A role for membrane-bound CD147 in NOD2-mediated recognition of bacterial cytoinvasion

Andreas Till[1,*], Philip Rosenstiel[1,2,*,‡], Karen Bräutigam[1], Christian Sina[3], Gunnar Jacobs[1], Hans-Heinrich Oberg[4], Dirk Seegert[5], Trinad Chakraborty[6] and Stefan Schreiber[1,3,‡]

[1]Institute for Clinical Molecular Biology, [3]1st Department for General Internal Medicine and [4]Institute of Immunology, University Hospital Schleswig-Holstein, Campus Kiel, Schittenhelmstr. 12, 24105 Kiel, Germany
[2]Max-Planck Institute for Molecular Genetics, Berlin, Germany
[5]CONARIS Research Institute AG, Kiel, Germany
[6]Institute for Medical Microbiology and Hygiene, University of Giessen, Germany
*These authors contributed equally to this work
‡Authors for correspondence (e-mails: p.rosenstiel@mucosa.de; s.schreiber@mucosa.de)

Summary

NOD2 is an intracellular receptor for the bacterial cell wall component muramyl dipeptide. Mutations in the leucine-rich repeat region of NOD2, which lead to an impaired recognition of muramyl dipeptide, have been associated with chronic inflammatory diseases of barrier organs such as Crohn disease, asthma and atopic eczema. In this study we identify CD147 (also known as BSG and EMMPRIN), a membrane-bound regulator of cellular migration, differentiation and inflammatory processes, as a protein interaction partner of NOD2. We demonstrate a complex influence of the CD147-NOD2 interaction on NOD2-dependent signaling responses. We show that CD147 itself acts as an enhancer of the invasion of *Listeria monocytogenes*, an intracellular bacterial pathogen. We propose that the CD147-NOD2 interaction serves as a molecular guide to regulate NOD2 function at sites of pathogen invasion.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/4/487/DC1

Key words: Inflammation, Crohn disease, Protein interaction, CD147 (BSG, EMMPRIN), NOD2 (CARD15), Cytoinvasion, *Listeria monocytogenes*

Introduction

The innate immune system represents the first line of defense of metazoans against an infection by bacterial pathogens. This primary response is mediated via the recognition of invariant pathogen-associated molecular patterns (PAMPs) by a limited set of germline-encoded receptors (Chamaillard et al., 2003a; Pandey and Agrawal, 2006). These include Toll-like receptors (TLRs), which are transmembrane proteins localized in the plasma membrane or in cytosolic vesicles, and NOD-like receptors (NLRs), which are cytosolic sensors for bacterial components and are capable of recognizing intracellular PAMPs.

NOD2, a prototypical member of the NLR family, comprises two N-terminal caspase recruitment domains (CARDs), a central nucleotide binding and oligomerization domain (NBD) and a C-terminal leucine-rich repeat (LRR) region which mediates the ability to recognize muramyl dipeptide (MurNAc–L-Ala–D-isoGln; MDP), a component of the peptidoglycan layer of bacterial cell walls (Chamaillard et al., 2003a; Morre et al., 2004). NOD2 is expressed constitutively by monocytes and macrophages, granulocytes and dendritic cells and is induced in intestinal epithelial cells upon pro-inflammatory stimuli (Ogura et al., 2001b; Rosenstiel et al., 2003). Sensing of MDP elicits a recruitment of the cytosolic adaptor protein RIP2 (also known as RIPK2, RICK and CARDIAK) and leads to activation of the canonical nuclear factor kappa B (NF-κB) pathway. By induction of proinflammatory cytokines and chemokines (e.g. IL8 and IL1β), NOD2-mediated MDP recognition results in systemic inflammatory responses to bacterial pathogens. However, the NOD2-dependent release of α- and β-defensins points to a pivotal role of NOD2 in the direct anti-microbial defense mechanisms of the intestinal epithelial barrier (Kobayashi et al., 2005; Wehkamp et al., 2004). Functional studies have shown that NOD2 in humans may be important for the recognition of a diverse range of pathogens including *Listeria* spp., *Pneumococci* (Opitz et al., 2004), *Mycobacterium tuberculosis* (Ferwerda et al., 2005) and *Helicobacter pylori* (Rosenstiel et al., 2006a).

Variants in the *NOD2* (*CARD15*) gene have been described to be genetically associated with a number of inflammatory barrier diseases (Costello et al., 2005). Missense or nonsense mutations within or close to the LRR region (i.e. SNP8: R702W, SNP12: G908R, SNP13: L1007fs[insC]) contribute to the development of Crohn disease (CD), a chronic relapsing-remitting inflammatory disorder of the digestive tract (Hampe et al., 2001; Hugot et al., 2001; Ogura et al., 2001a). The CD-associated mutations lead to a diminished NF-κB activation upon MDP stimulation (Bonen et al., 2003; Ogura et al., 2001b). This impairment in NOD2 function causes a complex barrier defect including facilitated entry of bacteria into epithelial cells through defective regulation of defensin expression, impaired bactericidal capacity and reduced secretion of cytokines ('loss of function'). Subsequently, these variants have been associated with other diseases, such as allergy, atopic eczema (Kabesch et al., 2003), colonic cancer (Kurzawski et al., 2004) and MALT-lymphoma associated with chronic *H. pylori* infection (Rosenstiel et al.,

2006a). The data substantiate the hypothesis that NOD2 may play a crucial role as a guarding molecule for interactions between host cells and bacteria on body surfaces, which may include outer and inner barrier organs.

Similar to other NLRs, upon activation, NOD2 constitutes a macromolecular effector platform: the 'nodosome'. It is tempting to speculate that the function of NOD2 is modulated by various interacting proteins resulting in different cellular outcomes, which depend on the composition of this effector complex. Recent reports point to a dynamic recruitment of the NOD2 complex to membrane compartments (Barnich et al., 2005a; Kufer et al., 2006).

In this study, a bacterial two-hybrid screen using a colonic cDNA library identified the transmembrane receptor CD147 (EMMPRIN) as a novel NOD2-interacting protein. We describe the regulation and functional contribution of CD147 in NOD2-mediated innate immune responses to MDP and assess its role for bacterial invasion into epithelial cells.

Results

CD147/EMMPRIN interacts with NOD2

To identify novel NOD2-interacting proteins, we performed a bacterial two-hybrid screening with NOD2 and NOD2 domains (CARDs, NBD, LRR) as bait proteins and a human colonic cDNA library cloned into the target vector (supplementary material Fig. S1). The screening identified CD147 as an interaction partner of NOD2 (eight independent positive clones for CD147: five identified by using full-length NOD2 as bait and three by a bait construct containing both CARDs). The interaction could be confirmed for full-length NOD2 and for constructs only expressing the two N-terminal CARD domains, but for neither the NBD nor the C-terminal LRR domain alone.

To verify our results in a eukaryotic cell system, we performed co-immunoprecipitation experiments in HEK293 cells using stable as well as transient overexpression with different constructs (for illustration see supplementary material Fig. S2A,B). In all experimental approaches we could confirm the interaction of CD147 with the N-terminal region of NOD2 as shown in Fig. 1. Using stably transfected HEK^{NOD2} cells and HEK^{mock} cells as control we found a specific band representing NOD2 in anti-CD147 immunocomplexes (Fig. 1A). No such band was detected using either control antibody or HEK^{mock} cells. Subsequent mapping of the interaction confirmed that neither the central NBD nor the C-terminal LRR domain alone or a combination of NBD and LRR could be precipitated with CD147. Both CARD domains seemed to be required for the interaction as a construct containing both CARDs, but not the individual CARDs alone, was able to interact with CD147 (Fig. 1B). In a reciprocal setup, only the intracellular domain (IC) of CD147 (CD147-IC), fused to enhanced green fluorescent protein (EGFP), was capable of precipitating with full-length NOD2 or both CARD domains (Fig. 1C), whereas no interaction was observed between CD147-IC and any other NOD2 domain. As expected, CD147 expression constructs lacking the intracellular part showed no interaction (data not shown). To provide evidence for an interaction of endogenous NOD2 and CD147, immunoprecipitation experiments using a myelomonocytic cell line THP1 were performed. In anti-CD147 precipitates we could identify a protein band of the predicted size of full-length NOD2 (Fig. 1D). A second band of slightly smaller molecular size was also detectable that may correspond to smaller NOD2 isoforms (King et al., 2007; Leung et al., 2006; Ogura et al., 2001b).
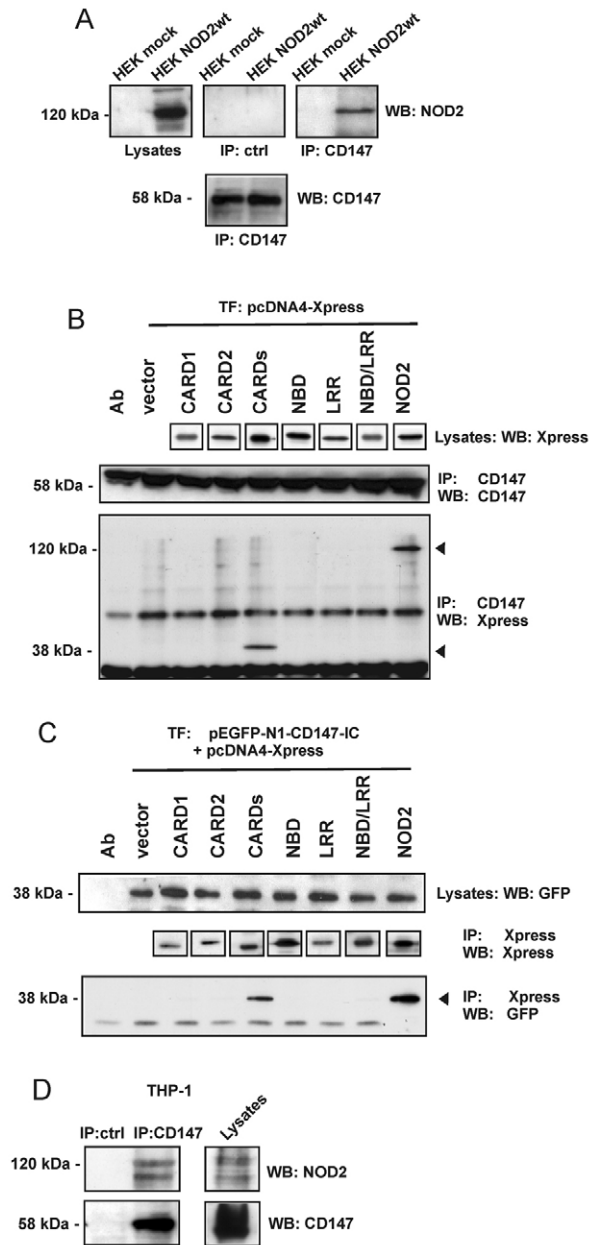

Fig. 1. The CARD domains of NOD2 interact with the intracellular stalk of CD147. (A) HEK293 cells stably transfected with NOD2 expression construct (HEK^{NOD2}) or empty vector (HEK^{mock}) were used for co-immunoprecipitation of CD147 and NOD2. CD147 immunocomplexes and whole cell lysates were assayed for endogenous and precipitated NOD2 and CD147. Precipitates from irrelevant antibody were used as specificity control (ctrl). (B) HEK293 cells were transfected with constructs containing Xpress-tagged NOD2 or NOD2 domains. After precipitation of endogenous CD147, co-precipitated NOD2 or NOD2 domains were visualized using anti-Xpress antibody. Only full-length NOD2 and a fusion protein containing both CARD domains were found in CD147 immunocomplexes (black arrowheads). (C) HEK293 cells were transfected with constructs containing Xpress-tagged NOD2 or NOD2 domains in combination with a construct containing the intracellular domain of CD147 (IC) fused to EGFP. After precipitation of Xpress-tagged NOD2 domains, EGFP-CD147-IC was identified in the immunocomplexes using anti-GFP antibody. Only full-length NOD2 and both CARD domains were able to precipitate with GFP-CD147-IC (indicated by black arrowheads). Antibody alone (Ab) was used as control. (D) CD147 immunocomplexes derived from THP1 monocytes were used to verify the interaction of CD147 with endogenous NOD2. Detection of two specific bands by use of NOD2 antibody points to the presence of more than one NOD2 isoform.

Taken together, we could identify the glycoprotein CD147 as a novel NOD2 interaction partner and could map the interaction site to the CARD-CARD domain of NOD2 and the IC of CD147.

Regulation of CD147 expression by proinflammatory stimuli

Analysis of tissue distribution of CD147 and NOD2 expression using human cDNA tissue panels revealed overlapping expression in colon and thymus, pointing to a putative physiological role of the interaction in these organs (supplementary material Fig. S3). For analysis of CD147 regulation under proinflammatory conditions, we performed stimulation experiments on THP1 cells with a variety of proinflammatory stimuli. As shown in the examples in Fig. 2A,B, the stimulation of THP1 cells with proinflammatory stimuli leads to an increased expression of CD147 mRNA and protein. TNF-α and IFN-γ (also known as TNFA, INFG, respectively; data not shown), and LPS alone showed only a minor effect, whereas MDP and the combination of TNF-α and IFN-γ produced a strong induction of CD147. Infection of THP1 cells with cytoinvasive *L. monocytogenes* (MOI=100) also results in a significant upregulation of CD147 mRNA levels. In addition, proinflammatory stimuli resulted in an accumulation of CD147 protein at the cell surface, as shown by FACS analysis (Fig. 2B). These results are consistent with a recent report describing an induction of CD147 by NF-κB and AP1 activating pathways in tumor-associated macrophages (Hagemann et al., 2005).

CD147 and NOD2 co-localize at the membrane of epithelial and myelomonocytic cells

To determine the site of complex formation between NOD2 and CD147, the subcellular localization of the two proteins in human cells was analyzed. HeLaS3 cells were transiently transfected with GFP-tagged human NOD2. CD147 was detected using a specific antibody. The CD147 signal was mainly confined to the cell membrane. GFP-NOD2 was partially localized in the cytoplasm, but also at the cell membrane (Fig. 3A) with a prominent signal in membrane ruffles and cellular spiculae as described previously (Barnich et al., 2005a; Kufer et al., 2006; Legrand-Poels et al., 2007; McDonald et al., 2005). The NOD2 signal clearly colocalizes with CD147 in the absence of bacterial infection. No membrane association was detectable for GFP protein itself (data not shown). To address the spatial distribution of the CD147-NOD2 interaction in the natural course of bacterial cytoinvasion we investigated GFP-NOD2-transfected HeLaS3 cells that were infected with the invasive *L. monocytogenes* strain EGD. Again, the CD147 staining exhibited a strong colocalization with NOD2, especially in fibrolamellar structures at the sites of bacterial entry (Fig. 3B, open arrowheads, insert).

Fluorescence microscopy was used to analyze the subcellular localization of NOD2 and CD147 in PMA-differentiated THP1 monocytes. As shown in Fig. 3C, staining of CD147 and NOD2 using specific antibodies showed a clear membrane association and a partial overlapping localization (arrowheads in Fig. 3C).

CD147 negatively regulates NOD2-dependent NF-κB activation and cytokine release

To analyze the role of CD147-NOD2 interaction in NOD2-induced signaling pathways, we performed reporter gene assays with an NF-κB-dependent luciferase system and enzyme-linked immunosorbent assays for detection of IL8 release upon stimulation with MDP. As expected, we found no MDP-induced
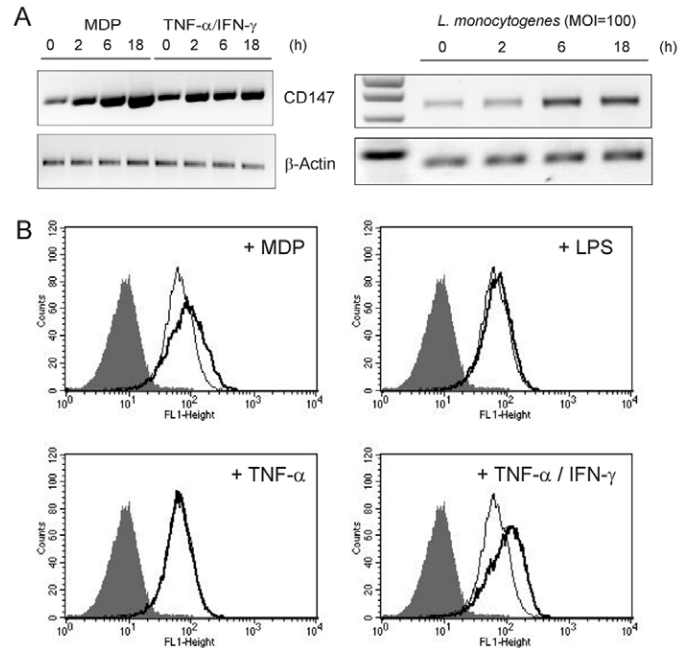

Fig. 2. Expression and regulation of CD147. (A) CD147 mRNA levels in monocytic THP1 cells are upregulated by stimulation with MDP, TNF-α plus IFN-γ and infection with *L. monocytogenes*. (B) THP1 monocytes were stimulated for 24 hours with different agents (thick line) or left untreated (thin line). Cell surface expression of CD147 protein was detected by FACS. Irrelevant antibody was used as specificity control (gray curve). A variety of proinflammatory stimuli (MDP, LPS, TNF-α/IFN-γ) lead to an increased expression of CD147. Note that TNF-α alone showed only a minor effect.

activation of NF-κB or IL8 release in HEK293 cells in the absence of NOD2 protein. As shown in Fig. 4A, the overexpression of either full-length CD147 or its IC lead to a two- to threefold reduction of basal and MDP-induced NF-κB activity in HEK^{NOD2} cells. However, the expression of the extracellular domain of CD147 showed nearly no effect. These data correlate with our results from co-immunoprecipitation studies and favor the concept of direct interaction of both CARDs with the intracellular part of CD147. In addition, the release of IL8 by MDP (10 μg/ml) was markedly reduced by ectopic expression of CD147 in a dose-dependent manner (Fig. 4B). However, no significant effect on the basal level of IL8 secretion was observed. It is important to note that the expression of NOD2 was not influenced by overexpression of CD147 fusion proteins or gene silencing, as confirmed by RT-PCR and immunoblotting (data not shown).

To further confirm the regulatory role of this interaction, we used gene silencing by introducing small hairpin RNAs directed against CD147. As shown in Fig. 5A, the transfection with expression constructs containing CD147 specific target sequences (CD147-si 1-3) resulted in a significant downregulation of CD147 protein whereas the unspecific control target sequence (ctrl-si) had no effect. NF-κB activation after stimulation of HEK293 cells stably overexpressing NOD2 with MDP (10 μg/ml) was strongly enhanced in cells transfected with shRNA targeting CD147 (Fig. 5B). Moreover, MDP-induced release of IL8 was significantly augmented by gene silencing of CD147 in the colonic epithelial cell line SW480 constitutively expressing endogenous NOD2 (Fig. 5C). Taken together, our results point to a role of CD147 as a negative regulator of NOD2 signaling after stimulation with MDP.
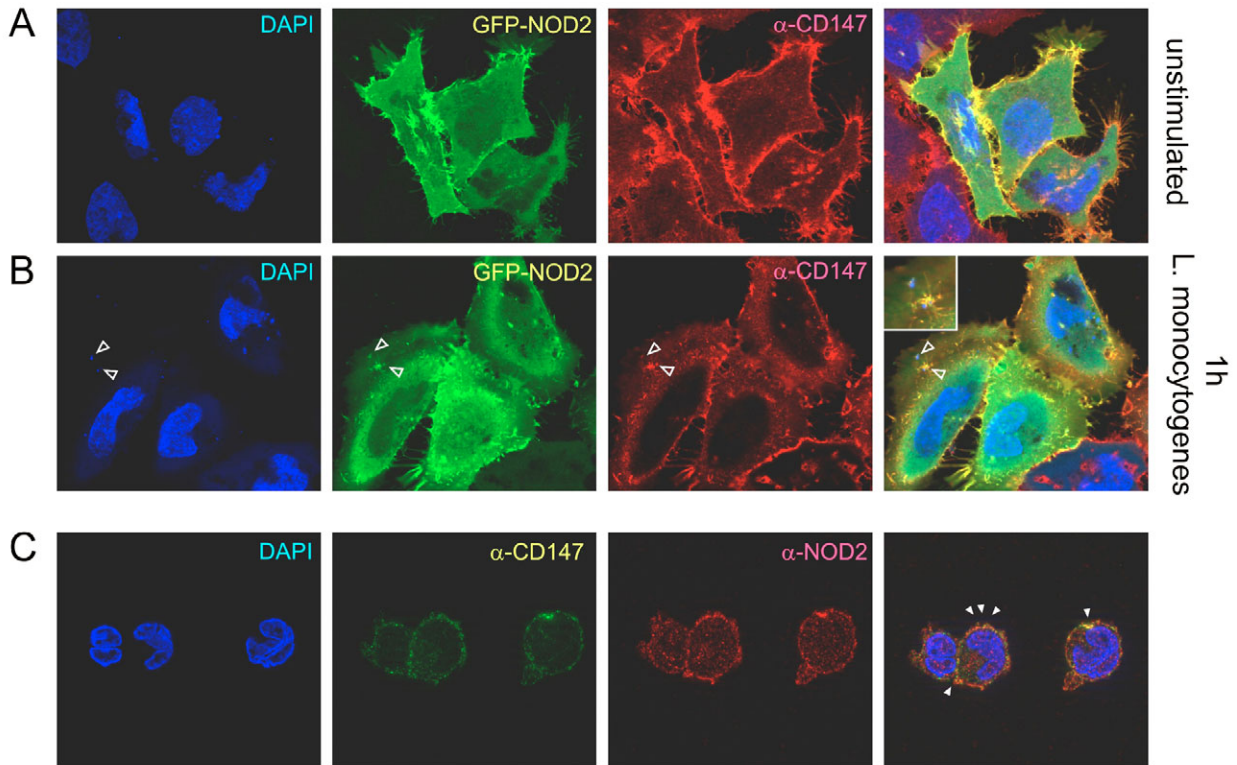
Fig. 3. Co-localization of CD147 and NOD2 at the membrane of epithelial and myelomonocytic cells. (A) HeLaS3 cells were transiently transfected with GFP-NOD2 (green) and stained for CD147 (red) and DAPI as a DNA counterstain (blue). (B) Transfected cells were infected with *L. monocytogenes* (MOI=100) and fixed after 1 hour. In the basal as well as in the infected state, NOD2 and CD147 colocalize in the membrane compartment. An accumulation of both NOD2 and CD147 in areas of early bacterial invasion is detectable (open arrowheads and inset in B). (C) Indirect immunofluorescence micrographs of PMA-differentiated THP1 myelomonocytic cells. Staining with specific antibodies against endogenous NOD2 (red channel) and CD147 (green channel) shows a partial colocalization at the cell membrane (arrowheads in merged image). Images are representative of at least three independent experiments. Original magnification: 100×.

CD147 expression enhances invasion of *Listeria* into epithelial cells

CD147 has been described as a key molecule involved in infection of human cells by HIV-II and SARS-CoV (Chen et al., 2005; Pushkarsky et al., 2001). By direct interaction of CD147 with viral nucleocapsid proteins, it facilitates the entrance of virus particles into mammalian cells. To evaluate the role of the CD147-NOD2 interaction in bacterial infection, we studied the invasion by *L. monocytogenes* into HEK293 cells using gentamicin protection assays. As shown in Fig. 6, NOD2 overexpression resulted in a reduced infection of HEK293 cells. This points to the role of NOD2 as a cytosolic surveillance protein and mediator of anti-microbial defense mechanisms. However, overexpression of CD147 resulted in an increased invasion into HEK293 cells, which could only partly be prevented by NOD2 overexpression (Fig. 6A). By contrast, when using HEK293 cells stably expressing hairpin RNAs targeting CD147, relative invasion of *Listeria* was significantly decreased (Fig. 6B). The same effect was achieved by preincubation of the cells with a specific antibody directed against the extracellular domain of CD147. Thus, CD147 expression appears to favor the invasion of *Listeria* into epithelial cells, which is counteracting the anti-bacterial defense mechanism of NOD2. Importantly, neither cell proliferation (as assessed by MTT test) nor apoptosis (determined by propidium iodide/annexinV FACS) were influenced by overexpression of NOD2 or CD147 under the described conditions (data not shown). Since the influence of

CD147 on bacterial entry might be explainable by differential regulation of host cell factors involved in cytoinvasion, we asked if modification of CD147 expression had any influence on known entry factors. Cytoinvasion of *L. monocytogenes* into non-professional phagocytic cells such as epithelial cells is induced by binding of bacterial cell surface proteins (internalins) to receptors on the host cell (Hamon et al., 2006). Internalin A (InlA) specifically binds to adherens junction protein E-cadherin (Mengaud et al., 1996), whereas InlB physically interacts with Met, the cognate receptor for hepatocyte growth factor, to induce phagocytosis (Shen et al., 2000). In order to dissect the entry mechanism relevant for our experimental system, we used *Listeria monocytogenes* wild-type and mutant strains genetically deficient in either InlA or InlB (ΔInlA, ΔInlB). Gentamicin-protection assays identified a major relevance of InlB for *Listeria* invasion of HEK293 cells since ΔInlB mutants showed an almost complete loss of invasion capacity whereas invasion by ΔInlA mutant strain was only marginally reduced (Fig. 7A). Interestingly, we found that neither overexpression nor RNAi-mediated knockdown of CD147 resulted in an altered cell surface expression of Met and E-cadherin (Fig. 7B). An indirect effect of CD147 by influencing entry factor expression may thus be excluded.

Our data point to a novel role of CD147 in cell invasion of *Listeria monocytogenes* into epithelial cells and emphasize the importance of further elucidating the mechanisms underlying CD147-NOD2 interactions.
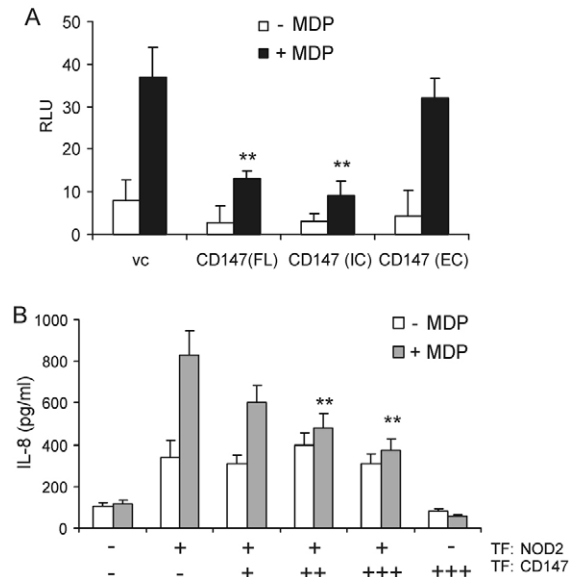
Fig. 4. CD147 negatively regulates NOD2-mediated NF-κB activation and IL8 release. (A) NOD2-dependent MDP-induced activation of NF-κB was determined by dual-luciferase assay. Overexpression of full-length CD147 (FL) or its intracellular domain (IC), but not of the extracellular domain (EC) lead to a decrease of NOD2-dependent NF-κB activation. (RLU, relative light units; vc, vector control; **$P<0.01$ for transfection of CD147 constructs versus vc). (B) Overexpression of full-length CD147 impairs NOD2-dependent IL8 release in a dose-dependent manner. HEK293 cells were transiently transfected with pcDNA4-Xpress-NOD2 and increasing amounts of pEGFP-N1-CD147 and were left untreated or were treated with MDP. Supernatants were assayed for IL8 by enzyme-linked immunosorbent assay. Transfection with empty vector pEGFP-N1 served as control (indicated by '–'). Data represent the mean ± s.d. of three independent experiments (**$P<0.01$ for transfection of CD147 construct versus control).

Discussion

The family of NOD-like receptors (NLRs, CATERPILLER) represents a major surveillance mechanism of the vertebrate innate immune system and has functional homologues in the invertebrate and plant kingdom (Chisholm et al., 2006; Nurnberger et al., 2004). Strikingly, recent advantages in finding genetic risk factors for inflammatory disorders have pointed to a pivotal role of these proteins in the etiopathogenesis of several autoimmune and inflammatory diseases (Chamaillard et al., 2003b; Tschopp et al., 2003). Evidence is emerging that NLR proteins build macromolecular complexes upon activation and that the composition of these complexes crucially modulates the net outcome of the ligand-induced signal. The NOD2 gene represents a major genetic risk factor for several chronic inflammatory disorders of barrier organs. Dissecting the molecular framework of proteins, which is necessary to modulate MDP detection and activation of signaling processes by NOD2, will contribute to the understanding of how NOD2 senses invasive and non-invasive bacteria at the forefront of epithelial barrier organs.

To date there are several proteins identified that directly interact with NOD2. Recruitment of RIP2 (also designated RICK, CARDIAK and RIPK2) to the CARD domains of NOD2 has been shown to be the critical effector mechanism for the activation of NF-κB downstream of MDP sensing. Other proteins described as NOD2 interaction partners are transforming-growth factor β (TGFβ)-activating kinase 1 (TAK1; also known as MAPK3K7) and GRIM-19 (also known as NDUFA13) (Barnich et al., 2005b).
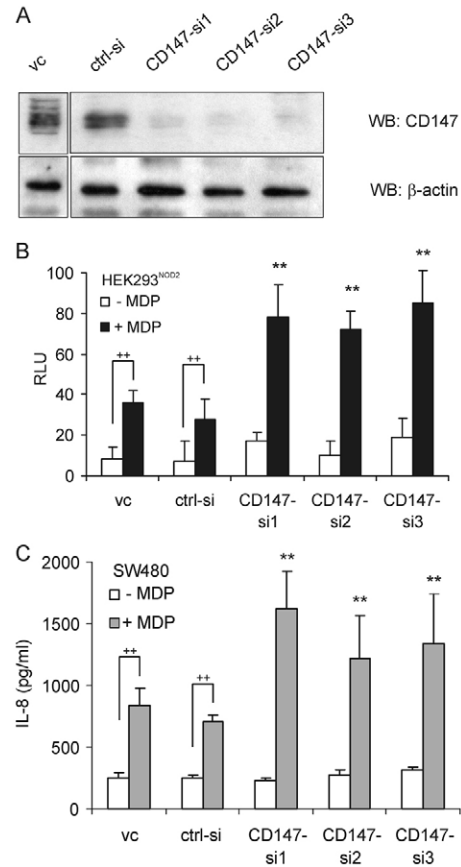

Fig. 5. Gene silencing of CD147 induces upregulation of NOD2-mediated NF-κB activation and IL8 release. HEK[NOD2] cells and SW480 cells expressing endogenous NOD2 were transfected with pSUPER.neo+GFP vectors containing three different target sequences for CD147 (CD147-si1 to si3) or irrelevant control sequence (ctrl-si). (A) After selection and sorting, HEK[NOD2] cells were assayed for CD147 expression by immunoblotting. Equal loading was monitored by concomitant detection of β-actin (lower panel). (B) Stably transfected shRNA HEK[NOD2] cells were stimulated with MDP or left untreated. Induction of NF-κB activity was determined as described in Materials and Methods. Data are expressed as relative luciferase activity (RLU, mean ± s.d.; $n=3$ independent experiments). (C) Transiently transfected SW480 cells were stimulated with MDP (50 μg/ml) overnight and assayed by ELISA for IL8 release. Gene silencing of CD147 augmented NF-κB activation and IL8 secretion in both transfected HEK293 cells and SW480 cells expressing endogenous NOD2 (++$P<0.01$ for MDP-stimulated versus unstimulated cells; **$P<0.01$ for CD147-si versus ctrl-si).

Recently, ERBIN (also known as ERBB2IP) has been identified by two independent groups as another NOD2-interacting protein by using biochemical screening methods and a yeast two-hybrid approach, respectively (Kufer et al., 2006; McDonald et al., 2005). ERBIN is a transmembrane protein involved in cellular polarity and receptor trafficking; the interaction is dependent on the CARD domain structure of NOD2, which negatively influences NOD2 signaling.

In this report, we identified CD147 as a novel interaction partner of NOD2, using bacterial two-hybrid screening. The interaction could be confirmed by co-immunoprecipitation assays and was mapped to both CARD domains of NOD2 and to the cytosolic part of CD147 as minimal necessary interaction structures. In this context, CD147 is similar to RIP2 and ERBIN using the CARD-CARD structure as protein interaction site, whereas TAK1 and
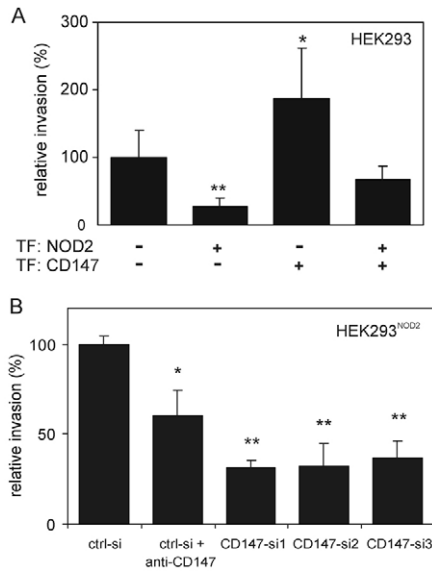
Fig. 6. Surface expression of CD147 modifies invasion of *L. monocytogenes* into epithelial cells. (A) HEK293 cells were transiently transfected with pcDNA4-Xpress-NOD2 and/or pEGFP-N1-CD147 and infected with *L. monocytogenes* for 1 hour. After killing of extracellular bacteria by gentamicin, cells were lysed and plated on solid medium. CFU of *Listeria* were expressed as relative invasion adjusted to the cell number (measured in parallel by MTT test). Transient overexpression of NOD2 led to a protection of HEK293 against infection, whereas this effect was abolished by parallel overexpression of CD147. CD147 overexpression alone enhanced the relative infection rate of *Listeria*. (B) HEK[NOD2] cells stably transfected with siRNA constructs targeting CD147 were used for gentamicin protection assays as described. Gene-silencing of CD147 strongly reduced the infection by *Listeria monocytogenes*. Antibodies directed against the extracellular part of CD147 inhibited invasion [relative invasion as a percentage of control; mean ± s.d. (*n*=3); *$P<0.05$, **$P<0.01$ for experiment versus control].
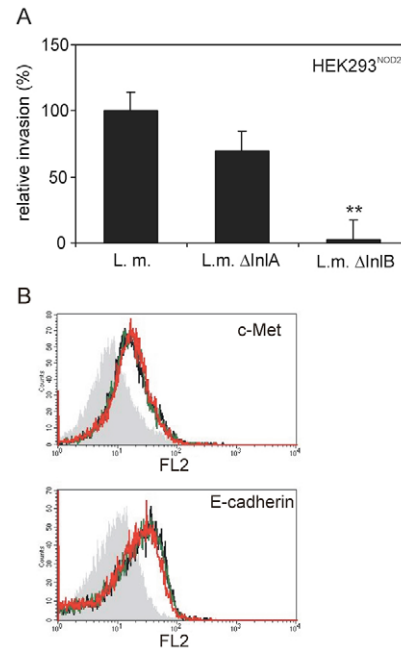

Fig. 7. CD147 does not influence expression of host cell entry factors. (A) Gentamicin protection assays with *Listeria monocytogenes* wild-type (L.m.) or mutant strains deficient in either internalin A or internalin B (ΔInlA, ΔInlB) were performed with HEK293[NOD2] cells as described in Materials and Methods. Deletion of InlB markedly reduced the ability of *Listeria* to invade into HEK293 cells, whereas deletion of InlA only had a marginal effect. Relative invasion is shown as percentage of CFUs compared to *Listeria* wild-type strain; data represent the mean ± s.d. (*n*=3); **$P<0.01$. (B) Cell surface expression of c-Met and E-cadherin. Cells stably transfected with pSUPER constructs silencing CD147 expression (red) or transiently transfected with EGFP-CD147 (green) were assayed for cell surface expression of Met (upper panel) or E-cadherin (lower panel) as described. Untransfected cells were used as positive control (black), cells treated with secondary antibody alone were used as specificity control (gray curve). Neither overexpression nor knock-down of CD147 influenced Met or E-cadherin expression level.

GRIM-19 have been shown to interact with the C-terminal part of NOD2. Interestingly, no homology of the IC of CD147 to known protein-protein interaction modules could be found. Complete deletion of the 40 amino acid IC abolished the interaction with NOD2. Which minimal structure of CD147-IC is responsible for the physical contact to the CARD-CARD structure, and if additional factors contribute to this interaction, remains to be analyzed.

CD147 is a transmembrane glycoprotein implicated in neuronal development, tumor progression and inflammation. CD147 is thought to exert its main physiological functions by homotypic or heterotypic protein-protein interactions [e.g. binding to extracellular cyclophilins (Renno et al., 2002; Yurchenko et al., 2006)]. The best characterized function of CD147 is production and activation of matrix-metalloproteases (MMPs) and involvement in cell adhesion (Choi et al., 2002; Lim et al., 1998; Schmidt et al., 2006). Remarkably, patients suffering from Crohn disease show enhanced expression levels of MMP1 and MMP9 in colonic tissue, especially in fistulae (Kirkegaard et al., 2004; Stumpf et al., 2005). Further studies will have to clarify if CD147 directly contributes to MMP dysregulation in chronic intestinal inflammation.

Regarding the subcellular localization of the interaction we demonstrate that a reasonable fraction of NOD2 protein is located close to the cell membrane and shows a merging distribution with CD147. In contrast to early reports claiming an exclusively cytosolic expression of NOD2, recent studies have presented evidence that membrane recruitment of NOD2 is not only a common event but is indispensable for proper sensing of MDP (Barnich et al., 2005a; Kufer et al., 2006; McDonald et al., 2005). We propose that proteins that relocalize NOD2 to the cell membrane might serve as molecular guides to allow a fast cellular response to bacterial invasion by procuring close proximity of NOD2 monomers and bacterial-derived MDP. We demonstrate a direct influence of cellular CD147 levels on the cytoinvasive properties of *Listeria monocytogenes* infection in HEK293 cells. A pivotal event in the invasion of mammalian cells by *Listeria* is the interaction of bacterial internalin B with Met on the host cell surface (Shen et al., 2000) resulting in reorganization of the local actincytoskeleton and clathrin-dependent endocytosis (Veiga and Cossart, 2005). Together with the recent report suggesting a functional association between NOD2 and components of the actin cytoskeleton (Legrand-Poels et al., 2007), our data point to a role of the CD147-NOD2 complex in the early phases of host-pathogen interaction. It was shown recently, that secretion of bacterial factors by *Helicobacter pylori* into the cytosol of gastric epithelial cells leads to activation of Met signaling and subsequent upregulation of matrix metalloproteases MMP2 and MMP9 (Oliveira et al., 2006). MMP-mediated degradation of components of the

extracellular matrix is assumed to favor bacterial cytoinvasion by allowing the pathogens to come in contact with and interact with host cells (Yanagisawa et al., 2005). Since CD147 has likewise been described to regulate expression and activity of MMPs, it is tempting to speculate that Met signaling and CD147-mediated MMP induction represent distinct cellular mechanisms abused by bacterial pathogens to facilitate cytoinvasion.

Interestingly, our results point to a role of CD147 as a negative regulator of NOD2 signaling after stimulation with MDP. Overexpression of full-length CD147 and of the intracellular domain (IC) lead to an impaired NF-κB response upon MDP stimulation, whereas a construct lacking the intracellular domain showed no effect. By contrast, RNAi-mediated knockdown of endogenous CD147 lead to a strong enhancement of MDP-driven NF-κB activity. The diminished release of IL8 after transient overexpression of CD147 in MDP-treated NOD2-expressing HEK293 showed that the effect is also detectable on the level of target gene expression. The finding that CD147 overexpression affects basal NF-κB activation, but obviously does not influence basal IL8 release might reflect the complex nature of chemokine regulation, since secretion of IL8 is not solely dependent on NF-κB but is affected by other signaling pathways, including MAPK, and epigenetic mechanisms (Kim et al., 2005; Schmeck et al., 2005). We would propose that the observed effects are due to a direct competition between CD147 and the essential signal adaptor RIP2 for binding to NOD2. Since both RIP2 and CD147 appear to interact via the CARD domain structure and RIP2-NOD2 interaction is required for activation of NF-κB, a stoichiometric competition of RIP2 and CD147 for the NOD2-interaction site would result in impaired NF-κB activation.

Evidence is emerging that NOD2 activation is tightly controlled using several cellular mechanisms in parallel. (i) Peroxiredoxin 4 (PRDX4) is an antioxidant enzyme located in the cytoplasm, which is upregulated by MDP in NOD2-overexpressing HEK293 cells and contributes to negative regulation of NOD2-dependent NF-κB activation (Weichart et al., 2006). (ii) The interaction of NOD2 with ERBIN has been demonstrated to block the transmission of the MDP-induced signal to the downstream effectors of NOD2. (iii) We have shown recently that an alternatively spliced form of NOD2 (designated NOD2-S) is induced by anti-inflammatory stimuli and acts as an endogenous inhibitor of NOD2 signaling by interfering with MDP-induced nodosome formation (Rosenstiel et al., 2006b). It is thus tempting to speculate that regulatory processes limiting the outcome of NOD2 dependent signaling – such as the newly identified interaction of NOD2 and CD147 – play a fundamental role in maintaining the cellular homeostasis.

This report, to our knowledge, is the first to show a functional connection between CD147 and innate immune receptors. Obtaining details about the molecular mechanism of the CD147-NOD2 interaction through atomic structure determination of the complex and subsequent functional studies of selected structure-directed mutants will be a crucial step in understanding the molecular processes involved in the recognition of cytoinvasive bacteria by NLRs. The dichotomy of the cellular regulatory network may help to facilitate the immunological balance after encounter of bacterial pathogens by the host's innate immune system. Detailed analyses of CD147 functions in the gastrointestinal tract might lead to new avenues for the treatment of inflammatory bowel diseases.

Materials and Methods

Bacterial two-hybrid screening
NOD2 full-length cDNA and single domains of NOD2 (CARDs, NBD, LRR) were amplified from leukocyte cDNA and ligated into the bait vector pBT (Stratagene, La Jolla, CA, USA) using standard cloning procedures (see supplementary material Fig. S1 for details). The construct pBT-NOD2-wt was used for generation of a bait-fusion plasmid containing the Crohn disease-associated variant NOD2-SNP13 (1007fs^{insC}) by site-directed mutagenesis as described elsewhere (Weichart et al., 2006). A human colonic cDNA library (Stratagene, La Jolla, CA) was inserted into target vector pTRG. The bacterial two-hybrid screening was performed using the BacterioMatch^{TM} Two-Hybrid System (Stratagene) according to the manufacturer's instructions. After cotransformation of pBT and pTRG plasmids and isolation of clones grown in the presence of the appropriate combination of antibiotics, plasmids were isolated using the MiniPrep kit from Qiagen (Hilden, Germany). The insert of the pTRG vector was identified by sequencing using an ABI3730 capillary sequencer (ABI, Foster City, CA, USA).

Cell lines and transfection
Human epithelial HEK293 cells (ACC305), cervical carcinoma HeLaS3 cells (ACC161), colonic carcinoma SW480 (ACC313) and human acute monocytic cell line THP1 (ACC16) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Isogenic HEK293 cell lines stably transfected with a human NOD2 expression construct (HEK^{NOD2}) or empty vector (HEK^{mock}) were previously described (Weichart et al., 2006). All cells were cultivated under standard conditions of 5% CO$_2$ and 37°C. Transfections were performed using Fugene 6^{TM} (Roche, Basel, Switzerland) according to the manufacturer's manual.

For generation of transfectants with stable RNA silencing of CD147, HEK^{NOD2} and HEK^{mock} cells were transfected with pSUPER constructs (described below), treated with 300 μg/ml G418/Geneticin (PAA Laboratories) for 4 weeks and separated by flow cytometry as described (see below).

Bacterial strains
The *Listeria monocytogenes* serotype 1/2a strain EGD was used as a model organism for cytoinvasion. EGD mutant strains deficient in either internalin A or internalin B (ΔInlA, ΔInlB) were used to further analyze the bacterial entry mechanism. Single colonies of bacteria were used for inoculation of LB medium and expanded by incubation at 37°C for 18 hours. Overnight cultures of *Listeria* were diluted 1:10 and grown for 3-4 hours to mid logarithmic phase. Bacteria were harvested by centrifugation, washed and resuspended in cell culture medium without antibiotics. For gentamicin protection assays, a multiplicity of infection (MOI) of 100 bacteria per cell was used as determined by plating serial tenfold dilutions onto LB agar plates.

Antibodies and reagents
Rabbit anti-NOD2 antibodies were purchased from Cayman (Cayman Chemical Company, Ann Arbor, MI) and Novus Biologicals (Littleton, CO). Goat anti-CD147 (EMMPRIN) antibody (K20) was from Santa Cruz (Santa Cruz, CA), monoclonal rabbit anti-CD147 antibody (ab666) was from Abcam Inc. (Cambridge, MA). Anti-green fluorescent protein (GFP) antibody was obtained from Clontech (Clontech, Palo Alto, CA). The antibody specific for the Xpress-Tag of expression plasmid pcDNA4-Xpress was from Invitrogen (Carlsbad, CA). Goat anti-Met antibody (AF276) was from R&D Systems Inc. (Minneapolis, MN), monoclonal mouse anti-E-cadherin antibody was from Axxora, LLC (San Diego, CA). All horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences (Piscataway, NJ). Cy3-conjugated or FITC-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Muramyl dipeptide (MurNAc-L-Ala-D-isoGln, MDP) was purchased from Bachem (Bubendorf, Switzerland). Phorbol myristate acetate (PMA) was from Sigma (Sigma-Aldrich Corp., St. Louis, MO). Purified lipopolysaccharide (LPS) was a kind gift from Prof. Ulrich Zähringer (Leibniz Research Center Borstel, Germany). Recombinant human TNF-α and IFN-γ were from R&D Systems.

Expression plasmids
Mammalian expression constructs for the different domains of NOD2 were constructed by insertion of the coding sequence of the appropriate domain into expression plasmid pcDNA4-Xpress (Invitrogen, Carlsbad, CA). Fusion constructs for CD147 and EGFP were generated by cloning of full-length CD147, the extracellular domain (EC; corresponding to amino acids 22-204) or the intracellular domain (IC; corresponding to amino acids 229-269) into the pEGFP-N1 vector (Clontech, Palo Alto, CA). For generation of EGFP-NOD2 fusion protein, the coding sequence for NOD2 was inserted into pEGFP-C3 using the *Hin*dIII and *Bam*HI restriction sites.

All constructs were sequence-verified, and expression constructs were tested in HEK293 and HeLaS3 cells by transient overexpression (supplementary material Fig. S2A,B).

Co-immunoprecipitation and western blots
HEK293 cells were transfected with expression constructs for NOD2 or NOD2 domains and CD147 or CD147 domains as indicated in supplementary material Fig.

S2A,B. THP1 cells and isogenic stable HEK[NOD2] as well as HEK[mock] cells were used as control cells for interaction of endogenous NOD2 and CD147. Co-immunoprecipitation experiments and western blots were performed as previously described (Till et al., 2005).

Gene silencing of CD147 by RNA interference
The mammalian RNAi vector system pSUPER (OligoEngine, Seattle, WA) was used for silencing of CD147 gene expression. Three different siRNA target sequences for CD147 were inserted into pSUPER.neo+GFP using *Bgl*II and *Hin*dIII restriction sites according to the manufacturer's manual. Target sequences were as follows: CD147_si1 5′-AAGACCTTGGCTCCAAGATAC-3′, CD147_si2 5′-AAGTCGT-CAGAACACATCAAC-3′, CD147_si3 5′-AAGATCACTGACTCTGAGGAC-3′. An unspecific target sequence was used as specificity control. For stable transfection, HEK[NOD2] and HEK[mock] were treated with G418 (300 μg/ml) for 4 weeks after transfection and selected for GFP expression using a FACSAria Cell-Sorting System (Beckton-Dickinson, San Jose, CA). Expression levels of CD147 were assayed by immunoblotting and flow cytometry as described (below). To analyze the effect of CD147 silencing on IL8 release downstream of endogenous NOD2, intestinal epithelial SW480 cells were used for transient transfection with the same constructs.

Immunofluorescence
HeLaS3 cells were seeded on sterile cover slides at 4×10^5 cells/ml in 6-well plates. The next day, cells were transfected with 1 μg/well pEGFP-C3-NOD2 or pEGFP-C3 mock vector and cultured for 24 hours. After replacement of cell culture medium by medium without antibiotics, cells were infected with *L. monocytogenes* at a MOI of 100 for 1 hour at 37°C, followed by fixation in 4% paraformaldehyde-PBS. After blocking, cells were stained for endogenous CD147 using a goat polyclonal antibody against CD147 at 1:200 and Cy3-conjugated anti-goat secondary antibody (Jackson Immuno Research; 1:200). Visualization of eukaryotic and bacterial DNA was performed by DAPI staining (Sigma-Aldrich GmbH, Munich, Germany).

For localization studies of endogenous NOD2, THP1 myelomonocytic cells were differentiated using PMA at 10 ng/ml for 24 hours. Cells were fixed using 4% paraformaldehyde-PBS, and adherent cells were stained with a polyclonal rabbit NOD2 antibody (Novus Biologicals, Littleton, CO; 1:200) and a Cy3-coupled secondary antibody (Jackson Immuno Research; 1:350). Specificity of the NOD2 antibody was determined using western blot analysis (data not shown). Endogenous CD147 was stained using monoclonal antibody ab666 (1:200) and FITC-conjugated secondary antibody (Jackson Immuno Research; 1:200).

After washing, cover slides 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).

FACS analysis
HEK293 and THP1 were grown at 5×10^5 cells/2 ml in a 6-well plate overnight, stimulated with 10 μg/ml MDP, 100 ng/ml purified LPS, 10 ng/ml TNF-α, IFN-γ (10,000 IU/ml) or a combination thereof (TNF-α/IFN-γ) and assayed for CD147. FACS analysis was performed using a FACScalibur (Becton-Dickinson, San Jose, CA). For analysis of cell surface expression of Met and E-cadherin on HEK293 cells transiently transfected with CD147-EGFP or stably transfected with pSUPER constructs, cells were stained using anti-Met or anti-E-cadherin antibodies and the appropriate secondary antibodies labelled with Cy3. For cytometric measurement, cells were gated for positive EGFP fluorescence and analyzed for Cy3 staining.

Isolation of mRNA and RT-PCR
Cells were seeded on 6-well plates at 4×10^5 cells/well and stimulated for the times indicated with MDP (10 μg/ml), purified LPS (100 ng/ml), TNF-α (10 ng/ml), IFN-γ (10,000 IU/ml) or a combination thereof. For infection with *Listeria*, cell medium was replaced by medium without antibiotics and cells were cultured in the presence of bacteria for 1 hour at 37°C. Medium was replaced by medium containing antibiotics and cells were cultured for the times indicated. Total RNA was isolated and reverse transcribed as described elsewhere (Waetzig et al., 2002). For analysis of tissue distribution pattern of CD147 and NOD2, commercially available human cDNA tissue panels (Clontech) were used (see supplementary material Fig. S3). Expression of CD147 and NOD2 was analyzed by RT-PCR using standard protocols. All samples were checked in parallel for β-actin mRNA expression. Amplified DNA fragments were analyzed on 1% agarose gels and documented using a BioDoc Analyzer (Biometra, Göttingen, Germany).

Dual luciferase reporter gene assay
Activation of the transcription factor NF-κB was determined using a dual-luciferase reporter gene kit (Promega, Madison, WI) according to the manufacturer's manual. Cells seeded on 96-well plates were transfected with 15 ng/well of pNF-κB_Luc plasmid (Stratagene, La Jolla, CA, USA) in combination with 5 ng/well of pRL-TK (Promega) and 30 ng/well of expression plasmids for full length (FL), intracellular domain (IC) or extracellular domain (EC) CD147. Empty vector (pEGFP-N1) was used as vector control (vc). For gene silencing experiments, HEK[mock] and HEK[NOD2] cells stably transfected with pSUPER small hairpin constructs (described above)

were used for transient transfection of reporter vectors. Cells were stimulated with 10 μg/ml MDP for 6 hours or left untreated. Cell lysates were analyzed on a Tecan Genios Pro microplate luminometer (Tecan Trading AG, Switzerland). All samples were measured in duplicates and all experiments were repeated independently at least three times. The results for NF-κB-driven firefly luciferase activity were normalized using the reference plasmid and expressed as relative light units (RLU).

ELISA
HEK293 cells ($10^4/100$ μl) were transfected with or without 3 ng pcDNA4-Xpress-NOD2 and increasing amounts of pEGFP-N1-CD147 ranging from 0 to 25 ng/well. The total amount of DNA used per well was equilibrated using pEGFP-N1. The next day, HEK293 cells were stimulated with MDP (10 μg/ml) or left untreated. Alternatively, SW480 cells ($10^4/100$ μl) transiently transfected with shRNA constructs targeting CD147 (50 ng/well) were stimulated overnight with MDP (50 μg/ml). Supernatants were collected after 24 hours, and release of human IL8 was measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Rochester, MN, USA). The results were expressed as pg cytokine/ml. To assure equal cell numbers, cell viability was checked using a colorimetric MTT test (Promega, Madison, WI).

Gentamicin protection assay
HEK293 cells were seeded on 12-well plates, incubated overnight and transiently transfected with pcDNA4-Xpress-NOD2, pEGFP-N1-CD147, a combination of both plasmids or empty vector (mock transfection). In an additional experimental setup, HEK[mock] and HEK[NOD2] cell populations stably transfected with pSUPER constructs were used. Where indicated, cells were pretreated with anti-CD147 mouse monoclonal antibody to selectively block CD147 activity. The cells were infected with *Listeria* using a MOI of 100 bacteria/cell. After 1 hour infection, extracellular bacteria were killed by replacing the medium with DMEM containing gentamicin (50 μg/ml) for 1 hour. Cells were then washed, lysed in 1% Triton X-100-PBS and plated on solid medium for counting of colony forming units (CFU). All experiments were performed in triplicate. Relative infection was calculated as a percentage of CFU from transfected to mock-transfected cells.

References
Barnich, N., Aguirre, J. E., Reinecker, H. C., Xavier, R. and Podolsky, D. K. (2005a). Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-{kappa}B activation in muramyl dipeptide recognition. *J. Cell Biol.* **170**, 21-26.

Barnich, N., Hisamatsu, T., Aguirre, J. E., Xavier, R., Reinecker, H. C. and Podolsky, D. K. (2005b). GRIM-19 interacts with nucleotide oligomerization domain 2 and serves as downstream effector of anti-bacterial function in intestinal epithelial cells. *J. Biol. Chem.* **280**, 19021-19026.

Bonen, D. K., Ogura, Y., Nicolae, D. L., Inohara, N., Saab, L., Tanabe, T., Chen, F. F., Foster, S. J., Duerr, R. H., Brant, S. R. et al. (2003). Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. *Gastroenterology* **124**, 140-146.

Chamaillard, M., Girardin, S. E., Viala, J. and Philpott, D. J. (2003a). Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. *Cell. Microbiol.* **5**, 581-592.

Chamaillard, M., Philpott, D., Girardin, S. E., Zouali, H., Lesage, S., Chareyre, F., Bui, T. H., Giovannini, M., Zaehringer, U., Penard-Lacronique, V. et al. (2003b). Gene-environment interaction modulated by allelic heterogeneity in inflammatory diseases. *Proc. Natl. Acad. Sci. USA* **100**, 3455-3460.

Chen, Z., Mi, L., Xu, J., Yu, J., Wang, X., Jiang, J., Xing, J., Shang, P., Qian, A., Li, Y. et al. (2005). Function of HAb18G/CD147 in invasion of host cells by severe acute respiratory syndrome coronavirus. *J. Infect. Dis.* **191**, 755-760.

Chisholm, S. T., Coaker, G., Day, B. and Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **124**, 803-814.

Choi, E. Y., Kim, D., Hong, B. K., Kwon, H. M., Song, Y. G., Byun, K. H., Park, H. Y., Whang, K. C. and Kim, H. S. (2002). Upregulation of extracellular matrix metalloproteinase inducer (EMMPRIN) and gelatinases in human atherosclerosis infected with Chlamydia pneumoniae: the potential role of Chlamydia pneumoniae infection in the progression of atherosclerosis. *Exp. Mol. Med.* **34**, 391-400.

Costello, C. M., Mah, N., Hasler, R., Rosenstiel, P., Waetzig, G. H., Hahn, A., Lu, T., Gurbuz, Y., Nikolaus, S., Albrecht, M. et al. (2005). Dissection of the inflammatory bowel disease transcriptome using genome-wide cDNA microarrays. *PLoS Med.* **2**, e199.

Ferwerda, G., Girardin, S. E., Kullberg, B. J., Le Bourhis, L., de Jong, D. J., Langenberg, D. M., van Crevel, R., Adema, G. J., Ottenhoff, T. H., Van der Meer,

J. W. et al. (2005). NOD2 and toll-like receptors are nonredundant recognition systems of Mycobacterium tuberculosis. *PLoS Pathog*. **1**, 279-285.

Hagemann, T., Wilson, J., Kulbe, H., Li, N. F., Leinster, D. A., Charles, K., Klemm, F., Pukrop, T., Binder, C. and Balkwill, F. R. (2005). Macrophages induce invasiveness of epithelial cancer cells via NF-kappa B and JNK. *J. Immunol*. **175**, 1197-1205.

Hamon, M., Bierne, H. and Cossart, P. (2006). Listeria monocytogenes: a multifaceted model. *Nat. Rev. Microbiol*. **4**, 423-434.

Hampe, J., Cuthbert, A., Croucher, P. J., Mirza, M. M., Mascheretti, S., Fisher, S., Frenzel, H., King, K., Hasselmeyer, A., MacPherson, A. J. et al. (2001). Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet* **357**, 1925-1928.

Hugot, J. P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J. P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C. A., Gassull, M. et al. (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411**, 599-603.

Kabesch, M., Peters, W., Carr, D., Leupold, W., Weiland, S. K. and von Mutius, E. (2003). Association between polymorphisms in caspase recruitment domain containing protein 15 and allergy in two German populations. *J. Allergy Clin. Immunol*. **111**, 813-817.

Kim, J. M., Kim, N. I., Oh, Y. K., Kim, Y. J., Youn, J. and Ahn, M. J. (2005). CpG oligodeoxynucleotides induce IL-8 expression in CD34+ cells via mitogen-activated protein kinase-dependent and NF-kappaB-independent pathways. *Int. Immunol*. **17**, 1525-1531.

King, K., Bagnall, R., Fisher, S. A., Sheikh, F., Cuthbert, A., Tan, S., Mundy, N. I., Rosenstiel, P., Schreiber, S., Mathew, C. G. et al. (2007). Identification, evolution, and association study of a novel promoter and first exon of the human NOD2 (CARD15) gene. *Genomics* **90**, 493-501.

Kirkegaard, T., Hansen, A., Bruun, E. and Brynskov, J. (2004). Expression and localisation of matrix metalloproteinases and their natural inhibitors in fistulae of patients with Crohn's disease. *Gut* **53**, 701-709.

Kobayashi, K. S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G. and Flavell, R. A. (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* **307**, 731-734.

Kufer, T. A., Kremmer, E., Banks, D. J. and Philpott, D. J. (2006). Role for erbin in bacterial activation of Nod2. *Infect. Immun*. **74**, 3115-3124.

Kurzawski, G., Suchy, J., Kladny, J., Grabowska, E., Mierzejewski, M., Jakubowska, A., Debniak, T., Cybulski, C., Kowalska, E., Szych, Z. et al. (2004). The NOD2 3020insC mutation and the risk of colorectal cancer. *Cancer Res*. **64**, 1604-1606.

Legrand-Poels, S., Kustermans, G., Bex, F., Kremmer, E., Kufer, T. A. and Piette, J. (2007). Modulation of Nod2-dependent NF-kappaB signaling by the actin cytoskeleton. *J. Cell Sci*. **120**, 1299-1310.

Leung, E., Hong, J., Fraser, A. and Krissansen, G. W. (2006). Splicing of NOD2 (CARD15) RNA transcripts. *Mol. Immunol*. **44**, 284-294.

Lim, M., Martinez, T., Jablons, D., Cameron, R., Guo, H., Toole, B., Li, J. D. and Basbaum, C. (1998). Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38. *FEBS Lett*. **441**, 88-92.

McDonald, C., Chen, F. F., Ollendorff, V., Ogura, Y., Marchetto, S., Lecine, P., Borg, J. P. and Nunez, G. (2005). A role for Erbin in the regulation of Nod2-dependent NF-kappaB signaling. *J. Biol. Chem*. **280**, 40301-40309.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R. M. and Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. *Cell* **84**, 923-932.

Morre, S. A., Ouburg, S., Klinkenberg-Knol, E. C., Mulder, C. J. and Pena, A. S. (2004). The true ligand of the NOD2 receptor is peptidoglycan instead of lipopolysaccharide: a schematic representation of ligand-receptor interactions and NF-kappa B activation. *Gastroenterology* **126**, 371-373.

Nurnberger, T., Brunner, F., Kemmerling, B. and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev*. **198**, 249-266.

Ogura, Y., Bonen, D. K., Inohara, N., Nicolae, D. L., Chen, F. F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R. H. et al. (2001a). A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* **411**, 603-606.

Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S. and Nunez, G. (2001b). Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J. Biol. Chem*. **276**, 4812-4818.

Oliveira, M. J., Costa, A. C., Costa, A. M., Henriques, L., Suriano, G., Atherton, J. C., Machado, J. C., Carneiro, F., Seruca, R., Mareel, M. et al. (2006). Helicobacter pylori induces gastric epithelial cell invasion in a c-Met and type IV secretion system-dependent manner. *J. Biol. Chem*. **281**, 34888-34896.

Opitz, B., Puschel, A., Schmeck, B., Hocke, A. C., Rosseau, S., Hammerschmidt, S., Schumann, R. R., Suttorp, N. and Hippenstiel, S. (2004). Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized Streptococcus pneumoniae. *J. Biol. Chem*. **279**, 36426-36432.

Pandey, S. and Agrawal, D. K. (2006). Immunobiology of Toll-like receptors: emerging trends. *Immunol. Cell Biol*. **84**, 333-341.

Pushkarsky, T., Zybarth, G., Dubrovsky, L., Yurchenko, V., Tang, H., Guo, H., Toole, B., Sherry, B. and Bukrinsky, M. (2001). CD147 facilitates HIV-1 infection by interacting with virus-associated cyclophilin A. *Proc. Natl. Acad. Sci. USA* **98**, 6360-6365.

Renno, T., Wilson, A., Dunkel, C., Coste, I., Maisnier-Patin, K., Benoit de Coignac, A., Aubry, J. P., Lees, R. K., Bonnefoy, J. Y., MacDonald, H. R. et al. (2002). A role for CD147 in thymic development. *J. Immunol*. **168**, 4946-4950.

Rosenstiel, P., Fantini, M., Brautigam, K., Kuhbacher, T., Waetzig, G. H., Seegert, D. and Schreiber, S. (2003). TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* **124**, 1001-1009.

Rosenstiel, P., Hellmig, S., Hampe, J., Ott, S., Till, A., Fischbach, W., Sahly, H., Lucius, R., Folsch, U. R., Philpott, D. et al. (2006a). Influence of polymorphisms in the NOD1/CARD4 and NOD2/CARD15 genes on the clinical outcome of Helicobacter pylori infection. *Cell. Microbiol*. **8**, 1188-1198.

Rosenstiel, P., Huse, K., Till, A., Hampe, J., Hellmig, S., Sina, C., Billmann, S., von Kampen, O., Waetzig, G. H., Platzer, M. et al. (2006b). A short isoform of NOD2/CARD15, NOD2-S, is an endogenous inhibitor of NOD2/receptor-interacting protein kinase 2-induced signaling pathways. *Proc. Natl. Acad. Sci. USA* **103**, 3280-3285.

Schmeck, B., Beermann, W., van Laak, V., Zahlten, J., Opitz, B., Witzenrath, M., Hocke, A. C., Chakraborty, T., Kracht, M., Rosseau, S. et al. (2005). Intracellular bacteria differentially regulated endothelial cytokine release by MAPK-dependent histone modification. *J. Immunol*. **175**, 2843-2850.

Schmidt, R., Redecke, V., Breitfeld, Y., Wantia, N., Miethke, T., Massberg, S., Fischel, S., Neumann, F. J., Schomig, A. and May, A. E. (2006). EMMPRIN (CD 147) is a central activator of extracellular matrix degradation by Chlamydia pneumoniae-infected monocytes. Implications for plaque rupture. *Thromb. Haemost*. **95**, 151-158.

Shen, Y., Naujokas, M., Park, M. and Ireton, K. (2000). InlB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. *Cell* **103**, 501-510.

Stumpf, M., Cao, W., Klinge, U., Klosterhalfen, B., Junge, K., Krones, C. J. and Schumpelick, V. (2005). Reduced expression of collagen type I and increased expression of matrix metalloproteinases 1 in patients with Crohn's disease. *J. Invest. Surg*. **18**, 33-38.

Till, A., Rosenstiel, P., Krippner-Heidenreich, A., Mascheretti-Croucher, S., Croucher, P. J., Schafer, H., Scheurich, P., Seegert, D. and Schreiber, S. (2005). The Met-196 → Arg variation of human tumor necrosis factor receptor 2 (TNFR2) affects TNF-alpha-induced apoptosis by impaired NF-kappaB signaling and target gene expression. *J. Biol. Chem*. **280**, 5994-6004.

Tschopp, J., Martinon, F. and Burns, K. (2003). NALPs: a novel protein family involved in inflammation. *Nat. Rev. Mol. Cell Biol*. **4**, 95-104.

Veiga, E. and Cossart, P. (2005). Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. *Nat. Cell Biol*. **7**, 894-900.

Waetzig, G. H., Seegert, D., Rosenstiel, P., Nikolaus, S. and Schreiber, S. (2002). p38 mitogen-activated protein kinase is activated and linked to TNF-alpha signaling in inflammatory bowel disease. *J. Immunol*. **168**, 5342-5351.

Wehkamp, J., Harder, J., Weichenthal, M., Schwab, M., Schaffeler, E., Schlee, M., Herrlinger, K. R., Stallmach, A., Noack, F., Fritz, P. et al. (2004). NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* **53**, 1658-1664.

Weichart, D., Gobom, J., Klopfleisch, S., Hasler, R., Gustavsson, N., Billmann, S., Lehrach, H., Seegert, D., Schreiber, S. and Rosenstiel, P. (2006). Analysis of NOD2-mediated proteome response to muramyl dipeptide in HEK293 cells. *J. Biol. Chem*. **281**, 2380-2389.

Yanagisawa, N., Geironson, L., Al-Soud, W. A. and Ljungh, S. (2005). Expression of matrix metalloprotease-2, -7 and -9 on human colon, liver and bile duct cell lines by enteric and gastric Helicobacter species. *FEMS Immunol. Med. Microbiol*. **44**, 197-204.

Yurchenko, V., Constant, S. and Bukrinsky, M. (2006). Dealing with the family: CD147 interactions with cyclophilins. *Immunology* **117**, 301-309.