DUOX2-derived reactive oxygen species are effectors of NOD2-mediated antibacterial responses

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Summary

Generation of microbicidal reactive oxygen species (ROS) is a pivotal protective component of the innate immune system in many eukaryotes. NOD (nucleotide oligomerisation domain containing protein)-like receptors (NLRs) have been implicated as phylogenetically ancient sensors of intracellular pathogens or endogenous danger signals. NOD2 recognizes the bacterial cell wall component muramyldipeptide leading to NFκB and MAPK activation via induced proximity signalling through the serine-threonine kinase RIP2. In addition to the subsequent induction of cytokines and antimicrobial peptides, NOD2 has been shown also to exert a direct antibacterial effect. Using a fluorescence-based ROS detection assay we demonstrate controlled ROS generation as an integral component of NOD2-induced signalling in epithelial cells. We demonstrate that the NAD(P)H oxidase family member DUOX2 is involved in NOD2-dependent ROS production. Coimmunoprecipitation and fluorescence microscopy were used to show that DUOX2 interacts and colocalizes with NOD2 at the plasma membrane. Moreover, simultaneous overexpression of NOD2 and DUOX2 was found to result in cooperative protection against bacterial cytoinvasion using the *Listeria monocytogenes* infection model. RNAi-based studies revealed that DUOX2 is required for the direct bactericidal properties of NOD2. Our results demonstrate a new role of ROS as effector molecules of protective cellular signalling in response to a defined danger signal carried out by a mammalian intracellular NLR system.

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Key words: Inflammation, ROS, NOD2 (*CARD15*), DUOX2, Crohn's disease, NFκB, Innate immunity

Introduction

NOD-like receptors (NLR) represent sensor proteins of the innate immune system that are characterized by a tripartite protein domain structure with a variable N-terminal effector binding domain, a central nucleotide-binding and oligomerisation domain (NBD, also referred to as NOD) and a C-terminal leucine-rich repeat region (LRR). The prototypic NLR family member NOD2 responds to the presence in the cytosol of muramyldipeptide (MDP), a breakdown product of bacterial cell walls (Girardin et al., 2003; Morre et al., 2004). Current molecular models of NOD2 activation suggest direct or indirect recognition of MDP via its LRR in the cytosol of epithelial or monocytic cells followed by NOD-mediated oligomerisation and direct interaction with receptor-interacting protein 2 (RIP2; also known as RICK), a cytosolic Ser/Thr kinase (Bertin et al., 1999; Girardin et al., 2001; Inohara et al., 1999). The assembly of the resulting NOD2 signalosome complex activates distinct signalling pathways such as nuclear factor κB (NFκB) and mitogen-activated protein kinase (MAPK) pathways (Rosenstiel et al., 2008). Moreover, genetic variants of *NOD2*, which are associated with Crohn's disease, a chronic relapsing-remitting inflammatory disorder affecting the whole gastrointestinal tract, result in impaired MDP recognition and diminished NFκB activation (Hampe et al., 2001; Hugot et al., 2001; Ogura et al., 2001a).

Interestingly, mammalian NLRs share similarities with a large family of pathogen-resistance (*R*-) genes in plants (Chisholm et al., 2006; Nurnberger et al., 2004). These 'plant NLRs' also contain a central NOD and a C-terminal LRR. Activation of the encoded R-proteins by pathogen infection induces a hypersensitive response (HR) (Ausubel et al., 1995; Hulbert et al., 2001). HR is an inborn protective response of plants against pathogen invasion. Upon pathogen sensing, a complex signal transduction cascade is initiated that involves kinase activation, modification of ion fluxes and generation of reactive oxygen species (ROS). Finally, cell death may be induced to restrict the growth of the pathogen. The initiated ROS production originates from the plant equivalent of the NAD(P)H oxidase (NOX) complex and resembles the 'oxidative burst' of mammalian neutrophils (Torres et al., 2002; Torres et al., 1998). In mammalian phagocytes, NOX complexes consist of at least six single proteins, two membrane-associated catalytic flavocytochrome *b* components [gp91^{phox} (NOX2) and p22^{phox}] and four regulatory components [p47 (NCF1), p67 (NCF2), p40 and Rac1] located in the cytosol (Sumimoto et al., 2004). Since homologues of NOX2 and its associated subunits have been shown to be expressed in a variety of different tissues and are activated by varying stimuli, NOX-mediated ROS production is nowadays regarded to be a general feature of many cells rather than a unique characteristic of phagocytes (Geiszt, 2006; Lambeth, 2004; Leto and Geiszt, 2006).

The NOX/DUOX superfamily consists of NOX1, 2, 3, 4 and 5, dual oxidase 1 and 2 (DUOX1 and DUOX2) (Bokoch and Knaus, 2003; Lambeth, 2004). For family members NOX1, NOX2 and NOX3 a variety of regulatory subunits, activators and adaptor

proteins have been described that specifically control the enzymatic activity (Banfi et al., 2003; Banfi et al., 2004; Bedard and Krause, 2007; Cheng et al., 2004; Takeya et al., 2003).

Within the DUOX subfamily an additional N-terminal extracellular peroxidase-homology domain is present and distinguishes this group of proteins from the NOX subfamily (Lambeth et al., 2007). Whereas most NOX enzymes release superoxide, it was shown that DUOX isoforms generate hydrogen peroxide (H_2O_2) in a Ca^{2+}-dependent manner (Bjorkman and Ekholm, 1984; Dupuy et al., 1989). Recently it was demonstrated that the activity of DUOX1 and DUOX2 is differentially regulated via specific phosphorylation by protein kinase A (DUOX1) or protein kinase C (DUOX2) (Rigutto et al., 2009).

DUOX proteins have been shown to be expressed in epithelial linings of barrier organs, including intestinal and bronchial epithelial cells (Krause, 2004). In particular, colonic epithelial cells express high levels of DUOX2 (Cheng and Lambeth, 2005; El Hassani et al., 2005; Rokutan et al., 2006). There are interesting lines of evidence describing NOX-mediated ROS formation as an integral part of the host defence system and inflammatory responses at mucosal surfaces. Direct physical interactions have been demonstrated for NOX family members and components of the Toll-like receptor (TLR) signalling cascade (Asehnoune et al., 2004; Kawahara et al., 2004; Park et al., 2004). In *Drosophila*, the DUOX orthologue dDUOX is indispensable for the clearance of bacterial infections of the gastrointestinal tract (Ha et al., 2005). Moreover, regulated hydrogen peroxide production by mammalian DUOX enzymes is required for the microbicidal lactoperoxidase response in airway epithelial cells (Forteza et al., 2005). However, ROS production and protein or lipid modifications induced by ROS have been accused of contributing to tissue damage in chronic inflammatory disorders, such as Crohn's disease (McKenzie et al., 1996).

Despite the profound consequences of either absent or excessive ROS generation in the intestinal tract, little is known about the molecular pathways controlling ROS production via NOX and/or DUOX enzymes. Given the link between plant NLRs and pathogen-induced ROS production we aimed to explore whether NOD2 signalling mediates ROS production and if this mechanism contributes to host defence.

Results

NOD2 facilitates ROS generation in intestinal epithelial cells involving DUOX2 activation

To test whether ROS are generated during NOD2 activation in epithelial cells, intracellular ROS production was measured in Caco-2 cells transfected with NOD2 overexpression construct or empty vector as control. Cells were loaded with 15 μM cH_2DCFDA (a redox-sensitive fluorescent dye), stimulated with 10 μg/ml MDP and monitored for fluorescence of the intracellular product cDCF. We observed a rapid NOD2-dependent ROS production within 20 minutes after MDP stimulation in Caco-2 cells. These findings are underscored by our observation that the Crohn's disease-associated NOD2 variant *NOD2*^{L1007fsinsC} (SNP13), which encodes for a truncated protein, results in diminished production of ROS after stimulation with MDP (Fig. 1A). Similar results were obtained by MDP stimulation of Caco-2 cells pre-stimulated with TNFα, which is known to upregulate endogenous NOD2 expression (Rosenstiel et al., 2003) (Fig. 1B). Furthermore, primary murine intestinal epithelial cells isolated from colon of *Nod2*-knockout mice (*Nod2*$^{-/-}$) or wild-type mice (WT) were monitored for ROS generation upon

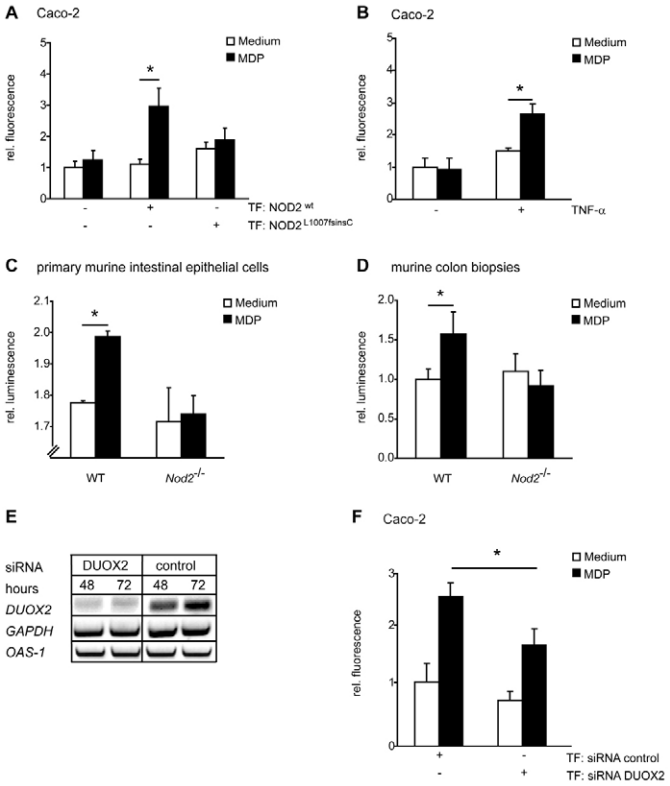

Fig. 1. ROS are involved in NOD2 signalling in epithelial cells. (A) ROS detection using cH_2DCFDA assay in Caco-2 cells transfected with plasmids encoding NOD2 wild type, the NOD2 variant NOD2^{L1007fsinsC} (SNP13) or vector control. The changes of intracellular ROS levels of MDP-stimulated cells in comparison with cells incubated in medium alone (mean ± s.d., n=6; *P<0.05). (B) ROS detection in Caco-2 cells pre-treated with TNFα and stimulated with 10 μg/ml MDP or medium alone (mean ± s.d., n=6; *P<0.05). A rapid NOD2-dependent ROS production within 20 minutes after MDP stimulation was detected. (C,D) Luminol chemiluminescence assay of primary murine epithelial cells isolated from colon biopsies (C) or total colon tissue samples (D) from transgenic *Nod2*$^{-/-}$ knockout and wild-type (WT) mice. The changes of intracellular ROS levels upon MDP stimulation in comparison with incubation in medium alone (mean ± s.d., n=3; *P<0.05). Genetic deficiency for *Nod2* abolished MDP-dependent ROS production. (E) RNAi-mediated knockdown of *DUOX2*. Caco-2 cells were transfected with a pool of three different siRNAs targeting *DUOX2* or appropriate control siRNA for 48 hours or 72 hours. Specific reduction of *DUOX2* mRNA levels was detected whereas no induction of *OAS-1* was observed, indicating lack of general off-target effects. (F) ROS detection using cH_2DCFDA assay. Caco-2 cells were either transfected with 10 nM DUOX2 siRNA (pool of three different siRNAs) or control siRNA. Cells were loaded with 15 μM cH_2DCFDA and stimulated with 10 μg/ml MDP for 30 minutes. ROS levels were quantified by measurement of cDCF fluorescence (mean ± s.d., n=5; *P<0.05). RNAi-mediated knockdown of *DUOX2* significantly decreased MDP-dependent ROS production.

MDP stimulation. In contrast to wild-type cells, intestinal epithelial cells from *Nod2*$^{-/-}$ mice showed no response to MDP stimulation with regard to intracellular ROS levels (Fig. 1C). Similar results were obtained using murine colonic organ cultures. Again, increased ROS generation was detectable in stimulated tissue samples from wild-type animals but not from *Nod2*$^{-/-}$ mice (Fig. 1D).

To further elucidate the role of endogenous DUOX2 in intestinal epithelial cell lines we chose an RNAi-based knockdown approach using DUOX2-specific siRNAs in Caco-2 cells. A reduction of *DUOX2* mRNA levels was observed using a pool of three different siRNAs targeting *DUOX2* (Fig. 1E) or three individual siRNAs

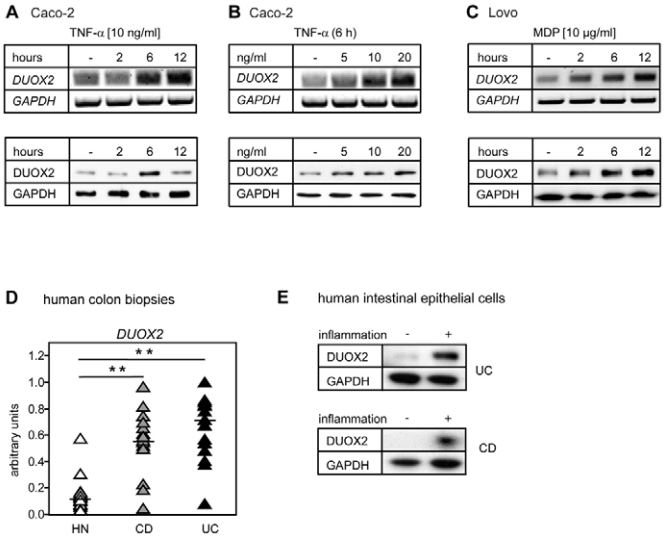

Fig. 2. DUOX2 is upregulated in intestinal epithelial cells under pro-inflammatory conditions. (A,B) Time- and dose-dependent upregulation of *DUOX2* mRNA (upper panel) and protein levels (lower panel) in Caco-2 cells after stimulation with TNFα. RT-PCR and immunoblotting was performed for *DUOX2* and for the housekeeping gene *GAPDH* (loading control). (C) Time-dependent upregulation of *DUOX2* mRNA (upper panel) and protein levels (lower panel) in intestinal epithelial Lovo cells after stimulation with MDP. (D) Expression of *DUOX2* in human colon. Total RNA was isolated from colonic biopsies of patients with Crohn's disease (CD), ulcerative colitis (UC) or healthy controls (HN; n=14 each). RT-PCR was performed for *DUOX2* and *GAPDH*, and relative expression of *DUOX2* was calculated as fold change compared with control (*GAPDH*) (mean ± s.d., n=14; $^{**}P<0.01$). *DUOX2* mRNA levels were significantly elevated in CD and UC compared with healthy controls. (E) Western blot analysis for DUOX2 protein levels in intestinal epithelial cells isolated from colonic biopsies of patients with CD or UC. DUOX2 levels were increased in epithelial cells isolated from inflamed colonic tissue compared with non-inflamed biopsy samples.

alone (supplementary material Fig. S1). Importantly, no induction of 2′,5′-oligoadenylate synthetase 1 (OAS1) mRNA was detected, indicating a lack of generalized off-target effects against double-stranded RNA. To determine whether ROS production was affected by this knockdown, siRNA-transfected Caco-2 cells were monitored for cDCF fluorescence upon MDP stimulation. As expected, RNAi-mediated knockdown of DUOX2 decreased the intracellular ROS generation after MDP addition ($P<0.05$; Fig. 1F).

Expression of *DUOX2* in intestinal epithelial cells

Intestinal epithelial cells express a variety of NOX family members including NOX1 and DUOX2 that are thought to represent essential components of the host mucosal surface defence (El Hessian et al., 2005; Kikuchi et al., 2000). In this study we were interested in the specific role of DUOX2 as a potential source of NOD2-induced ROS production. As a consequence, we investigated *DUOX2* expression under pro-inflammatory conditions and observed a time- and dose-dependent increase in *DUOX2* mRNA and protein levels following TNFα stimulation in Caco-2 cells (Fig. 2A,B). Furthermore, MDP stimulation also elevated endogenous DUOX2 expression in Lovo cells both on the mRNA and on the protein level (Fig. 2C). Next, we analyzed expression levels of *DUOX2* in human colonic biopsies of patients suffering from Crohn's disease (CD) or ulcerative colitis (UC), and of healthy normals (HN). Interestingly, we found a significant increase in *DUOX2* mRNA expression in CD as well as in UC patients compared to healthy controls (Fig. 2D). To narrow down the analysis on the regulation of *DUOX2* in epithelial cells and to avoid bias by immune cell invasion, we next investigated DUOX2 protein expression in intestinal epithelial cells (IECs) isolated from biopsies of either inflamed or non-inflamed colon tissue. As demonstrated in Fig. 2E, DUOX2 protein levels were increased in IECs originating from inflamed tissue in comparison to non-inflamed tissue.

In conclusion, upregulation of *DUOX2* seems to be linked to inflammatory bowel diseases, supporting previous reports on differential expression of NOX/DUOX family members in Crohn's disease and *Helicobacter pylori*-induced intestinal inflammation (Csillag et al., 2007; Rokutan et al., 2008; Szanto et al., 2005).

NOD2 interacts and colocalizes with DUOX2

To determine whether a direct physical interaction between NOD2 and the DUOX2 complex is detectable in analogy with reports demonstrating protein interaction between TLR components and NOX family members (Asehnoune et al., 2004; Kawahara et al., 2004; Park et al., 2004), we performed colocalization and coimmunoprecipitation studies. Coexpression of EGFP-tagged NOD2 with DUOX2-HA and its essential activator DUOXA2 in HEK293 cells revealed a colocalization at the plasma membrane (Fig. 3A). Next, we investigated the intracellular distribution of endogenous DUOX2 and NOD2 in Caco-2 cells. Caco-2 cells were pre-stimulated with TNFα to upregulate endogenous *NOD2* expression as reported before (Rosenstiel et al., 2003). Since a recent study demonstrated that MDP administration results in NOD2 translocation to the plasma membrane in monocytes, probably by Rac1-dependent mechanisms (Eitel et al., 2008), we pretreated Caco-2 cells with MDP to enforce membrane association of NOD2. As expected, endogenous DUOX2 and NOD2 were found to colocalize in spot-like patterns in the cytoplasm as well as at the plasma membrane (Fig. 3B). In comparison with unstimulated cells, MDP-stimulated cells had a slightly more pronounced colocalization at the membrane (data not shown).

Direct physical interaction between NOD2 and DUOX2 proteins was analyzed by coimmunoprecipitation. Precipitation of overexpressed HA-tagged DUOX2 resulted in co-precipitation of FLAG-tagged NOD2 in HEK293 cells (Fig. 3C). Moreover, endogenous DUOX2 could be precipitated with endogenous NOD2 in pre-stimulated Caco-2 cells (Fig. 3D) thus demonstrating that NOD2 and DUOX2 represent integral parts of a common multi-protein complex.

To map the region of NOD2 that might be essential for the observed interaction with DUOX2, we used overexpression of individual domains of NOD2 [caspase recruitment domains (CARDs), NBD, LRR] followed by coimmunoprecipitation. As shown in Fig. 3E, only full-length NOD2 and the LRR domain of NOD2 directly interact with DUOX2 whereas neither the CARDs nor the NBD alone showed evidence for interaction.

DUOX2 and NOD2 cooperatively facilitate antibacterial action

Since NOX/DUOX-derived ROS are effective antimicrobial effector molecules as part of the innate immune response (Rada and Leto, 2008) we asked if NOD2-dependent activation of DUOX2 might participate in direct antibacterial mechanisms. To gain further insight into the crosstalk between NOD2 activation and DUOX2-derived ROS in direct bacterial killing, quantitative cytoinvasion assays were applied after different pre-treatment conditions. Using carboxyfluorescein succinimidyl ester (CFSE)-labelled *Listeria monocytogenes* and FACS analysis, bacterial cytoinvasion was

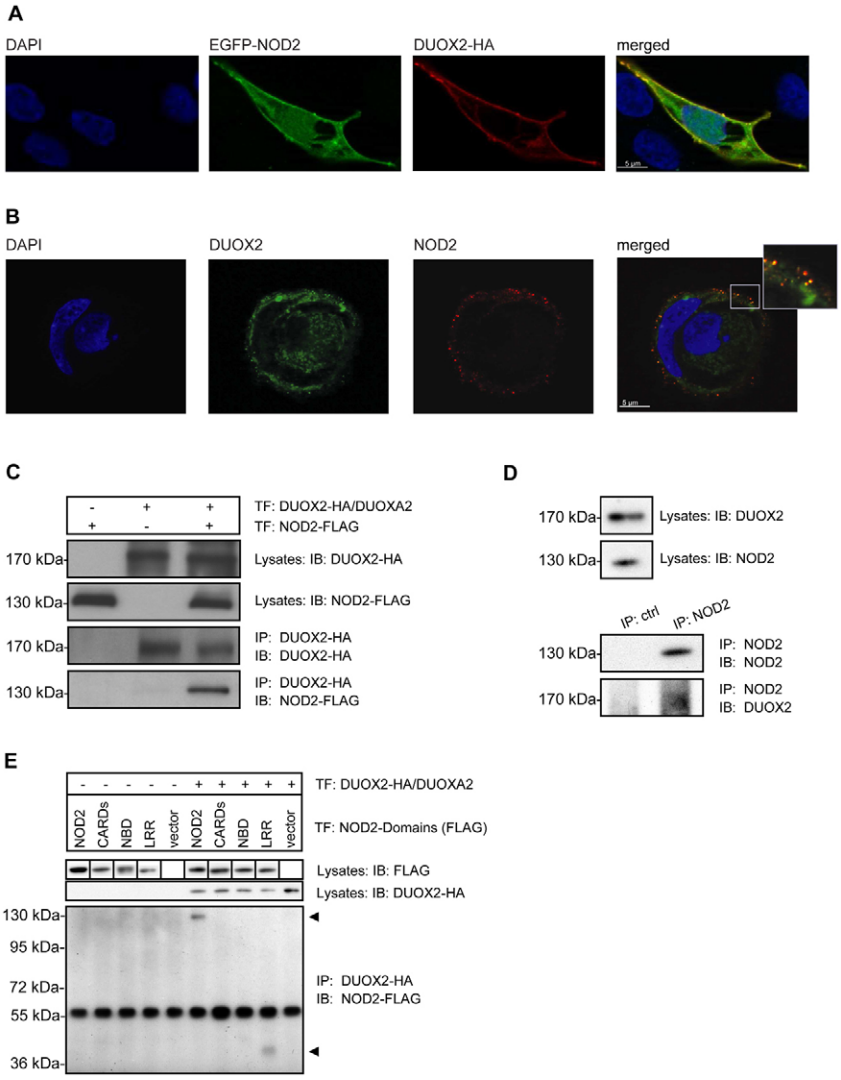

Fig. 3. NOD2 interacts and colocalizes with DUOX2. (A) HEK293 cells coexpressing EGFP-NOD2, DUOX2-HA and the DUOX2 activator DUOXA2 were fixed, incubated with anti-HA and anti-mouse-Cy3 secondary antibodies and counterstained using DAPI. (B) Caco-2 cells pre-treated with TNFα (5 ng/ml) for 24 hours and stimulated with MDP (10 μg/ml) for 30 minutes were probed for endogenous DUOX2 and NOD2 using goat anti-DUOX2 and rabbit anti-NOD2 antibodies and respective secondary antibodies (FITC-anti-goat-Ig, Cy3-anti-rabbit-Ig) and counterstained with DAPI. Colocalization of NOD2 and DUOX2 was detected for both overexpressed and endogenous proteins. (C) Coimmunoprecipitation of NOD2 and DUOX2. DUOXA2, DUOX2-HA and NOD2-FLAG constructs were used for overexpression in HEK293 cells. Immunocomplexes (anti-HA) and cell lysates were assayed for the presence of co-precipitated proteins as indicated. (D) Coimmunoprecipitation of endogenous NOD2 and DUOX2 in Caco-2 cells. Cells were pre-treated as described before. Immunocomplexes (anti-NOD2) and cell lysates were assayed for presence of DUOX2 as indicated. An irrelevant antibody was used as specificity control (ctrl). Endogenous DUOX2 was found to co-precipitate with endogenous NOD2. (E) Coimmunoprecipitation of NOD2 domains and DUOX2. HEK293 cells were transfected with constructs for overexpression of FLAG-tagged NOD2 or NOD2 domains in combination with HA-tagged DUOX2/DUOXA2. After precipitation of DUOX2-HA, FLAG-tagged NOD2 domains were identified in the immunocomplexes using anti-FLAG antibody. Only full-length NOD2 and the LRR domain (arrowheads) could be demonstrated to interact with DUOX2.

monitored in HEK293 cells stably transfected with plasmids encoding NOD2 ($NOD2^{wt}$), the Crohn's disease- associated NOD2 variant $NOD2^{L1007fsinsC}$ (SNP13) or empty vector. As expected, wild-type-NOD2 conferred significant protection against bacterial cytoinvasion whereas overexpression of $NOD2^{L1007fsinsC}$ gave only reduced protection (Fig. 4A). Overexpression of DUOX2 in parallel with its essential activator DUOXA2 partially protected mock-transfected cells from bacterial cytoinvasion. Interestingly, the NOD2-dependent protective effect was significantly enhanced by additional overexpression of DUOX2 and DUOXA2 (DUOX2/DUOXA2). In line with this observation, we found a remarkable increase in intracellular ROS formation after infection with *L. monocytogenes* by parallel overexpression of NOD2 and DUOX2/DUOXA2 (Fig. 4B). As documented in Fig. 4C, RNAi-mediated knockdown of endogenous DUOX2 resulted in increased bacterial cytoinvasion and in abrogation of the cytoprotective effect conferred by NOD2 in Caco-2 cells (Fig. 4C). These data further emphasize the prominent role of functional crosstalk between NOD2 and DUOX2 for initiation of defence mechanisms against pathogenic bacteria.

Activation of NFκB signalling and subsequent induction of antibacterial target genes is a well characterized downstream event of NOD2 activation. Since generation of H_2O_2 also activates the NFκB signalling cascade (reviewed by Gloire et al., 2006) we investigated the role of DUOX2 on NOD2-mediated NFκB activation and downstream signalling events. We found that overexpression of DUOX2/DUOXA2 in HEK293 increased NOD2-dependent activation of an NFκB reporter gene whereas knockdown of endogenous DUOX2 in Caco-2 cells resulted in significantly reduced NFκB activation compared with controls (Fig. 5A,B). Importantly, we observed similar effects on the target gene level by analyzing IL-8 secretion in response to MDP (Fig. 5C,D).

Taken together, these findings demonstrate a synergistic mechanism in which pathogens are both sensed and attacked through NOD2 activation, the antibacterial action of which is reinforced by interacting with ROS-generating DUOX2.

Discussion

From this study we provide evidence that MDP-dependent activation of the NODosome signalling complex, which is composed of the NLR member NOD2 and its ligand MDP, results in rapid generation of intracellular ROS. This observation is in line with recent findings that link other NLR family members to intracellular redox homeostasis: NLRX1 was found to contribute to mitochondrial ROS

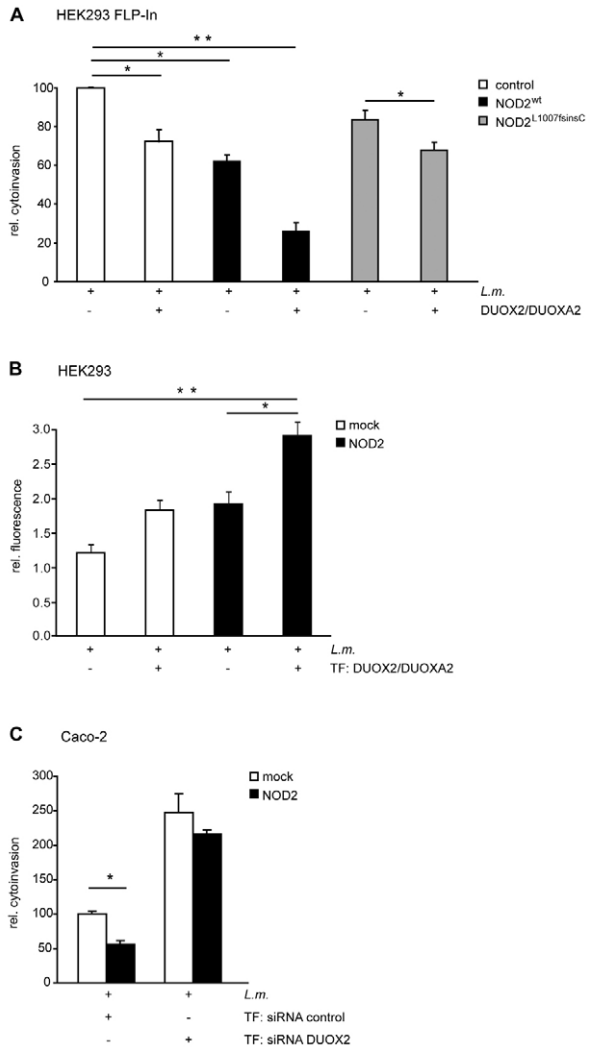

Fig. 4. NOD2 and DUOX2 exhibit synergistic effects on bactericidal activity. (A) Quantification of bacterial cytoinvasion using CFSE-labelled *Listeria monocytogenes*. HEK293 cells were stably transfected with plasmids encoding NOD2 ($NOD2^{wt}$), the Crohn's disease-associated NOD2 variant $NOD2^{L1007fsinsC}$ (SNP13) or empty vector as control and transiently co-transfected with DUOX2/DUOXA2. Cells were incubated with labelled bacteria and analyzed for fluorescence using flow cytometry (mean ± s.d., n=3; *P<0.05 and **P<0.01). Both NOD2 and DUOX2 had a protective effect against bacterial cytoinvasion, which was potentiated by simultaneous coexpression, indicating a cooperative effect. (B) ROS detection using cH_2DCFDA assay in NOD2, DUOX2/DUOXA2- or vector control-transfected HEK293 cells. The changes of intracellular ROS levels of cells infected with *Listeria monocytogenes* (multiplicity of infection=100) in comparison to cells incubated with medium alone (mean ± s.d., n=6; *P<0.05 and **P<0.01). Overexpression of DUOX2/DUOXA2 enhanced NOD2-dependent ROS formation. (C) Quantification of bacterial cytoinvasion using CFSE-labelled *Listeria monocytogenes*. Caco-2 cells were transfected with a pool of three different siRNAs targeting DUOX2 or control siRNA and co-transfected with NOD2 overexpression plasmid or empty vector (mock). After 48 hours, cells were infected with fluorescently labelled bacteria and analyzed by flow cytometry (mean ± s.d., n=3; *P<0.05). Gene silencing of DUOX2 abrogated NOD2-mediated protection against bacterial cytoinvasion.

production (Tattoli et al., 2008) and NACHT-, LRR- and PYD-domain-containing protein 3 (NALP3) inflammasome assembly and activation was shown to be triggered by ROS (Dostert et al., 2008). To date the molecular crosstalk between NLR-mediated signalling and inflammasome-dependent caspase activation is still unclear. Given the data presented here, NOD2-dependent ROS formation may represent the molecular link for the integration of NOD2- and NALP-mediated signalling. Intracellular ROS level elevated by NOD2 activation might affect the net outcome of NOD2-mediated signals (NFκB activation), but may also serve as a direct anti-bacterial effector and as an amplifier of inflammasome formation. Revealing the identity of a potential direct molecular ROS sensor linking redox status and innate immune receptor signalling via NALP-type NLRs represents an intriguing challenge for future studies.

The data presented here indicate that DUOX2 is a major source of NOD2-dependent ROS generation. Since it is well accepted that intestinal epithelial cells express a variety of NOX family members and other oxidases (e.g. NOX1, NOX5, COX2), we cannot rule out that other enzyme complexes contribute to the effects observed here. Nevertheless, the profound effects of specific siRNAs targeting DUOX2 underscore the prominent role of DUOX2 in regulation of NOD2 signalling. Interestingly, it has been shown previously that the *Drosophila* orthologue of DUOX2 is of significant importance for host defence against pathogenic bacteria and gut immunity (Ha et al., 2005). In addition, DUOX2 is described to be upregulated in gastric biopsies of monkeys infected with *Helicobacter pylori*, a pathogenic bacterium causatively involved in chronic inflammation of the gastric cavity (Hornsby et al., 2008). This is noteworthy since we have recently reported the effects of genetic variants of *NOD1* and *NOD2* on the clinical outcome of *H. pylori* infection with respect to mucosa-associated lymphoid tissue (MALT) lymphoma (Rosenstiel et al., 2006a). Thus unravelling the exact contribution of DUOX2 and other components of the NOX/DUOX superfamily to the regulation of innate immune responses at the intestinal epithelial interface represents a major challenge of future studies.

In this study we demonstrate that NOD2 physically interacts with DUOX2, which is comparable to data demonstrating complex formation between NOX enzymes and Toll-like receptors (TLRs) (Asehnoune et al., 2004; Kawahara et al., 2004; Park et al., 2004). Using domain-specific deletion constructs of NOD2 we mapped the essential domain for this interaction to the LRR of NOD2. This finding is somewhat surprising since most interaction partners identified so far bind to the N-terminal portion of the NOD2 protein. For example, the serine/threonine kinase RIP2 mediates NOD2-dependent NFκB activation through homophilic CARD-CARD interaction (Ogura et al., 2001b). Negative regulation of NOD2 activation by its interaction partner Erbin also requires binding to the CARD domains (Kufer et al., 2006; McDonald et al., 2005). Recently, our group has identified the immunoglobulin superfamily member CD147 as a novel interaction partner of NOD2 and has demonstrated its regulatory role in bacterial defence via interaction with both CARDs (Till et al., 2008). However, the transforming growth factor (TGF)-β-activated kinase 1 (TAK1) has been shown to be involved in reciprocal crosstalk with NOD2 via binding to the LRR region (Chen et al., 2004). These data and recent reports demonstrating that both the two CARDs and the LRR are required for association of NOD2 with Rac1 in membrane ruffles (Eitel et al., 2008; Legrand-Poels et al., 2007) highlight the complexity of the protein networks that contribute to regulation of NLRs.

Our data emphasize a cooperative role for NOD2 and DUOX2 activation for procuring antibacterial activity. In addition to the fact that NOD2 activation results in NFκB-mediated induction of antimicrobial target genes such as defensins (Kobayashi et al.,

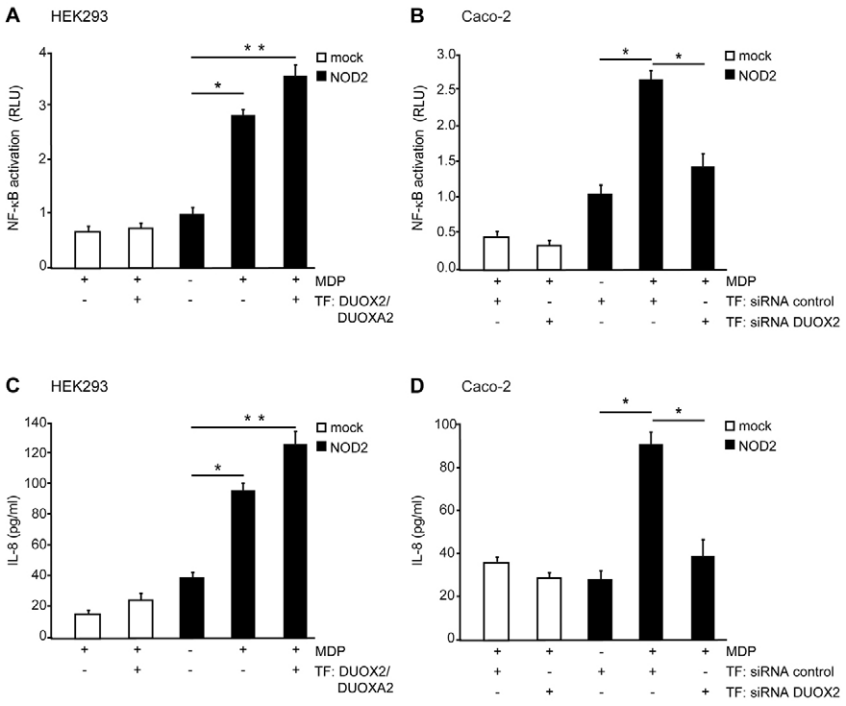

Fig. 5. Role of DUOX2 in NOD2-dependent signalling. (A,B) Reporter gene assay for NOD2-dependent NFκB activation. (A) HEK293 cells were co-transfected with a NFκB-dependent luciferase reporter gene construct (pNFκB-LUC), pRL-TK *Renilla* plasmid for normalization and an overexpression plasmid for NOD2 or empty vector (mock). Cells were additionally transfected with DUOX2/DUOXA2 and stimulated with 10 μg/ml MDP for 6 hours as indicated. Luciferase activity was measured as described and expressed as relative light units (RLU; mean ± s.d., n=3; *P<0.05). Overexpression of DUOX2 enhanced NOD2-dependent NFκB activation. (B) Alternatively, Caco-2 cells were transfected with a pool of three different siRNAs targeting DUOX2 or control siRNA for 48 hours. Additionally cells were transfected with pNFκB-LUC, pRL-TK and an overexpression plasmid for NOD2 (or empty vector as mock control) and stimulated with 10 μg/ml MDP for 6 hours as indicated. Luciferase activity was measured as described (mean ± s.d., n=3; *P<0.05). Knockdown of DUOX2 diminished NOD2-dependent NFκB activation. (C,D) ELISA for NOD2-dependent IL-8 secretion. (C) HEK293 cells were transfected with overexpression plasmid for NOD2 or empty vector (mock), co-transfected with DUOX2/DUOXA2 and stimulated with 10 μg/ml MDP for 6 hours as indicated. IL-8 secretion was measured by ELISA (mean ± s.d., n=3; *P<0.05). Overexpression of DUOX2 enhanced NOD2-dependent IL-8 secretion. (D) Alternatively, Caco-2 cells were transfected with a pool of three different siRNAs targeting DUOX2 or control siRNA for 48 hours and transfected with an overexpression plasmid for NOD2 or empty vector (mock). Cells were stimulated with 10 μg/ml MDP for 6 hours and IL-8 secretion was measured as described (mean ± s.d., n=3; *P<0.05). Knockdown of DUOX2 diminished NOD2-dependent IL-8 secretion.

2005; Voss et al., 2006; Wehkamp et al., 2004), direct antibacterial properties of NOD2 have been demonstrated (Hisamatsu et al., 2003; Till et al., 2008). Here we show that coexpression of DUOX2 and NOD2 results in increased protection against bacterial cytoinvasion that even exceeds the protection conveyed by NOD2 expression alone. In line with these observations, knockdown of DUOX2 impairs the protective effect of NOD2 during infection with *Listeria*. We conclude that DUOX2 acts as a molecular switch that enhances the protective properties of NOD2 by both direct (generation of bactericidal ROS) and indirect mechanisms (by amplifying NFκB signalling). Since this functional link between NLR signalling and intracellular redox homeostasis might not be restricted to DUOX2 and NOD2, further studies are warranted that comprehensively characterize the role of NOX and DUOX family members as integral parts of the innate defence program.

Our data demonstrate that infection of HEK293 cells with cytoinvasive *Listeria* induces ROS generation by mechanisms involving both NOD2 and DUOX2. Several lines of evidence link pathogenic bacteria to ROS generation in non-phagocytic cells. For example, it has been shown recently that *Mycobacterium ulcerans* induces ROS formation in epidermal keratinocytes via TLR-dependent pathways (Lee et al., 2009). By contrast, fermentation products derived from commensal bacteria of the gut flora also induce ROS production in intestinal epithelial cells resulting in neddylation of cullin 1 and suppression of NFκB signalling (Kumar et al., 2007; Kumar et al., 2009). Thus generation of ROS is a common event, initiated by both harmless bacteria and pathogens. It is tempting to speculate that an integration of different cellular signalling events is required to mediate the appropriate response that ultimately assures homeostatic conditions between host epithelia and bacterial flora. Dissection of the exact molecular mechanisms underlying this delicate discrimination process will be of general importance for understanding the complex crosstalk between host organism and microbiota.

Recent findings suggest that the redox equilibrium is modulated by NOD2-dependent induction of antioxidant enzymes, such as upregulation of peroxiredoxin 4 (PDX4) (Weichart et al., 2006). With respect to the association between truncated protein variants of NOD2 (e.g. $NOD2^{L1007fsinsC}$) and Crohn's disease, we assume that the observed dysfunction of these NOD2 variants is at least in part caused by disturbing counter-regulatory antioxidant responses. Indeed, our observation that DUOX2 is upregulated in human inflammatory bowel disease, may infact reflect a futile attempt to restore barrier function. It does, however, reveal the two aspects of ROS: although controlled ROS generation is a protective response against pathogens, excessive ROS formation leads to tissue destruction, ultimately resulting in inflammatory disorders.

We hypothesize that the mechanism suggested here opens new avenues for the understanding of immune responses initiated by the colonic epithelium: NOD2 activates multiple layers of host defence, including ROS generation, NFκB activation and cytokine and/or antimicrobial peptide secretion. Given the remarkable evolutionary conservation of NLRs, it is tempting to speculate that NLR-dependent ROS formation and sensing may represent an ancient defence mechanism, which is similar to the hypersensitive reaction induced by cytoplasmic *R*-genes in plants.

Taken together, our data point to a formerly unrecognized role of NOD2-dependent ROS formation, using the delicate equilibrium of the cellular redox status as amplification and effector mechanism of innate defence programs.

Materials and Methods

Mice, cell culture and bacteria

Mice genetically deficient for NOD2 (*Nod2*$^{-/-}$) were established on a C57BL/6 background and were originally obtained from the Jackson Laboratory. For ex vivo studies, colonic epithelial cells were isolated from fresh colon biopsies as described previously (deSchoolmeester et al., 2006). Human embryonal kidney HEK293 cells (ACC 305) and human colon adenocarcinoma cell lines Caco-2 (ACC 169) and Lovo (ACC 350) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). HEK293-FLP-IN cells stably transfected with plasmids encoding NOD2 (NOD2^{wt}), the Crohn's disease-associated NOD2 variant NOD2^{L1007fsinsC} (SNP13) or empty vector were described before (Weichart et al., 2006). The *Listeria monocytogenes* serotype 1/2a strain EGD was used as a model organism for cytoinvasion.

ROS detection

Intracellular ROS generation was determined by the use of the redox-sensitive fluorescent dye cH_2DCFDA (5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; Invitrogen, Karlsruhe, Germany) or luminol (Amersham Biosciences, Freiburg, Germany) as described by Cheng and Lambeth (Cheng and Lambeth, 2004).

Expression plasmids

Expression vector for NOD2 (pcDNA3-NOD2) was kindly provided by G. Nunez (Ann Arbor, MI). Overexpression construct pCEV-NOD2 has been described before (Rosenstiel et al., 2006b). EGFP-NOD2 fusion protein was generated by inserting the NOD2 coding sequence into pEGFP-C3 vector using the *Hin*dIII and *Bam*HI restriction sites. Overexpression constructs for individual NOD2 domains (CARDs, NBD, LRR) have been reported previously (Till et al., 2008). Overexpression constructs for DUOX2, DUOX2-HA and DUOXA2 have been described previously (Grasberger and Refetoff, 2006).

Transfection of human cell lines

One day before transfection, cells were seeded at a density of 4×10^5 cells/ml (six-well plates) or 2×10^4 (96-well plates). Transfection of plasmid DNA was carried out using Fugene 6^{TM} (Roche, Basel, Switzerland). Transfection of siRNA was performed using siPORT Amine (Ambion, Austin, TX, USA). All transfections were conducted according to the manufacturer's instructions.

Patient samples

Mucosal colonic biopsies were extracted as previously described (Waetzig et al., 2002). The control biopsies were derived from patients without pathological conditions (n=14). Inflammatory bowel disease samples were taken from the inflamed colon of relapsing, active patients with Crohn's disease (n=14, Crohn's disease activity index >200) or ulcerative colitis (n=14, colitis activity index >6) during colonoscopy for routine clinical evaluation. None of the patients had received immunosuppressive drugs (glucocorticoids, azathioprine, 6-mercatopurine, methotrexate, cyclosporine) within the previous 4 weeks. All patient-related procedures were approved by the university hospital's ethics committee. All patients agreed to participate by giving informed consent at least 24 hours before the study.

Isolation of primary human intestinal epithelial cells

Primary human colonic epithelial cells were isolated from mucosal colonic biopsies directly after sampling. Briefly, the biopsies were washed twice in Ca^{2+}-, Mg^{2+}-free, Hanks' balanced salt solution (HBSS; PAA, Colbe, Germany) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The biopsies were then incubated for 60 minutes at 37°C in 5 ml of HBSS supplemented with 1 mg/ml collagenase-dispase (Roche Diagnostics, Mannheim, Germany) with constant rotation. Epithelial cells were collected by centrifugation of supernatant at 100 ***g*** for 10 minutes and directly used for protein extraction.

Immunofluorescence microscopy

HEK293 or Caco-2 cells were seeded on sterile coverslips and transfected with pEGFP-NOD2, pcDNA3.1-DUOX2-HA, pcDNA3.1-DUOXA2 or the respective control vectors. The next day, cells were washed, fixed in 4% paraformaldehyde-PBS and blocked for 1 hour in 1% BSA-PBS. Cells were stained using rabbit polyclonal anti-HA antibody (1:500, Clontech, Palo Alto, CA) and Cy-3-conjugated anti-rabbit secondary antibody (1:500, ImmunoResearch Laboratories Inc., West Grove, PA). DAPI was used for DNA counterstaining.

For colocalization studies of endogenous NOD2 with DUOX2, Caco-2 cells were pre-stimulated with 5 ng/ml TNFα overnight to induce upregulation of NOD2 and DUOX2 expression. In addition, cells were stimulated with MDP (10 μg/ml, 30 minutes) to induce membrane recruitment of NOD2. After fixation, cells were stained with rabbit polyclonal anti-NOD2 antibody (1:250; Cayman Chemical Company, Ann Arbor, MI) and goat polyclonal anti-DUOX2 antibody (1:250; Santa Cruz, Santa Cruz, CA). For visualization, FITC-conjugated anti-goat or Cy3-conjugated anti-rabbit antibodies were used. DAPI was used for DNA counterstaining. Coverslips were mounted onto glass slides and examined using a Zeiss AxioImager.Z1 apotome fluorescence microscope and the AxioVision Imaging software (Carl Zeiss MicroImaging Inc., Thornwood, NY).

Coimmunoprecipitation and immunoblotting

HEK293 cells were seeded in 10-ml dishes at 4×10^6 cells/dish, grown overnight and transfected with expression constructs for full-length NOD2-FLAG, the respective domains of NOD2 (CARDs-FLAG, NBD-FLAG, LRR-FLAG) and DUOX2-HA in combination with DUOXA2. For precipitation of endogenous proteins, Caco-2 cells were pre-treated as described above. The next day, cells were washed with ice-cold PBS, scraped off the plates, and pelleted by centrifugation. Cells were lysed by vortexing three times for 45 seconds in 1000 μl of buffer containing 1% Triton X-100 and a mixture of protease and phosphatase inhibitors (Sigma-Aldrich Corp., St Louis, MO). After pre-clearing by incubation with 20 μl protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and centrifugation, DUOX2-HA complexes were precipitated using rat anti-HA antibody (Roche, Basel, Switzerland). Endogenous proteins were precipitated using the appropriate antibodies (mouse monoclonal anti-NOD2 antibody, Novus; goat polyclonal anti-DUOX2 antibody, Santa Cruz). Precipitates were washed, denatured by boiling in 20 μl Laemmli buffer, and separated by SDS-PAGE. After transfer onto polyvinylidene difluoride membranes (Millipore, Billerica, MA), western blots were performed as previously described (Till et al., 2005), and samples were probed with the respective antibodies against (co-) precipitated proteins (mouse anti-FLAG, Stratagene, La Jolla, CA; rabbit anti-NOD2, Cayman; rabbit anti-DUOX2, Abcam, Cambridge).

To analyze the expression of endogenous DUOX2 protein, cell samples and biopsies were lysed in Laemmli buffer and processed as described previously (Till et al., 2005). After denaturing SDS gel-electrophoresis and electrotransfer onto polyvinylidene difluoride membranes, proteins were detected using the following antibodies: goat anti-DUOX2, mouse anti-GAPDH (both purchased from Santa Cruz) in combination with appropriate secondary antibodies.

Isolation of mRNA and RT-PCR

RNA from cell lines or from human colonic biopsies was isolated using the RNeasy kit (Qiagen, Hilden, Germany). 250 ng of total RNA were reverse transcribed using the Advantage-RT for PCR kit (Clontech, Palo Alto, CA). Expression of target genes was analyzed using sequence-specific primers and standard PCR procedures. All samples were checked for GAPDH cDNA to confirm the use of equal amounts of cDNA. The amplified DNA fragments were analyzed on 1% agarose gels and subsequently documented using a BioDoc Analyzer (Biometra, Göttingen, Germany). For each gene, the number of cycles was chosen directly above detection threshold. The following primer sequences were used: DUOX2-F 5′-ACGCAGCTCTGTGT-CAAAGGT-3′, DUOX2-R 5′-TGATGAACGAGACTCGACAGC-3′; OAS1-F 5′-TGGCTCCTCAGGCAAGGGC-3′, OAS1-R 5′-TGGTACCAGTGCTTGACTAG-GCGG-3′; GAPDH-F 5′-CCAGCCGAGCCACATCGC-3′, GAPDH-R 5′-AT-GAGCCCCAGCCTTCTCCAT-3′.

Bacterial invasion

Bacterial cytoinvasion was analyzed using FACS analysis of CFSE-labelled bacteria by modification of a procedure described by Pils et al. (Pils et al., 2006).

Dual luciferase reporter gene assay

To quantify NFκB activation, a dual-luciferase reporter gene assay (Promega, Madison, WI) was used according to the manufacturer's instructions. Cells were grown until 50-70% confluency on 96-well plates and transfected with the respective siRNAs. After 48 hours, cells were transfected with 12 ng/well of pNFκB_Luc plasmid (Stratagene, La Jolla, CA), 3 ng/well of pRL-TK reference plasmid and 2 ng/well of pcDNA3.1-NOD2 expression plasmid or empty vector. The next day, cells were stimulated with 10 μg/ml MDP or medium alone for 6 hours. Cell lysates were analyzed on a Tecan GeniosPro microplate reader (Tecan Trading AG, Crailsheim, Germany). All experiments were measured in triplicate. NFκB activation was calculated as relative light units (RLU) by normalizing firefly luciferase activity to reference activity (*Renilla* luciferase).

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