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Nucleotide Oligomerization Domains 1 and 2: Regulation of Expression and Function in Preadipocytes

Thorsten Stroh,* Arvind Batra,* Rainer Glauben,* Inka Fedke,* Ulrike Erben,* Anjo Kroesen,† Markus M. Heimesaat,‡ Stefan Bereswill,§ Stephen Girardin,¶ Martin Zeitz,* and Britta Siegmund2*†

Translocation of bacteria into the mesenteric fat during intestinal inflammation and the expression of functional TLR1–9 in murine preadipocytes and adipocytes suggest an active role for these cells in innate immunity. The present study focuses on nucleotide oligomerization domains 1 and 2 representing intracellular pattern recognition receptors that sense motifs derived from bacterial peptidoglycans. On mRNA level nucleotide oligomerization domain 1 was found to be constitutively expressed in the preadipocyte cell line 3T3/L1 and in primary preadipocytes isolated from murine mesenteric fat, while nucleotide oligomerization domain 2 was only weakly expressed by these cells. Treatment with lactyl-tetra-diaminopimelic acid, muramyl dipeptide, LPS, IL-1β, and TNF-α did not affect cellular nucleotide oligomerization domain 1 mRNA amounts. Except muramyl dipeptide, all factors significantly increased nucleotide oligomerization domain 2 mRNA in mesenteric fat preadipocytes after 4 h. However, specific stimulation of nucleotide oligomerization domain 1 induced IL-6 synthesis in preadipocytes from wild-type or TLR2/4-deficient mice. Confirming nucleotide oligomerization domain 1 mRNA in mesenteric fat preadipocytes after 4 h. However, specific stimulation of nucleotide oligomerization domain 1 induced IL-6 synthesis in preadipocytes from wild-type or TLR2/4-deficient mice. Confirming nucleotide oligomerization domain 1 specificity, transfection of nucleotide oligomerization domain 1-specific small interfering RNA significantly blocked the effect of lactyl-tetra-diaminopimelic acid on IL-6 production. With specific inhibitors and a NF-κB reporter plasmid, nucleotide oligomerization domain 1-mediated activation of NF-κB was shown to be responsible for the induction of IL-6 in preadipocytes. In addition, expression of functional nucleotide oligomerization domain 1 could be confirmed in primary human preadipocytes. In summary, we here identified preadipocytes as a novel cell population expressing nucleotide oligomerization domains 1 and 2. Not regulated on transcriptional level, nucleotide oligomerization domain 1 in preadipocytes serves as a sensor for bacterial degradation products and triggers proinflammatory effector responses. Thus, our results further strengthen the allocation of the mesenteric fat and especially of preadipocytes to the innate immune system. The Journal of Immunology, 2008, 181: 3620–3627.

The fat body in Drosophila has been attributed to the immune defense system (1), while the adipose tissue in vertebrates has long been considered primarily as energy storage. Recent data indicate that the adipose tissue also not only participates in the regulation of the immune system but also might be part of it. More than 50 adipocyte-derived mediators so-called adipokines have been identified so far (2). In vitro and in vivo data provide evidence that adipokines like leptin, adiponectin, and visfatin contribute to immune regulation (3).

In addition, preadipocytes and adipocytes were found to exert properties characteristic for cells of the immune system. For instance, preadipocytes have the potential to differentiate into the macrophage lineage and have been shown to phagocytose (4). Our group demonstrated that adipocytes and preadipocytes express functional TLR in a leptin-dependent manner (5) suggesting that these cells might belong to the innate immune system.

A second group of intracellular pattern recognition receptors, which include nucleotide oligomerization domains 1 and 2, comprise nucleotide-binding sites and leucine-rich repeats (6). Nucleotide oligomerization domain 1 is primarily expressed by various epithelial cell lines (7). Lactyl-tetra-diaminopimelic acid (LT-DAP), a motif mainly found in peptidoglycans from Gram-negative bacteria, has been identified as nucleotide oligomerization domain 1-specific ligand (8). Functional studies revealed that proinflammatory responses are triggered by invasive enteropathogens like Shigella flexneri and enteroinvasive Escherichia coli (9, 10) but also by the noninvasive pathogen Helicobacter pylori (11) via nucleotide oligomerization domain 1 signaling. Expression of nucleotide oligomerization domain 2 is more restricted to cells of the myeloid lineage (12). The peptidoglycan muramyl dipeptide (MDP), derived from the cell wall of Gram-positive bacteria, represents a specific ligand for nucleotide oligomerization domain 2 (13). Recent studies summarize the function of nucleotide oligomerization domain 2 as an adjuvant receptor in cooperation with TLRs for activation of the adaptive immune system (14). For both receptors, an interaction with the R lip2/RICK/CARDIAK kinase through their caspase recruitment domain occurs after ligand binding resulting in an activation of the transcription factor NF-κB (12, 15).

Phenotypically, in animal models, chronic inflammation has been associated with a hypertrophy of the fat tissue surrounding...
the draining lymph nodes (16). Remarkably, in Crohn’s disease, the hypertrophy of the mesenteric fat attached to the inflamed segments of the intestine represents a characteristic finding. However, the significance of this hypertrophy for the disease course remains unknown at the moment (17). Mutations in nucleotide oligomerization domain 1 have been associated with asthma and inflammatory bowel disease (18, 19), while homozygous mutations in nucleotide oligomerization domain 2 have been associated with atopy at the moment (17). Mutations in nucleotide oligomerization domain 1 have been associated with asthma and inflammatory bowel disease (18, 19), while homozygous mutations in nucleotide oligomerization domain 2 have been associated with a 20- to 40-fold increase in the risk of developing Crohn’s disease (20–22).

In transmural inflammations of the large or small intestine in Crohn’s disease, bacterial translocation into the mesenteric fat tissue has been described (17) suggesting direct access of bacteria-derived molecules to preadipocytes and adipocytes. To further define a role of the local fat in intestinal inflammation as a part of the innate immune system, the present study investigated expression and function of nucleotide oligomerization domains 1 and 2 in primary preadipocytes from the mesenteric fat of wild-type (WT) and TLR 2/4-deficient mice (TLR2/4Δ/Δ).

Materials and Methods

Reagents

FCS was obtained from LINARIS Biologische Produkte. Further cell culture reagents were from PAA Laboratories and cell culture plates from Nunc. Isulin was from Aventis Pharma, hydrocortisone from Pharmacia, and MDP, γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), γ-D-glutamyl-lysine (iE-Lys), 17-DMAG, as well as ultra-pure LPS were from InvivoGen. Human TNF-α, murine M-CSF, murine IL-1β, and murine IFN-γ were used as recombinant proteins from PeproTech. LT-DAP was provided by the Department of Laboratory Medicine and Pathobiology of the University of Toronto (Toronto, Canada). Reagents for reverse transcription and for standard and quantitative PCR were obtained from Invitrogen and Promega. Primer for PCR were designed using the OligoPerfect designer software (Invitrogen) (Table I) and were manufactured by TIB MolBiol. The NF-κB-luciferase reporter plasmid and the plasmid pEGFP-N1 were from and from BD Pharmingen, respectively. All other chemicals were obtained from Sigma-Aldrich. Endotoxin contamination of all reagents used in cell culture settings was excluded using the Limulus amebocyte lysate assay (Hemochrom Diagnostica).

Mice

Animal protocols were approved by the regional animal study committee of Berlin, Germany. Six- to eight-week-old female C57BL/6J and C57BL/10 WT mice were bred under specific pathogen-free conditions at the “Forschungsinstitut für Experimentelle Medizin,” Charité Berlin, Germany. C57BL/10 TLR 2/4-deficient mice (TLR2/4Δ/Δ) were a kind gift from M. Freudenberg (Max-Planck-Institut für Immunobiologie, Freiburg, Germany) and bred and kept under the same conditions as the respective WT control animals. In experiments with cells from TLR2/4Δ/Δ mice, C57BL/10 mice were used as WT control. In all other experiments preadipocytes isolated from C57BL/6J were referred to as WT. The animals were housed at controlled temperature with light-dark cycles, fed standard mouse chow pellets, had access to tap water from bottles, and were acclimatized before being studied. Upon the end of an experimental period, mice were killed by cervical dislocation under CO2 anesthesia.

Isolation of mouse preadipocytes and cell culture

Mesenteric adipose tissue was excised, minced, and washed in HBSS. Tissue was digested within 25 min at 37°C in HBSS containing 1.5 mg/ml collagenase II, 3.5% BSA, and 550 μg/ml glucose. The cell suspension was subsequently washed through a 100 μm-nylon net (BD Pharmingen), sedimented at 200 g for 10 min, and washed twice in DMEM/HAMS F-12 containing 10% FCS and penicillin/streptomycin (100 U/ml each). After 24-h incubation in 48-well plates (5 × 10^5 cells/well), nonadherent cells were discarded and adherent cells were propagated to establish preadipocyte cell lines that were used up to the 10th passage. Cells of the murine preadipocyte cell line 3T3L1 (ATCC CL-173) were propagated in DMEM containing 10% FCS and penicillin/streptomycin (100 U/ml each).
The potential to give rise to fully differentiated adipocytes from all preadipocyte cell lines used in this study was confirmed by lipid droplet staining with Oil red O after incubation with insulin, dexamethasone, and 3-isobutyl-1-methylxanthine as described previously (23). All obtained primary cells were periodically tested for mycoplasma contamination using a commercial PCR test (Minerva).

Patients, isolation of human preadipocytes, and cell culture
Mesenteric adipose tissue was obtained from patients undergoing intestinal surgery performed at the Charité University Hospital in Berlin. All patients provided informed consent. Samples from 10 tumor patients (noninvolved areas) were investigated. This study was approved by the local ethics committee of the Charité Universitätsmedizin Berlin. Procedure of isolating preadipocytes was identical with the preparation of preadipocytes of mice. The potential to give rise to fully differentiated adipocytes from all human preadipocytes was performed as described previously (24).

Generation of bone marrow-derived macrophages
Bone marrow-derived macrophages were generated according to standard protocols (25). In brief, femurs from C57Bl/6J mice were dissected and flushed with DMEM. Once dispersed by passing through a 25-gauge needle, the cells were cultured overnight in plastic dishes (Ø 10 cm) in DMEM containing 10% FCS. Nonadherent cells transferred to new plastic dishes were cultured with 10 ng/ml M-CSF. For 7 days, medium was completely exchanged every other day.

Standard and quantitative RT-PCR
Cells were incubated with MDP (10 μg/ml), LT-DAP (200 nM), LPS (1 μg/ml), IL-1β (100 ng/ml), or TNF-α (100 ng/ml) at 37°C and were harvested after 1, 4, and 8 h as indicated. Total RNA (1.5 μg) isolated from detached cells using the RNAeasy kit (Qiagen) was reversely transcribed. Standard PCR was performed in a thermal cycler with three independent heating blocks (Biomera) in a total volume of 50 μl with 27, 35, and 20 cycles for nucleotide oligomerization domains 1 and 2 and GAPDH, respectively, using specific oligonucleotides (Table I) as primers. PCR products were electrophoretically separated in an agarose gel (2%) and were visualized after ethidium bromide staining. Quantitative PCR containing 5 pmol of sense and antisense primers and SYBR Green were set up in duplicates in total volumes of 20 μl. Specificity of the LightCycler (Roche) PCR over 40 cycles was confirmed by the molecular mass of the products and a melting curve analysis. Data analyzed according to a standard curve generated from a pool of all samples tested were normalized to coamplified GAPDH (26), and changes were calculated in relation to nontreated controls of the respective time point.

Absolute quantification of nucleotide oligomerization domains 1 and 2 expression using plasmid standards
Murine nucleotide oligomerization domains 1 and 2 cDNA were amplified by standard PCR from WT cells. Sequences cloned into the vector pCR2.1 (Invitrogen) were verified in comparison to GenBank accession no. NM_172729 and NM_145857, respectively. Plasmid DNA was linearized by standard PCR from WT cells. Sequences cloned into the vector pCR2.1 (Invitrogen) were verified in comparison to GenBank accession no. NM_172729 and NM_145857, respectively. Plasmid copy numbers were calculated from the concentration and molecular mass of the plasmid DNA. Single-use aliquots of these were stored at −80°C. The PCR mixture contained 4 μl plasmid standard or the cDNA generated of 2 × 103 cells, 5 pmol of sense and antisense primers, 2.5 pmol of FAM/TAMRA-labeled and platinum qPCR supermix (Invitrogen) in a total volume of 20 μl. Polymerase activation and target amplification were performed using the following protocol: 4 min at 95°C, 40 cycles at 95°C for 25 s and 60°C for 45 s. A series of 10-fold dilutions from 2 × 109 to 2 × 103 copies of the purified plasmid DNA identified the threshold of detection and generated a standard curve for quantification.

Transient transfection of preadipocytes
Detached cells (2 × 104) were resuspended in 100 μl electroporation buffer containing 90 mM phosphate buffer (pH 7.2), 10 mM MgCl2, and 50 mM glucose before 4 μg pEGFP-N1 were added. In a cuvette for electroporation with a gap of 2 mm (Biozym), cells were subjected to a single square pulse at 600 V for 600 μs. After the electroporation, cells were left to rest for 3 min before being transferred in prewarmed DMEM containing 10% FCS. Finally, 1.5 × 105 cells in 1 ml DMEM containing 10% FCS in 24-well plates were treated with various ligands as indicated for 24 h.

Cytokine production
Murine preadipocytes and macrophages were incubated with MDP, LT-DAP, and LPS as described above. Cell-free culture supernatants were collected and stored at −80°C. Subsequently, concentrations of IL-6, TNF-α, and IL-1β were determined by specific ELISA using the EIA kit (BD Pharmingen) within a range of 15–1000 pg/ml. Human preadipocytes were stimulated with the specific agonist for human nucleotide oligomerization domain 1 (iE-DAP) (50 μg/ml) (27) or with iE-Lys (50 μg/ml), which is not recognized by nucleotide oligomerization domain 1. Supernatants were collected after 24 and 48 h. Concentration of IL-6 was determined by specific ELISA using EIA kit (BD Pharmingen) within a range of 15–300 pg/ml.

FIGURE 1. Baseline expression of nucleotide oligomerization domains 1 (NOD1) and 2 (NOD2) in preadipocytes. Copy numbers of nucleotide oligomerization domains 1- and 2-specific mRNA per cell was determined by quantitative PCR using plasmid standards and specific fluorescence probes. Bars represent the mean of n = 4 ± SEM.
Bradford reagent (Bio-Rad). Samples were mixed for 5 s. Luciferase activity was measured for 0.5 s using a Mithras LB 940 luminescence reader (Berthold Technologies). NF-κB transfection. Alternatively, MDP (10 ng/ml) was added immediately after transfection. Cells were incubated for 24 h, washed twice with PBS, and lysed in 80 μl of reporter lysis buffer (Promega). Protein concentrations were determined using the Bradford reagent (Bio-Rad). Samples (20 μl) were transferred into a white 96-well plate, 60 μl of luciferase substrate were added, and the reactions mixed for 5 s. Luciferase activity was measured for 0.5 s using a Mithras LB 940 luminescence reader (Berthold Technologies). NF-κB activation state was estimated in relative light units corresponding to equal protein amounts.

Assessment of NF-κB activation using luciferase assay

Cells transiently transfected with the NF-κB-luciferase reporter plasmid received MDP (20 μg/ml), LT-DAP (200 nM), or LPS (1 μg/ml) 24 h after transfection. Alternatively, MDP (10 μg/ml) was present during the pulse and LPS (1 μg/ml) was added immediately after transfection. Cells were incubated for 24 h, washed twice with PBS, and lysed in 80 μl of reporter lysis buffer (Promega). Protein concentrations were determined using the Bradford reagent (Bio-Rad). Samples (20 μl) were transferred into a white 96-well plate, 60 μl of luciferase substrate were added, and the reactions mixed for 5 s. Luciferase activity was measured for 0.5 s using a Mithras LB 940 luminescence reader (Berthold Technologies). NF-κB activation state was estimated in relative light units corresponding to equal protein amounts.

Statistical analysis

Significance of differences between treatment and control groups was determined by the Kruskal Wallis nonparametric test using Prism 4 software for Windows (GraphPad Software).

Results

Murine preadipocytes from the mesenteric fat constitutively express nucleotide oligomerization domain 1 and only minor nucleotide oligomerization domain 2

First, we asked for baseline expression levels of nucleotide oligomerization domains 1 and 2 and compared preadipocytes from the mouse embryonic fibroblast-derived cell line 3T3L1 with primary preadipocytes derived from murine mesenteric fat. Nucleotide oligomerization domain 1 mRNA could be detected in all primary preadipocytes analyzed. Remarkably, nucleotide oligomerization domain 1 mRNA expression in WT and in TLR2/4−/− preadipocytes was expressed to higher levels as in macrophages. To determine the differences in expression, the copy number of nucleotide oligomerization domains 1 and 2 per cell was evaluated (Fig. 1). WT preadipocytes expressed ~4 copies of nucleotide oligomerization domain 1 mRNA per cell while TLR2/4−/− preadipocytes expressed ~13 copies/cell. In macrophages, only one nucleotide oligomerization domain 1 mRNA copy per cell could be measured. Baseline expression of nucleotide oligomerization domain 2 mRNA in the 3T3L1 cell line and in primary preadipocytes was very low. Statistically, only 5% of primary preadipocytes and 30% of macrophages expressed one molecule of nucleotide oligomerization domain 2 mRNA per cell.

These data confirm a constitutive expression of both nucleotide oligomerization domains 1 and 2 in primary cells, indicating a potential physiological role in vivo.

Expression of nucleotide oligomerization domain 2, but not 1, mRNA is regulated by specific ligands and proinflammatory cytokines

Next, effects of bacterial cell wall-derived compounds and of cytokines representing a proinflammatory milieu were questioned.
Preadipocytes from the mesenteric fat of WT mice and 3T3L1 cells were stimulated with either the specific ligands for nucleotide oligomerization domains 1 and 2, TLR4, or with the proinflammatory cytokines IL-1β or TNF-α, respectively. Nucleotide oligomerization domains 1 and 2 mRNA expression was evaluated after 1, 4, and 8 h of stimulation as indicated (Fig. 2). In the 3T3L1 preadipocyte cell line, neither the nucleotide oligomerization domain 1-specific stimulus LT-DAP, the nucleotide oligomerization domain 2-specific stimulus MDP, nor the TLR4-specific stimulus LPS resulted in an increase of nucleotide oligomerization domain 2 mRNA expression. However, stimulation with the proinflammatory cytokine TNF-α was followed by a significant up-regulation after 1 and 4 h, while IL-1β did not alter baseline nucleotide oligomerization domain 2 expression.

Remarkably, regulation of nucleotide oligomerization domain 2 mRNA expression was different in primary preadipocytes isolated from the mesenteric fat of WT mice. In this study, nucleotide oligomerization domain 1-specific stimulation with LT-DAP resulted in a significant increase after 4 h. Furthermore, LPS stimulation was followed by an ∼40-fold increase of nucleotide oligomerization domain 2 mRNA expression. The LPS mediated up-regulation of nucleotide oligomerization domain 2 mRNA was neither affected by the neutralization of TNF-α nor of IL-1β, but was sensitive to inhibition of the NF-κB-pathway (data not shown). Comparable to the 3T3L1 preadipocytes, MDP did not change nucleotide oligomerization domain 2 mRNA expression in these cells. However, in WT preadipocytes, both proinflammatory cytokines resulted in a profound up-regulation of nucleotide oligomerization domain 2 mRNA after 1 and 4 h, respectively.

In contrast to nucleotide oligomerization domain 2 mRNA, nucleotide oligomerization domain 1 mRNA expression in neither 3T3L1 nor WT preadipocytes was changed significantly after stimulation with either LT-DAP, MDP, LPS, IL-1β, or TNF-α, respectively. In contrast, an up-regulation of nucleotide oligomerization domain 1 mRNA expression could be achieved after stimulation with IFN-γ (data not shown).

**Induction of IL-6 by specific activation of nucleotide oligomerization domain 1 in primary preadipocytes**

To further characterize the functionality of nucleotide oligomerization domains 1 and 2 in preadipocytes, either the 3T3L1 preadipocyte cell line or preadipocytes isolated from the mesenteric fat of WT or TLR2/4−/− mice were stimulated with LT-DAP or MDP. For any functional analysis with defined derivates from the bacterial cell wall, it was crucial to exclude LPS contamination within the experimental system. Thus, preadipocyte cell lines deficient in TLR2/4 were established and included as control. TLR4 stimulation was included as positive control. IL-6, known to be produced in large amounts by preadipocytes, was selected for end-point determination. As indicated by Fig. 3A, nucleotide oligomerization domain 1-specific stimulation resulted in a significant increase of IL-6 in WT and TLR2/4−/− preadipocytes. Stimulation with LPS was followed by an up-regulation of IL-6 synthesis in 3T3L1 preadipocytes as well as in WT cells, while no increase was observed in TLR2/4−/− preadipocytes. Under comparable conditions, nucleotide oligomerization domain 2-specific stimulation with MDP did not result in an IL-6...
Nucleotide oligomerization domain 1-specific siRNA partially abrogates IL-6 production in preadipocytes

Concentrating on the nucleotide oligomerization domain 1 specificity of LT-DAP in preadipocytes, the RNAi technique was applied. Achieved knockdown of nucleotide oligomerization domain 1 by specific siRNA in comparison to a noncoding siRNA was ~70% after 24 h and ~50% after 48 h as analyzed by quantitative PCR (data not shown). Cells transfected with the nucleotide oligomerization domain 1-specific siRNA (800 nM) and subsequently stimulated with LT-DAP showed a significant decrease in IL-6 production in comparison to stimulated cells transfected with a noncoding control siRNA (Fig. 4). However, the response to LPS stimulation was not affected by transfection with either nucleotide oligomerization domain 1-specific siRNA or noncoding control siRNA, respectively, hence confirming the specificity of the knockdown (Fig. 4). These data verify that nucleotide oligomerization domain 1 in preadipocytes is specifically activated by LT-DAP.

IL-6 production is mediated by nucleotide oligomerization domain 1-induced NF-κB activation in preadipocytes

We next addressed the mechanism by which nucleotide oligomerization domain 1 activation results in an increase of IL-6 production. In the presence of the NF-κB inhibitor 17-DMAG, the IL-6 production following nucleotide oligomerization domain 1-specific stimulation was significantly decreased (Fig. 5A). To further evaluate whether nucleotide oligomerization domain 1-specific stimulation results in an activation of NF-κB in preadipocytes, a NF-κB reporter plasmid assay was performed. In this study, nucleotide oligomerization domain 1-specific stimulation induced a strong NF-κB activation in the 3T3L1 preadipocyte cell line as well as in the WT preadipocytes (Fig. 5B). Remarkably, NF-κB activation in LT-DAP-stimulated preadipocytes was comparable to LPS-stimulated cells. No NF-κB activation occurred in any of the cell lines investigated after stimulation with the nucleotide oligomerization domain 2 ligand MDP (Fig. 5B).

Expression and function of nucleotide oligomerization domains 1 and 2 in human preadipocytes

To allow a transfer of the murine data into the human system, preadipocytes isolated from the mesenteric fat of patients undergoing intestinal surgery were investigated. As evaluated by RT-PCR, nucleotide oligomerization domain 1 was expressed constitutively in human preadipocytes (Fig. 6A) while nucleotide oligomerization domain 2 could be detected in only 2 of 10 samples (data not shown). After nucleotide oligomerization domain 1-specific stimulation with iE-DAP the baseline IL-6 production was significantly up-regulated whereas no increase occurred in the presence of the negative control iE-Lys which is not recognized by nucleotide oligomerization domain 1, thus underlining the functionality of nucleotide oligomerization domain 1 in human preadipocytes. Stimulation with the nucleotide oligomerization domain 2 ligand MDP resulted in no change of IL-6 production (Fig. 6B).

Discussion

Preadipocytes have initially been described as precursors of adipocytes and thus as cells of the endocrine and metabolic system. Recent data indicate an additional function for this cell population since they are not only capable of phagocytosis but, furthermore, have been shown to express functional TLRs (5). With the present study, the expression and function of the nucleotide-binding site domain-1 pattern recognition receptor family localized in the cytoplasm, were characterized in preadipocytes.

In contrast to TLRs that are mostly associated with the plasma membrane or, in some cases, with lysosomal vesicles, both nucleotide oligomerization domains 1 and 2 are expressed predominantly in the cytoplasm (28). Concordant with this intracellular localization, both receptors do not recognize native microbial products but rather fragments that are derived from the degradation of bacterial cell wall peptidoglycans (13, 27, 29, 30). Both receptors have so far been reported to be expressed by APC such as macrophages and dendritic cells but not in B or T cells (12, 28, 31).

Nucleotide oligomerization domain 1 is expressed by epithelial cell lines including intestinal epithelia-derived cell lines and primary intestinal epithelial cells, while nucleotide oligomerization domain 2 mRNA is detectable in most epithelial cell lines, the protein is below detection limits (32, 33). In primary cells, nucleotide oligomerization domain 2 expression seems to be restricted
to Paneth cells, located at the base of the intestinal crypt (34). Thus, preadipocytes are a novel cell type expressing nucleotide oligomerization domains 1 and 2, nucleotide oligomerization domain 1 at significantly higher levels than nucleotide oligomerization domain 2. Interestingly, in TLR2/4−/− preadipocytes, nucleotide oligomerization domain 2 expression is significantly enhanced when compared with WT preadipocytes.

The regulation of nucleotide oligomerization domains 1 and 2 expression by proinflammatory cytokines is well described. Nucleotide oligomerization domain 1 is constitutively expressed and can be up-regulated by IFN-γ but not by TNF-α (35). Accordingly, also in preadipocytes constitutive high expression of nucleotide oligomerization domain 1 is not affected by nucleotide oligomerization domain 1-, nucleotide oligomerization domain 2-, or TLR4-specific stimuli or by the proinflammatory cytokines IL-1β and TNF-α, respectively. Differently, nucleotide oligomerization domain 2 baseline protein expression in epithelial cells is low; TNF-α induces an up-regulation that is further enhanced in the presence of IFN-γ (36). Nucleotide oligomerization domain 2-specific stimulation has been reported to result in an activation of NF-κB and subsequent up-regulation of nucleotide oligomerization domain 2 itself (12, 36). Our data indicate that, comparable to epithelial cells, nucleotide oligomerization domain 2 expression in preadipocytes is up-regulated by the proinflammatory stimuli LPS, IL-1β, and TNF-α. In addition, specific stimulation of nucleotide oligomerization domain 1 resulted in an increased nucleotide oligomerization domain 2 expression, thus confirming the previously described nucleotide oligomerization domain 2-dependent regulation by activation of the NF-κB pathway expression for the first time in preadipocytes (12).

Nucleotide oligomerization domains 1 and 2 recognize specific peptidoglycans. For instance, LT-DAP has been established as a specific ligand for murine nucleotide oligomerization domain 1 while MDP has been identified as specific ligand for nucleotide oligomerization domain 2, respectively (27, 29). MDP is part of the cell wall of Gram-positive and -negative bacteria. Consequently, nucleotide oligomerization domain 2 functions as sensor of most, if not all, bacteria. Nucleotide oligomerization domain 1 mainly senses degradation products from Gram-negative bacteria (27, 37, 38). To activate nucleotide oligomerization domains 1 or 2, the specific ligands need to reach the respective nucleotide oligomerization domain protein in the cytosol. For APC, it has been suggested that phagocytosis might participate in this process (39, 40), which could also represent the critical mechanism for the uptake of bacterial Ags by preadipocytes (4). In line with this hypothesis, we observed that electroporation facilitated the access of the specific ligands to nucleotide oligomerization domain 1 (data not shown).

Stimulation of preadipocytes with the nucleotide oligomerization domain 1-specific ligand LT-DAP resulted in increased IL-6 production. In line with the findings that expression of nucleotide oligomerization domain 1 is only one copy per cell, it was not surprising that LT-DAP did not induce IL-6 in bone marrow-derived macrophages. These differences to data found in the literature might be attributed to the isolation protocol for peritoneal derived macrophages. These differences to data found in the literature might be contributed by nucleotide oligomerization domain 2 baseline protein expression in epithelial cells is low; TNF-α induces an up-regulation that is further enhanced in the presence of IFN-γ (36). Nucleotide oligomerization domain 2-specific stimulation has been reported to result in an activation of NF-κB and subsequent up-regulation of nucleotide oligomerization domain 2 itself (12, 36). Our data indicate that, comparable to epithelial cells, nucleotide oligomerization domain 2 expression in preadipocytes is up-regulated by the proinflammatory stimuli LPS, IL-1β, and TNF-α. In addition, specific stimulation of nucleotide oligomerization domain 1 resulted in an increased nucleotide oligomerization domain 2 expression, thus confirming the previously described nucleotide oligomerization domain 2-dependent regulation by activation of the NF-κB pathway expression for the first time in preadipocytes (12).

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In contrast to nucleotide oligomerization domain 1, the expression of nucleotide oligomerization domain 2 in unstimulated primary preadipocytes was very low but could be up-regulated by proinflammatory stimuli. Although an immediate role of nucleotide oligomerization domain 2 in preadipocytes remained unclear, it has to be different from nucleotide oligomerization domain 1. As described for epithelial cell lines, nucleotide oligomerization domain 2-specific stimulation with MDP did neither induce IL-6 production nor result in NF-κB activation (7). Activation of NF-κB via nucleotide oligomerization domain 2 has so far only been described for nucleotide oligomerization domain 2 over-expressing transfected epithelial cells (8, 13). The copy numbers of each receptor per cell might provide the explanation to this finding. The copy number for nucleotide oligomerization domain 1 is ~2.5-fold higher than the copy number for nucleotide oligomerization domain 2, thus one can conclude that a higher copy number is required for a response that is underlined by the data acquired in nucleotide oligomerization domain 2 over-expressing transfected epithelial cells (8, 13).

The synergistic effect of LPS and MDP shown earlier for bone-marrow-derived macrophages (42, 43) lacked in preadipocytes.

What might be the in vivo significance of nucleotide oligomerization domain 1 in preadipocytes as sensor for bacterial wall fragments? Preadipocytes from the mesenteric fat tissue are of particular interest for two reasons: although cause and meaning remain unclear, the hypertrophy of the mesenteric fat tissue surrounding the inflamed segments of the intestine is a characteristic finding in Crohn’s disease (17). During intestinal inflammation, bacterial translocation in the mesenteric fat tissue is well-described, thus allowing for a direct activation of preadipocytes through bacteria or bacterial wall fragments. Keeping also in mind that homozygous mutations in nucleotide oligomerization domain 2 are associated with a 20- to 40-fold increased risk in developing Crohn’s disease (18, 19), the understanding of the relevance of these receptors within the mesenteric fat tissue seems to be crucial.

In conclusion, with the present work, we demonstrate for the first time nucleotide oligomerization domains 1 and 2 expression in preadipocytes. We provide evidence that nucleotide oligomerization domain 1 here serves as a sensor for bacteria and is capable to induce an effector responses via NF-κB activation. These results further strengthen the role of the mesenteric fat tissue and of preadipocytes within the innate immune system.

Disclosures
The authors have no financial conflict of interest.

References


