeostasis and for the etiology of CD.

Materials and Methods

Cell culture experiments, cloning, and transfections

Human epithelial HeLa S3 cells (ACC 161), intestinal epithelial HT-29 cells (ACC 299), Caco-2 cells (ACC 169), SW480 cells (ACC 313) and myelomonocytic THP-1 cells (ACC 16) were purchased from the German Collection of Microorganisms and Cell Cultures. SW620 and SW948 cells were a gift from H. Kalthoff (Department of Surgical Oncology, University Hospital Schleswig-Holstein, Kiel, Germany).

For the *DMBT1* promoter studies, we amplified a 906-bp product comprising bp -1 to -906 from human genomic DNA using the primers 5′-CGCTCGAGCTTCCAAGGTGAGGTTACTAGTACT-3′ and 5′-GCAAGCTTAATATAAAGGAAGTGAGGGC-3′ The product was cloned into the pGL3-basic plasmid (Promega) in front of a luciferase reporter gene. For NF-κB activity measurements, the synthetic construct pNF-κB Luc (Stratagene) was used. Luciferase activity was normalized against *Renilla* activity under the control of a constitutive promoter (pRL-TK; Promega). Transfections were performed using Fugene 6 (Roche) according to the manufacturer's protocol, and the relative luciferase activities were determined as described in Ref. 30. The MD2 expression construct was constructed by amplifying the whole open reading frame of MD2 from leukocyte cDNA (Clontech). The resulting fragment was cloned into the pcDNA4TOPO vector (Invitrogen Life Technologies). The TLR4 expression plasmid was a gift from M. Rehli (University of Regensburg, Regensburg, Germany) and has been described before (31). All constructs were sequence verified in an ABI3700 sequencer (Applied Biosystems) before use. An expression plasmid encoding a mutant human IκBα which cannot be phosphorylated upon stimulation (IκBα-SR) was described earlier (32) and was obtained from Clontech. TNF-α was purchased from R&D Systems. MDP was from Bachem. Ultrapure LPS from *S. enterica* serovar Friedenau was a gift from H. Brade (Research Center Borstel, Borstel, Germany).

Isolation of primary epithelial cells

Epithelial cell preparation was conducted using a standard protocol as described (30). The number and viability of the crypt epithelial cells were determined by 0.1% trypan blue exclusion. The purity of the epithelial cell preparation was confirmed by routine H&E staining, showing >92% of epithelial cells. For stimulation experiments, pIECs (1×10^6) were resuspended in 1000 μl of minimal essential medium supplemented with Earle's salts, 20% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamicin. The cells were incubated at 37°C in air with 10% CO_2. Cell viability was again determined by trypan blue exclusion after 12 h of culture.

Table I. *Clinical characteristics of the CD patient groups and controls*[a]

	Controls	CD NOD2 wt	CD NOD2 Mutated
N	19	22	20
Age (median)	39	34	38
Gender (F/M)	10/9	12/10	10/10
Median CDAI	N/A[b]	264 (220–320)[c]	280 (225–310)
Disease duration	N/A	10	13
Treatment			
5-Aminosalicylates	N/A	19	17
Corticosteroids	N/A	10	9
Azathioprine	N/A	8	8

[a] Characteristics of the two CD patient groups (NOD2 wild-type genotype or carrying the CD-associated NOD2 SNP13 allele), and controls. In the case of non-inflamed hospitalized controls, colonoscopy was performed for cancer surveillance, diarrhea, and anemia but revealed no significant colonic pathology.

[b] N/A, Not applicable.

[c] Numbers in parentheses, Range.

RNA isolation and RT-PCR

Total RNA was isolated using the RNeasy kit from Qiagen. Total RNA (500 ng) was reverse transcribed as described elsewhere (33). DMBT1-specific primers for PCR: DMBT1, sense 5′-TGGGACATTGAGGTGCAAAAC-3′; DMBT1, antisense 5′-TGGGACATTGAGGTGCAAAAC-3′. Expected amplicon length: 537 bp. PCR: Denaturation for 5 min at 95°C; 27 cycles of 30 s at 95°C, 20 s at 60°C, 90 s at 72°C; final extension for 10 min at 72°C. To confirm the use of equal amounts of RNA in each experiment, all samples were monitored in parallel for β-actin mRNA expression. Endpoint analyses of all amplified DNA fragments was done by separation on 1% agarose gels and subsequent analysis with a BioDoc analyzer (Biometra) for densitometric measurement.

Patient samples for real-time PCR

For the quantitative real time-PCR studies, we used total RNA extracted from snap-frozen colonic biopsies. The biopsy bank from the outpatient clinic of the Department of General Internal Medicine, University Hospital Schleswig-Holstein (Kiel, Germany) was screened for CD patients with active ileocolonic disease carrying the NOD2 SNP13 (*L1007fsinsC*) mutation. All patient samples (pairs of matching DNA/colonic biopsy) and phenotype information were pseudonymized before the procedure. After determination of the NOD2/CARD15 status, 42 CD specimens were selected for further study (22 NOD2/CARD15 wild-type (wt), 14 NOD2/CARD15 SNP13 heterozygotes, 6 NOD2/CARD15 SNP13 homozygotes). The 42 CD patients had a similar CD activity index range and were pretreated with 5-aminosalicyates, corticosteroids ($\leq$20 mg), or azathioprine according to their clinical requirements without any systematic differences between patients with NOD2 mutated or wild-type genotypes. Medication was stable for 4 wk before sampling. Patient characteristics are given in Table I. Nineteen hospitalized patients with a routine colonoscopy lacking significant pathology were randomly selected as controls. All biopsies were sampled from the colon; samples were taken from macroscopically and microscopically inflamed and uninflamed mucosa, if possible. The inflammatory activity was independently scored by two investigators. The diagnosis was based on standard criteria using radiological and endoscopic findings in every case. All patient-related procedures were approved by the university hospital ethics committee. All patients agreed to participation by giving informed consent at least 24 h before the study.

Quantitative real-time PCR

Single-stranded cDNA synthesis was done using 300 ng of total RNA and oligodepxythymidylate priming according to standard procedures. For the quantitative real-time PCR (TaqMan), we used 1/60 of the single-stranded cDNA (corresponding to 5 ng of reversely transcribed total RNA) per reaction and Taqman assays for β-actin (human) and *DMBT1* (Hs00244838_m1; Applied Biosystems) using the following cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles including 15 s at 95°C and 1 min at 60°C. All PCRs were done in triplicate. Signal detection was conducted using an ABI Prism 7900HT detection system (Applied Biosystems), and gene expression levels were determined by interpolation of threshold cycle (C_t) values to a standard curve generated from a dilution series of human small intestine cDNA. C_t values for *DMBT1* were normalized against C_t values obtained for the housekeeping gene β-actin. Statistically significant differences between experimental groups were determined using a Mann-Whitney U test. $p < 0.05$ was considered significant.

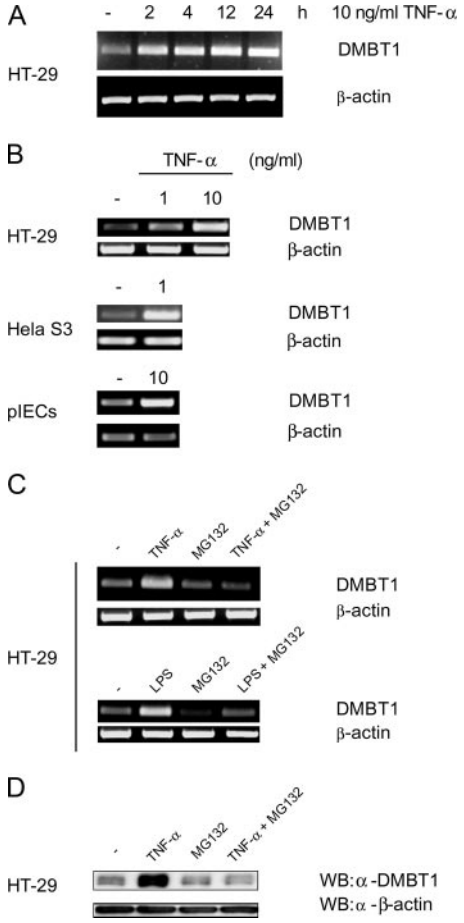

FIGURE 1. Detection of DMBT1 mRNA by RT-PCR and Western blot in IECs. *A*, Kinetics of DMBT1 expression in HT-29 cells treated with TNF-α (10 ng/ml). *B*, Stimulation of HT-29, HeLa-S3 (control) and pIECs with different concentrations of TNF-α (1–10 ng/ml) for 12 h. *C*, Stimulation of HT-29 for 12 h with TNF-α (10 ng/ml), LPS from *S. enterica* (100 ng/ml), and/or the proteasome inhibitor MG132. β-Actin was amplified in parallel in all experiments from the same RNA samples and analyzed on a separate gel (*bottom panels*). *D*, Corresponding Western blot analysis of HT-29 cells stimulated with TNF-α and/or MG132. DMBT1 was detected using the anti-DMBT1h12 Ab. The loading of equal amounts of proteins was controlled by stripping of the blots and reprobing with an anti-β-actin Ab. Representative example of five independent experiments.

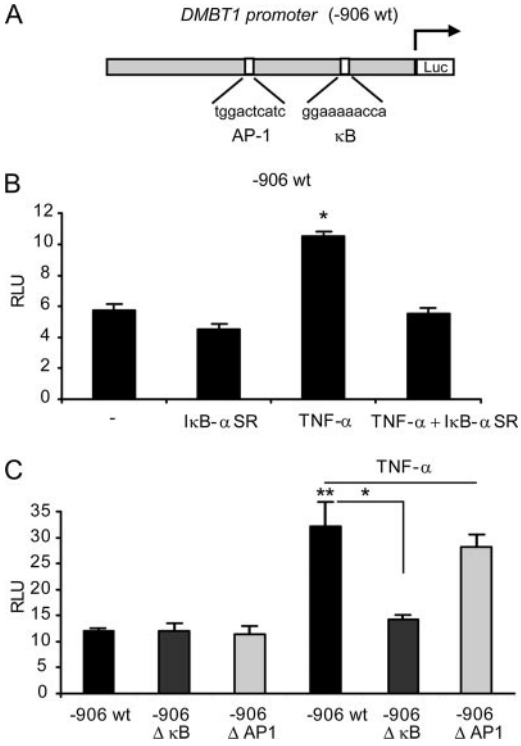

FIGURE 2. *A*, Illustration of the pGL3B-DMBT1wt^{-906} construct and location of putative NF-κB- and AP-1-binding elements. *B*, Down-regulation of the TNF-α response of the DMBT1 promoter (−906) by NF-κB inhibition (overexpression of an IκBα superrepressor; IκBα-SR) in HeLa cells. Cells transfected with the pGL3-Basic plasmid (vector) served as negative controls. *C*, Effect of the deletion of the putative κB ($\Delta\kappa$B)- or AP1-element (ΔAP-1) in the reporter construct upon TNF-α treatment in HeLa cells. *, $p < 0.05$, **, $p < 0.01$. RLU, Relative luciferase unit.

Western blot analyses

Western blots were performed as previously described (33). For detection of human DMBT1, we applied a monoclonal anti-DMBT1h12 Ab (4). For signal detection, we used a chemoluminescence detection kit (Amersham Biosciences) as recommended by the supplier. A monoclonal β-actin control Ab was purchased from Sigma-Aldrich and used according to the manufacturer's instructions.

In situ hybridization and immunohistochemical analyses

Paraformaldehyde-fixed (4% w/v in PBS), paraffin-embedded tissue sections (3–4 μm) were prepared and analyzed with polyclonal antiserum anti-DMBT1p84 (1/100), which was previously confirmed for its specificity (M. M. Renner, G. Bergmann, I. Krebs, C. End, S. Lyer, F. Hilberg, B. Helmke, N. Gassier, F. Autschbach, F. Bikker, O. Strobel-Freidekind, S. Gronert-Sum, A. Benner, S. Blaich, R. Wittig, M. Hudler, A. J. Ligtenberg, J. Madsen, U. Holmskov, V. Annese, A. Latiano, P. Schirmacher, A. V. Nieuw Amerongen, M. D'Amato, P. Kloschis, M. Hafner, A. Poustka, and J. Mollenhauer, manuscript in preparation). Tissue specimens were resected for diagnostic or therapeutic purposes. For the in situ and immunohistochemistry studies these comprised formalin-fixed paraffin-embedded distal ileum and proximal colon sections from 41 CD patients (20 female and 21 male samples) from Department of Pathology, Heidelberg. As controls served disease-free sections comprising 101 samples from distal ileum/proximal colon (controls: 36 female and 65 male samples) from non-IBD patients. For in situ hybridization, a 0.4-kb fragment of the *DMBT1* cDNA (SID5 to SID6) was subcloned into the pCRII-TOPO vector (Invitrogen Life Technologies). One microgram of *Xho*I or *Bam*HI-linearized plasmid DNA was transcribed in vitro using SP6 polymerase (antisense strand) or T7 polymerase (sense strand), respectively, in the presence of digoxygenin-labeled UTP. Afterward, we proceeded as described earlier using a hybridization temperature of 47°C and an NEL700 detection kit (PerkinElmer). The immunohistochemical studies were conducted using the protocol previously described for the mAb anti-DMBT1h12 and a hematoxylin counterstaining (6) except that we used a polyclonal antiserum anti-DMBT1p84, an automated Ventana Discovery stainer (Ventana Medical Systems), and a DAP-Map staining kit according to the instructions recommended by the supplier.

Recombinant expression and purification of human DMBT1

Detailed protocols on molecular cloning, the generation of stably transfected cell lines, and the purification of recombinant human DMBT1 (rhDMBT1) have been published elsewhere (34). Briefly, the largest known *DMBT1* open reading frame, corresponding to the *DMBT1*/8kb.2 variant (EMBL accession number AJ243212), was cloned into the pT-Rex-DEST30 vector (Invitrogen Life Technologies) under the control of a tetracycline-inducible promoter.

Gentamicin protection assay

Bacterial invasion was assessed by a gentamicin protection assay. HT-29 cells were seeded at a concentration of $\sim 3 \times 10^5$ in 24-well plates. Medium was replaced with fresh antibiotic-free medium 24 h before the invasion assay. Overnight bacterial cultures grown in Luria-Bertani (LB) medium were washed twice with PBS, resuspended in HBSS, and added to target cells in a multiplicity of infection ratio of 100. Infected cells were incubated at 37°C for 2 h to allow bacteria to enter the epithelial cells. After infection, cells were washed twice with PBS, and fresh medium containing

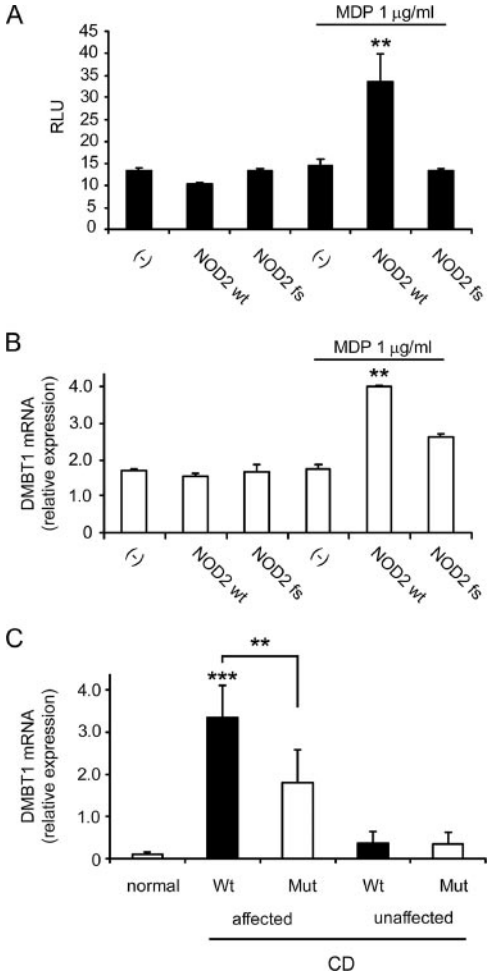

FIGURE 3. DMBT1 mRNA expression in response to NOD2 signaling in vitro and in vivo. *A*, The DMBT1 promoter is *trans*-activated in HT-29 cells by NOD2-mediated MDP sensing. A plasmid carrying the NOD2 L1007fs (fs; SNP13) does not lead to DMBT1 promoter activation upon MDP stimulation. *B*, Real time PCR analysis of NOD2-mediated signaling on DMBT1 mRNA expression in HT-29 cells. *C*, Colonic mRNA expression levels of DMBT1 in controls and Crohn's disease patients. Values are means of relative transcript levels (SEM). ***, $p < 0.001$; **, $p < 0.01$ (Mann-Whitney *U* test) Wt, NOD2 wild-type; Mut, NOD2 mutation L1007fs (SNP13); RLU, relative luciferase unit.

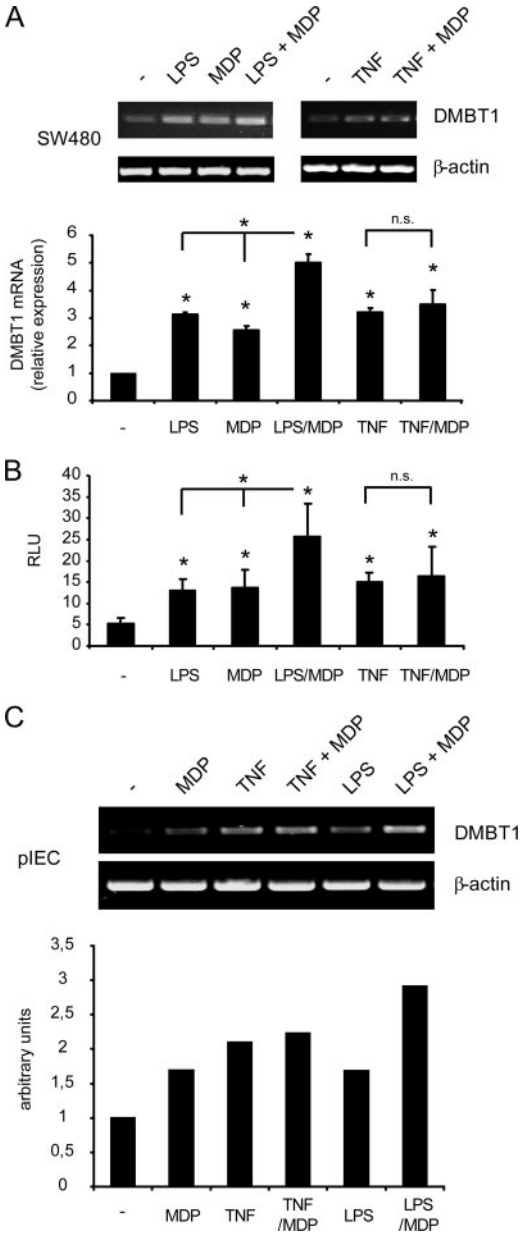

FIGURE 4. LPS and MDP synergistically regulate DMBT1 mRNA expression in intestinal epithelial cells. *A*, MDP-responsive SW480 IECs were stimulated with LPS (100 ng/ml), MDP (1 μg/ml), and TNF-α (10 ng/ml) alone or in combination for 12 h, and RT-PCR for DMBT1 was performed. β-Actin was amplified in parallel from the same RNA samples and analyzed on a separate gel (*bottom panel*). The bar graph represents relative transcript levels; results are means + SD of three independent experiments. *B*, *trans* activation of the DMBT1 promoter (−906)-driven reporter gene construct was assessed in the same cell line by dual luciferase assay (mean + SD; ∗, $p < 0.05$). *C*, DMBT1 and β-actin transcript levels from isolated pIECs treated with LPS (100 ng/ml), MDP (1 μg/ml), and TNF-α (10 ng/ml) alone or in combination were analyzed by RT-PCR. Representative of two independent experiments performed in duplicate. The histograms represent means of the densitometric analysis. RLU, Relative luciferase unit.

gentamicin (50 μg/ml) was added. Cells were incubated in medium supplemented with gentamicin for an additional 1.5 h to assure killing of extracellular bacteria.

In parallel assays, the same bacterial cultures used for stimulation of cells were incubated with gentamicin for 1.5 h and inoculated in LB medium to confirm the absence of extracellular bacteria. After incubation with gentamicin, cells were washed twice with PBS and lysed with 1% Triton X-100 for 5 min. The lysates were collected and plated on LB agar. Plates were placed at 37°C overnight, and colonies were counted the next day. Vitality of cells before and after the infection was assessed by trypan blue exclusion assay. Invasion was calculated on the basis of number of CFUs recovered from each well and expressed as mean ± SEM.

Small interfering RNA (siRNA) transfection

SW480 intestinal epithelial cells were transfected with three different siRNAs against DMBT1 (Invitrogen Stealth Select HSS102811, HSS102812, and HSS102813) or a scrambled control without any homology to known transcripts. After 8 h, transfection medium was replaced with regular cell culture medium. After 72 h, gentamicin protection assays were performed as described.

IL-8 ELISA

Supernatants of THP-1/HEK 293 cultures were collected after 24 h with or without stimulation with LPS (100 ng/ml). IL-8 was measured by ELISA (R&D Systems) according to the manufacturer's protocol. The results were expressed as picograms of cytokine per 5×10^5 cells.

Statistical analysis

Normality of the data was checked by calculating Lilliefors probabilities based on the Kolmogorov-Smirnov test. FACS and cell culture data followed a normal distribution; their significances were determined by the *t* test for independent samples. Statistical significance of the nonnormally

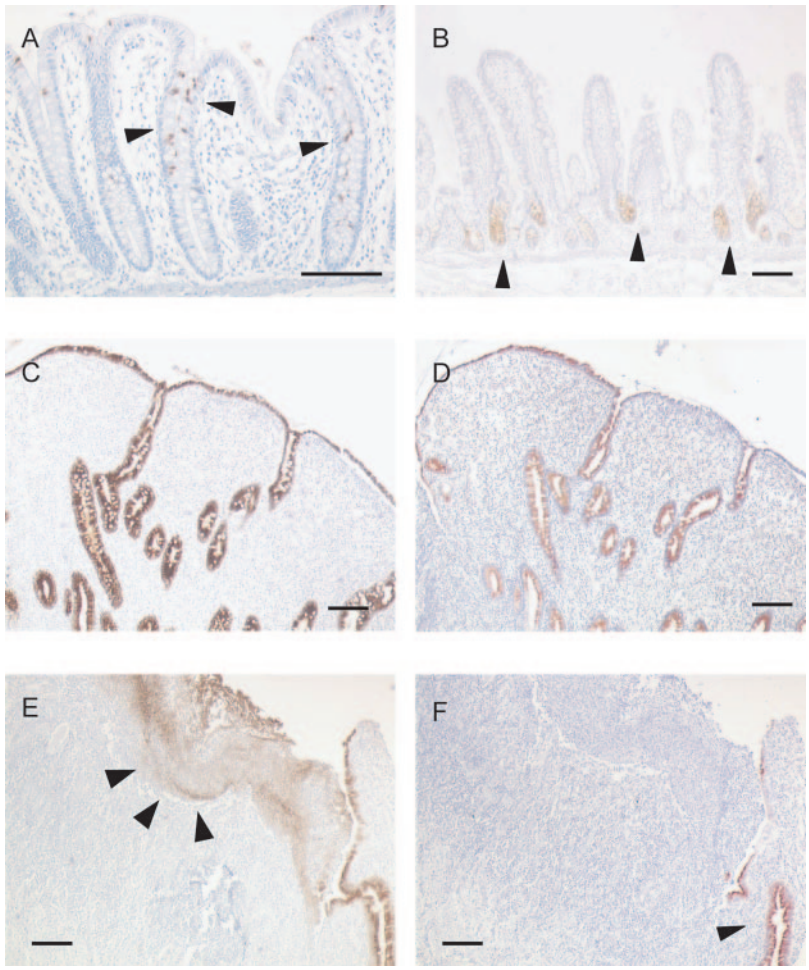

FIGURE 5. DMBT1 expression and localization in the human normal and IBD tissues. Depicted are immunohistochemical analyses (anti-DMBT1p84; *A*, *B*, *C*, and *E*) and mRNA in situ hybridization. *D* and *F*, Normal colon (scale bar, 200 μm, *A*). Arrowheads, Focal expression of DMBT1 in colonic epithelial cells. Normal ileum (scale bar, 200 μm, *B*). DMBT1 up-regulation in colonic epithelial cells in CD (scale bars, 200 μm, *C–F*). Association of DMBT1 produced by epithelial cells with debris at the surface of an ulcer (CD case; scale bars, 200 μm, *E*). The in situ signal is missing in the noncellular debris (*F*). Representative pictures from 41 CD patients and 101 disease-free controls.

distributed patient data was tested using the Mann-Whitney *U* test. Experiments and measurements were replicated at least three times.

Results

DMBT1 up-regulation in IECs by proinflammatory stimuli

To study a potential link between DMBT1 and inflammatory pathways, we first monitored DMBT1 expression in epithelial cell lines and pIECs stimulated with TNF-α. Time course studies with HT-29 colonic adenocarcinoma cells revealed that 10 ng/ml TNF-α results in a robust DMBT1 up-regulation as early as 2 h after the treatment, which is still detectable after 24 h (Fig. 1*A*). TNF-α stimulation consistently resulted in an up-regulation of the DMBT1 mRNA in epithelial HeLa S3 cells, as well as in pIECs (Fig. 1*B*); a similar up-regulation was detected in SW480, SW620, and SW948 colonic epithelial cells (data not shown).

In addition, the bacterial cell wall component LPS caused an up-regulation of DMBT1 expression in HT-29 cells (results included in Fig. 1*C*). Addition of the proteasome inhibitor MG132, which blocks NF-κB activation via inhibition of the proteasomal decay of the endogenous NF-κB inhibitor IκBα inhibited both LPS- and TNF-α-induced DMBT1 up-regulation. To demonstrate the up-regulation on the protein level, Western blot experiments were conducted in HT-29 cells. An MG132-sensitive increase in DMBT1 protein levels could also be detected after 12 h of stimulation with 10 ng/ml TNF-α (Fig. 1*D*). Basal DMBT1 mRNA levels were not detectable in unstimulated myelomonocytic THP-1 cells (even when using 40 cycles instead of 27 cycles in intestinal epithelial cells). Only after costimulation with 50 ng/ml LPS and 1 μg/ml MDP or 50 ng/ml TNF-α was a weak up-regulation of the transcript observed (data not shown).

These data suggested that DMBT1 is inducible in epithelial cells by proinflammatory stimuli, which possibly includes activation via the NF-κB pathway.

TNF-α-induced activation of the DMBT1 promoter depends on the activation of NF-κB

To confirm the involvement of NF-κB in the regulation of *DMBT1*, we next transfected a luciferase reporter gene construct driven by the *DMBT1* promoter (pGL3B-DMBT1^{-906}) (Fig. 2*A*) into HeLa S3 cells. Determination of luciferase levels demonstrated TNF-α inducibility of the *DMBT1* promoter, which could be suppressed by cotransfection of a vector encoding a mutant IκBα, which cannot be phosphorylated and degraded upon stimulation and thus serves as a superrepressor of NF-κB activation (Fig. 2*B*). At bp -323 of the DMBT1 promoter, computational analyses identified a DNA element with high homology to the NF-κB binding consensus sequence (5′-GGGGAAGCCC-3′, κB in Fig. 2*A*). Deletion of the κB-site resulted in significantly decreased TNF-α-induced *trans* activation of the *DMBT1* promoter (Fig. 2*C*). By contrast, deletion of a putative AP-1 binding site at bp -439 (sequence; AP-1 in Fig. 2*A*) had no significant effect on either basal or TNF-α-responsive *DMBT1* promoter activity (Fig. 2*C*). In conclusion, these data demonstrate that TNF-α responsiveness of DMBT1 is mediated by NF-κB.

DMBT1 regulation in the inflamed intestinal mucosa: influence of the NOD2 genotype

We next investigated whether DMBT1 expression might also be regulated by NOD2, which activates the canonical NF-κB pathway after recognition of cytosolic MDP. HT-29 cells were transiently

transfected with the luciferase reporter vector driven by the DMBT1 promoter and plasmids encoding wild-type NOD2 or the CD-associated NOD2 L1007fsinsC (SNP13) variant. Stimulation of the cells with the NOD2-ligand MDP (1 μg/ml) resulted in an up-regulation of DMBT1 luciferase activity only in the NOD2 wt, but neither in the NOD2 L1007fsinsC nor in the cells transfected with an empty vector (Fig. 3*A*). The results were confirmed by quantitative real-time PCR, where a significant up-regulation of DMBT1 mRNA also could be demonstrated only in the NOD2 wt-transfected cells upon MDP stimulation (Fig. 3*B*).

To investigate whether the intestinal DMBT1 expression levels are also affected by the respective NOD2 genotypes, we performed quantitative real-time PCR in colonic samples from CD patients and normal controls (Table I). Of 42 patients, 20 were positive for the L1007fsinsC (SNP13) variant (14 heterozygotes and 6 homozygotes).

In noninflamed colonic tissue, no differences in DMBT1 expression levels were detected in CD patients compared with controls. In inflamed colonic CD samples from the NOD2 wt group, DMBT1 mRNA level were increased >3-fold (***, $p < 0.001$). However, CD patients with the NOD2 SNP13 variant failed to show increased DMBT1 expression in inflamed colonic tissue (Fig. 3*C*). The level of DMBT1 expression was significantly lower in NOD2-mutated than in wt CD patients (**, $p < 0.01$; Fig. 3*C*). There was a tendency toward a lower DMBT1 expression in the SNP13 homozygous vs the heterozygous individuals; however, the difference between the two groups did not reach significance ($p > 0.05$; data not shown). The mRNA expression levels of proinflammatory cytokines (TNF-α and IL-8) did not differ significantly between the affected CD groups (wt and SNP13), thus ruling out a bias from different inflammatory activities among the experimental groups (data not shown).

Additive effects of NOD2 and TLR4-dependent signaling lead to an up-regulation of DMBT1

To test whether a cross-talk between NOD2 activation and other proinflammatory signaling pathways is involved in the regulation of *DMBT1* expression and may be responsible for the differences observed in the patient samples, we investigated the effect of LPS and TNF-α alone and in combination with MDP in the MDP-responsive IEC line SW480. An additive up-regulation of DMBT1 transcript levels was observed after 12 h of stimulation with both LPS (100 ng/ml) and MDP (1 μg/ml). The simultaneous application of TNF and MDP did not result in an additive regulation of DMBT1 transcript levels (Fig. 4*A*). Similar results were obtained when SW480 cells were transfected with the luciferase reporter gene construct pGL3B-DMBT1^{-906}. LPS and MDP act in cooperation to induce luciferase activity, whereas the combination of TNF-α and MDP failed to show a cooperative effect (Fig. 4*B*).

Using purified primary intestinal epithelial cells, we demonstrate that simultaneous application of LPS (100 ng/ml) and MDP (1 μg/ml) for 12 h considerably enhanced *DMBT1* mRNA expression compared with stimulation with individual agonists alone. No increase of DMBT1 mRNA above levels with TNF-α alone was observed by costimulation with TNF-α and MDP (Fig. 4*C*). The results demonstrate that the NOD2 agonist MDP and the TLR4 ligand LPS additively induce DMBT1 expression in intestinal epithelial cells.

Localization of DMBT1 expression in the human intestine

By immunohistochemical analyses using a polyclonal DMBT1 antiserum and mRNA in situ hybridization, we identified epithelial cells as the main source of DMBT1 expression in the human intestine (Fig. 5). Immunoreactivity was confined to single cells or few crypts within the epithelium of the proximal and the distal colon (Fig. 5*A*). In the ileum, staining was localized in the lower crypts, where also Paneth cells reside, which are known to constitutively express *NOD2/CARD15* (Fig. 5*B*). No staining was observed in cells of the lamina propria. Similar results were obtained for RNA in situ hybridization (data not shown). Sections of biopsies from inflamed regions of CD patients showed a strong up-regulation in the entire surface epithelium and intense staining of debris covering ulcer lesions (representative examples in Fig. 5, *C* and *E*). Interestingly, the pattern seen in CD ulcers seems to reflect the deposition of secreted DMBT1 in debris, given that only the adjacent epithelium was positive in the respective in situ hybridization (Fig. 5, *D* and *F*).

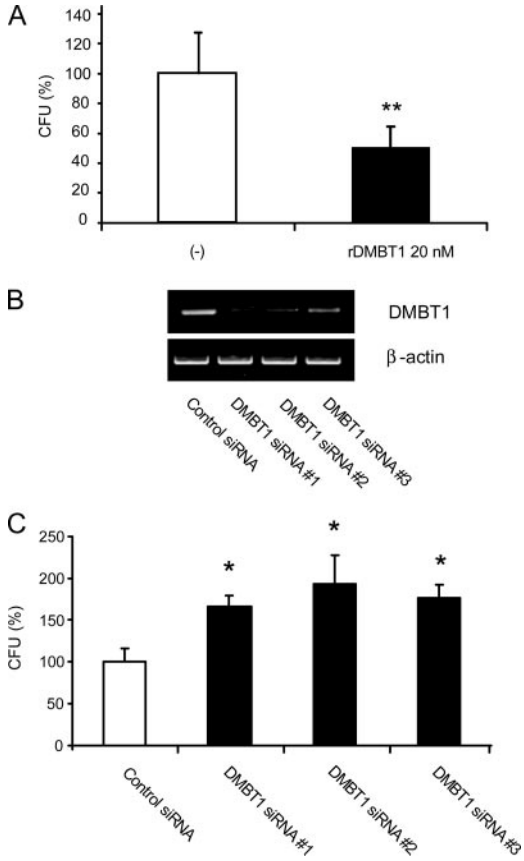

FIGURE 6. Gentamicin protection assay. *A*, HT-29 cells were infected with invasive *S. enterica* in the absence or presence of 20 nM rhDMBT1, and intracellular bacteria were determined after killing of extracellular bacteria. **, $p < 0.0077$. SW480 cells were transfected with control siRNA (*ctrlsiRNA*) or three different siRNAs targeting DMBT1 (*DMBT1si1–3*). After 72 h, DMBT1 knockdown was controlled by RT-PCR (*B*) and cells were infected with invasive *S. enterica* (multiplicity of infection, 100). Invasion of bacteria was assessed by a gentamicin protection assay (*C*; *, $p < 0.02$). Values are the means of five independent experiments each done in triplicate.

DMBT1 inhibits bacterial cytoinvasion in intestinal epithelial cells

Based on its ability to bind bacteria, DMBT1 was proposed to either prevent infection by aggregation of bacteria or to promote infection by allowing for bacterial adhesion; however, mechanistic analyses with regard to its role in bacterial cytoinvasion or innate immune recognition have not yet been conducted. To address the consequences of DMBT1 up-regulation in IECs through proinflammatory stimuli, we next studied the effect of rhDMBT1 on bacterial invasion and innate immune sensing in vitro.

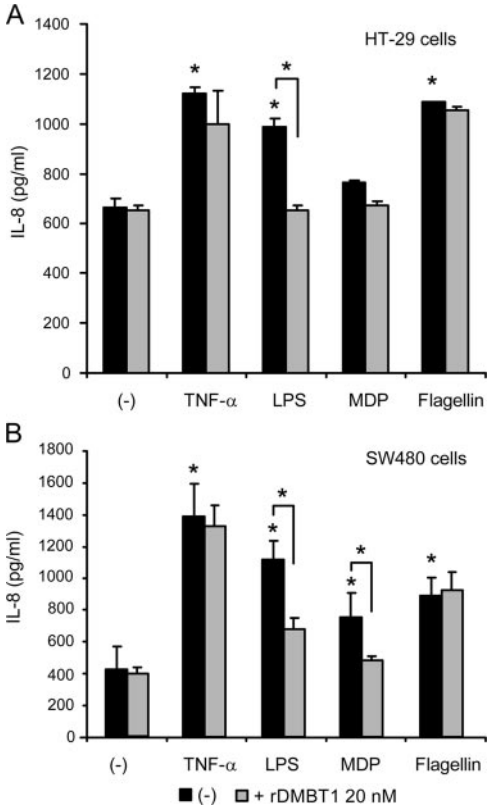

FIGURE 7. Influence of DMBT1 on IL-8 secretion of HT-29 (*A*) and SW480 (*B*) IECs. Cells were stimulated with TNF-α, LPS, MDP, or flagellin (200 ng/ml) in the presence or absence of 20 nM rDMBT1. Secretion of IL-8 was detected by ELISA. Values are expressed as mean + SEM. Values are means of three independent experiments in triplicate. ∗, $p < 0.02$.

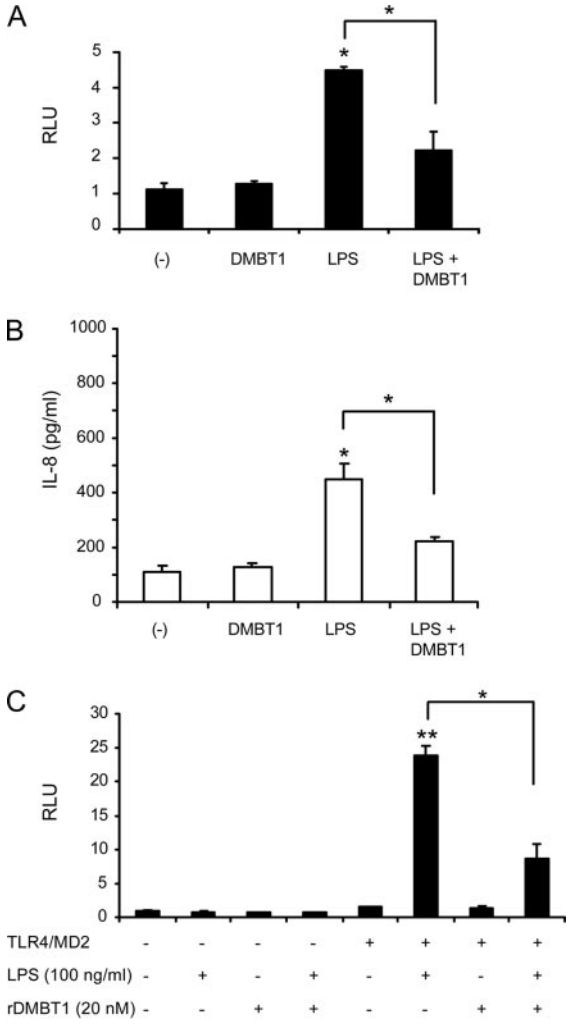

FIGURE 8. DMBT1 inhibits TLR4-mediated NF-κB activation and expression of IL-8 in myelomonocytic cells. *A*, LPS (*S. enterica*)-induced NF-κB-activation is inhibited by rhDMBT1 (20 nM) in THP-1 cells (dual luciferase assay). *B*, ELISA analyses of IL-8 production by THP-1 cells. rhDMBT1 (20 nM) inhibits IL-8 production induced by *S. enterica* LPS (100 ng/ml). $n = 3$ in triplicate; ∗, $p < 0.05$. *C*, NF-κB reporter assays in HEK293 cells. Upon cotransfection of TLR4 and MD-2, HEK293 cells become LPS responsive. Addition of 20 nM rDMBT1 inhibits LPS-induced TLR4/MD-2-mediated NF-κB activation, demonstrating that DMBT1 is a negative regulator of TLR4-induced signaling. RLU, Relative luciferase units; results are means of five independent experiments (SD); ∗∗, $p < 0.01$; ∗, $p < 0.05$).

At a concentration of 20 nM (lowest concentration tested), rhDMBT1 resulted in a visible aggregation of enteroinvasive *S. enterica* serovar Typhimurium (data not shown). Moreover, in gentamicin protection assays, rhDMBT1 was able to reduce intracellular invasion of intestinal epithelial HT-29 cells by ~50% (∗∗, $p = 0.0077$ according to the two-tailed Student *t* test; Fig. 6*A*). Similar results were obtained in the IEC lines Caco2 and SW480 (data not shown). The domains present within DMBT1 have no known degrading or lytic activities (12). Accordingly, rhDMBT1 did not reduce growth rates of *E. coli* or *S. enterica* when applied onto culture plates, and rhDMBT1 did not enhance bacterial lysis in vitro (data not shown).

To further demonstrate the role of endogenous *DMBT1* expression on bacterial invasion, we used a siRNA-based approach to down-regulate the basal levels of DMBT1 in SW480 IECs (Fig. 6*B*). A significant increase of bacterial invasion was observed with all individual siRNAs when compared with a nonsense siRNA.

DMBT1 acts as an immune exclusion factor and inhibits TLR4-mediated recognition of LPS

To test the effect of DMBT1 on innate immune recognition of bacterial pathogen-associated molecular patterns (PAMP), we next studied NF-κB-activation and cytokine secretion induced by LPS (from *S. enterica*), flagellin, and MDP. As a specificity control, the cytokine TNF-α was used. In two IEC lines (HT-29 and SW480) the addition of recombinant rhDMBT1 significantly inhibited the LPS-induced release of the NF-κB-dependent cytokine IL-8, whereas no effect on TNF-α- and flagellin-induced IL-8 secretion was observed (Fig. 7). In SW480 cells, which are known to express functional NOD2 and to respond to MDP, addition of recombinant DMBT1 also led to a significant decrease of MDP-induced IL-8 secretion (Fig. 7*B*).

Similar effects of rhDMBT1 were detected in the myeloid cell line THP-1. The addition of rhDMBT1 significantly inhibited LPS-induced NF-κB activation and IL-8 release of THP-1 cells (Fig. 8, *A* and *B*) pointing to a possible paracrine role of IEC-derived DMBT1 also on other cell types present in the intestinal mucosa.

TLR-4 and its cofactor MD-2, both expressed by THP-1 cells, trigger NF-κB activation and IL-8 secretion after binding of *Salmonella* LPS, raising the possibility that extracellular DMBT1 might inhibit TLR-4 signaling. By cotransfection of TLR-4 and MD-2, LPS-unresponsive HEK293 cells became responsive, as determined by an NF-κB reporter assay (Fig. 8*C*). Addition of 20 mM rhDMBT1 inhibited LPS-induced NF-κB activation in the TLR-4/MD-2-positive HEK293 cells (Fig. 8*C*).

We thus conclude that secretion of DMBT1 may prevent bacterial invasion into IECs and acts as a mucosal immune exclusion factor by inhibiting TLR4- and NOD2-mediated NF-κB activation and IL-8 production.

Discussion

Barrier integrity of the intestinal mucosa is maintained by a combination of mechanical, biochemical, and immunological mechanisms. The salient finding of the present study is the observation that DMBT1, a secreted scavenger receptor cysteine-rich protein with proposed functions in tumor suppression and binding of a broad range of bacteria, is a downstream target of NOD2 that mediates pathogen defense, exerts anti-inflammatory effects, and is inactivated by NOD2 mutation in the intestine of CD patients.

We present in vivo evidence that the PAMP receptor NOD2 plays a decisive role for DMBT1 expression in the inflamed intestinal mucosa. CD patients with the NOD2 mutation L1007fsinsC display a significant deficit in the induction of DMBT1 in the inflamed mucosa. This finding is not due to a milder inflammatory activity given that CD activity index, endoscopic appearance, and mucosal TNF-α levels were similar in both NOD2 wt and L1007fsinsC patients.

Interestingly, we show that MDP and LPS cooperatively up-regulate *DMBT1* gene expression in IECs. Taken together with recent reports demonstrating the importance of the NOD2/TLR4 cross-talk in the induction of inflammatory responses, it is tempting to speculate that disturbed additive signals of NOD2 and TLR4 are responsible for the decreased mucosal DMBT1 mRNA levels in the L1007fsinsC patients.

Recent findings on the importance of NOD2-mediated pathogen sensing for the intestinal barrier integrity have shed new light on the pathophysiology of CD and parallel our observations regarding DMBT1 expression (35). Genetic variations in the ligand-recognition domain of NOD2, which have been linked to susceptibility for CD, are associated with diminished levels of ileal and colonic α-defensins in the inflamed mucosa (36). Interestingly, *Nod2*-deficient mice exhibit lower expression levels of cryptdins, the murine orthologs of human α-defensins, and exhibit a higher susceptility to oral infection with *Listeria monocytogenes* (37). NOD2 regulates IL-8 expression and secretion in IECs (30) and mediates intracellular bactericidal activity possibly via interaction with GRIM-19, a protein with homology to the NADPH dehydrogenase complex (38). These findings taken together with experiments reported here suggest that NOD2 is at the apex of a protective program, which regulates the delicate balance between host intestinal epithelium and the mucosal flora.

DMBT1 has been shown to bind and aggregate bacteria and to inhibit viral infection (11, 12, 39). Thus, ligand binding and aggregation is a recurrent motif that DMBT1 shares with other archaic SRCR proteins (40–44). We show that aggregation of *S. enterica* by DMBT1 diminishes the cytoinvasive capacity into IECs (Fig. 6) and that DMBT1 inhibits LPS- and MDP-induced IL-8 secretion (Fig. 7). Whereas intestinal epithelial cells seem to be the primary source of DMBT1 in intestinal inflammation, the results obtained from myelomonocytic THP-1 cells suggest that secreted DMBT1 may have profound effects on other cell populations of the mucosa by neutralizing the biological activity of the bacterial PAMPs LPS and MDP.

We demonstrate in HEK293 and THP-1 cells that interaction of DMBT1 with LPS specifically inhibits TLR4-induced NF-κB reporter gene activity. Because DMBT1 itself was also shown to be regulated by LPS and MDP via NF-κB, it is likely that DMBT1 represents the extracellular part of an autoregulatory feedback loop, the purpose of which is to maintain mucosal homeostasis by inhibition of bacterial invasion and down-regulation of inflammation.

The common molecular determinants of the interaction of DMBT1 with its ligands remain yet to be determined; however, a complex, discontinuous interaction with Ag I/II family polypeptides, comprising cell wall anchored adhesins of streptococci, has been suggested. It has also been proposed that DMBT1 interacts with the surfactant protein D, pointing to a putative recognition of self-structures. It will be important to understand the exact basis of DMBT1-mediated pattern recognition to further understand its role in mucosal homeostasis. Denial of access to epithelial cells and/or inhibition of an inflammatory response by interaction of DMBT1 with bacterial components in the mucus layer of the intestine represent conceivable candidate mechanisms for the protective effects that were observed in vitro. Further support for this hypothesis is delivered from recent studies that identified an in vivo up-regulation of *Dmbt1* or its ichthyal ortholog in response to colonization of germfree animals by commensal bacteria or *H. pylori* infection (45–47).

The constitutive up-regulation of *DMBT1* in CD might on the one hand be a generic response to an activation of the transcription factor NF-κB, which is thought to play a key role in the etiopathogenesis of chronic intestinal inflammation (48–50). However, it may also represent an unavailing attempt to restore the disrupted epithelial barrier in patients with CD. Interestingly, the defective induction of DMBT1 in the inflamed mucosa of patients carrying the L1007fsinsC (SNP13) NOD2 variant might also for the first time point to a specific defect in epithelial NF-κB activation. This hypothesis would suggest that secreted DMBT1 from IECs is a protective factor for the integrity of the intestinal barrier and that a defective induction of DMBT1, e.g., by genetically defective NOD2-mediated recognition of intraepithelial bacteria, would contribute to an aggravation of the underlying barrier disruption. Because polymorphisms in the *DMBT1* gene result in interindividually different numbers of bacterial binding domains (4, 6, 51), genetic analyses of the *DMBT1* locus in CD patients are urgently required to determine as to whether these alterations may modulate disease risk.

In summary, we show that DMBT1 is a downstream target of innate immune receptors at the first frontline of mucosal defense and acts as an anti-inflammatory immune exclusion molecule. The response to bacterial cell wall components via an activation of the proinflammatory transcription factor NF-κB is phylogenetically conserved from sea urchins to mammals. Our results further emphasize that disruption of this promiscuous pathway may paradoxically aggravate chronic inflammatory processes by compromising the complex cross-talk of the mucosal flora and the intestinal epithelium. Understanding the evolutionary origin of PAMP recognition and the phylogeny of NF-κB-activated protective cellular programs in intestinal epithelial cells may thus be of pivotal importance for future development of anti-inflammatory therapies.

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Disclosures

The authors have no financial conflict of interest.

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