NOD2 receptors in adenopituitary folliculostellate cells: expression and function

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Abstract

Folliculostellate cells (FS cells) are non-endocrine cells from the pituitary gland that respond to bacterial endotoxins by producing cytokines. In immune cells, an important component of bacterial recognition are the toll-like receptors (TLRs). Previously, we showed that FS cells express TLR4. The TLR4 ligand lipopolysaccharide (LPS) stimulates interleukin-6 (IL6) production through nuclear factor κ B (NFKB) induction. Binding of IL6 to gp130 receptor activates signal transducer and activator of transcription 3 (STAT3), an important mediator of inflammatory response. Another family involved in innate immune response following bacterial infection is the nucleotide-binding oligomerisation domain (NOD) intracellular receptor family. Herein, we describe for the first time the expression and function of NOD receptors in human pituitary and FS TtT/GF cell line. The NOD2 agonist muramyl dipeptide (MDP) increased *Nfkb1*-transcriptional activity, -protein expression and IL6 secretion in TtT/GF

cells. Furthermore, these effects were potentiated by the combination of MDP and LPS. Silencing NOD2 abolished the action of LPS on NFKB transcriptional activity and IL6 production, indicating that, in TtT/GF cells, TLR4 transduces its signal through NOD2 receptor. We show here that in TtT/GF cells, *Nod2* overexpression or stimulation by MDP increased STAT3 transcriptional activity. Furthermore, silencing STAT3 inhibited basal, LPS and MDP stimulated NFKB protein expression and overexpression of protein inhibitor of activated STAT3 (*Pias3*) markedly decreased basal NFKB activity. These data suggest that in TtT/GF cells, STAT3 acting upstream to NFKB mediates NOD2 receptor signalling pathway. In conclusion, the present study demonstrates that NOD molecules play a modulatory role in the pituitary by regulating the function and activation of FS cells in response to bacterial components.

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Introduction

The neuroendocrine and the immune systems are interconnected anatomically and functionally. Both express and respond to common regulatory molecules such as cytokines, hormones, neuropeptides and neurotransmitters (Besedovsky & Del 1992, 2002). Recent publications support the direct participation of folliculostellate (FS) cells in the immune–endocrine network. These cells are star-shaped and follicle-forming cells from the anterior pituitary gland (Renihart & Farquhar 1953), which share properties with antigen presenting cells such as dendritic cells and macrophages by expressing S100 protein and major histocompatibility complex class II (Nakajima *et al.* 1980, Allaerts *et al.* 1996). Immune cells mediate the response to multiple bacterial cell wall components through a series of pattern recognition molecules expressed on the cell surface such as toll-like receptors (TLRs; Takeda *et al.* 2003). In response to cytokines released by activated immune cells,

FS cells produce several growth factors and cytokines, which modulate hormonal production by pituitary endocrine cells (Vankelecom *et al.* 1989, Koike *et al.* 1997, Bilezikjian *et al.* 2003, Herkenham 2005).

In addition, we have recently reported that the established FS cell line TtT/GF can be directly activated by bacterial components due to the expression of TLR4 on the cell surface (Lohrer *et al.* 2000, Tichomirowa *et al.* 2005). In these cells, the TLR4 ligand lipopolysaccharide (LPS, Gram-negative bacteria-derived endotoxins) activates the nuclear factor κ B (*Nfkb*), which induces the production of several cytokines such as interleukin-6 (IL6), a member of the gp130 family, which stimulates the production and secretion of ACTH, GH and prolactin (Arzt *et al.* 1993, 1999, Medzhitov & Janeway 1997, Aderem & Ulevitch 2000, Lohrer *et al.* 2000, Arzt 2001, Gloddek *et al.* 2001). The signal transducer and activator of transcription 3 (STAT3) was initially identified as an IL6 dependent transcription factor important for mediating the inflammatory response, tumorigenesis and

anti-apoptotic effects on cells. In addition to its ability to form complexes to itself and other STATs there are several reports showing that STAT3 and NF κ B interact with each other (Battle & Frank 2002). NF κ B p65 homodimers bind to phosphotyrosine STAT3 monomers. This interaction appears to increase transcriptional activity of genes driven by specific κ B elements (Yoshida *et al.* 2004). Moreover, inhibition of NF κ B decreased IL6 production and STAT3 phosphorylation in squamous cell carcinoma (Squarize *et al.* 2006).

More recently, the nucleotide-binding oligomerisation domain (NOD)-like receptors family involved in the recognition of bacterial products within the cytosol have been detected in both immune and non-immune cell types (Girardin *et al.* 2003a, Ting & Davis 2005). The protein NOD1 (also called CARD4) recognises the dipeptide γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP; Chamaillard *et al.* 2003, Girardin *et al.* 2003b), which is produced by most Gram-negative and specific Gram-positive bacteria (Hasegawa *et al.* 2006). In contrast, NOD2 (also called CARD15) is activated by muramyl dipeptide (MDP), a component of all types of peptidoglycans (Girardin *et al.* 2003c, Inohara *et al.* 2003).

In the immune system, the interaction of NOD1 and NOD2 with its specific ligand initiates a cascade of signalling inducing NF κ B activation and consequently cytokine production such as IL6, IL1 β (IL1 β), IL8, and tumour necrosis factor- α (Ogura *et al.* 2001, Chin *et al.* 2002, Kobayashi *et al.* 2002, Fritz *et al.* 2005). In addition, NOD2- and TLR4-agonists act in a synergic manner to induce cytokine secretion and dendritic cell maturation (Takada *et al.* 2002, Traub *et al.* 2004, Fritz *et al.* 2005).

In this study, we demonstrate for the first time the expression of NOD1 and NOD2 intracellular receptors in the pituitary gland and in TtT/GF cells. The present work is focused on the function of NOD2.

Herein, we show a synergic action of NOD2 molecules with TLR4 receptor on the induction of IL6 by NF κ B in TtT/GF cells. Moreover, our data suggest that TLR4 might transduce its signals through NOD2 receptors. Notably, our results suggest that not only NF κ B but also STAT3 participates in the NOD2 signalling pathway. These findings demonstrate that TLR and NOD receptors signalling pathways may significantly modulate the function of TtT/GF cells during bacterial infection and inflammation.

Material and Methods

Reagents

N-acetylmuramyl-D-alanyl-D-isoglutamine (MDP-LD; L-isoform), *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP-DD; D-isoform, inactive) and iE-DAP were purchased from Invitrogen. LPS (*Escherichia coli* serotype 055:B5) was obtained from Sigma-Aldrich.

Human pituitary

Samples from five human pituitary glands were obtained from autopsy cases without any evidence of endocrinological disease, with postmortem delay between 8 and 12 h. The tissues were snap-frozen and stored at -80°C . This study was performed after approval of the Ethics Committee of the Medical Faculty of the University of Munich (Ethic grant Nr. 141-07).

Rat pituitary

Female Sprague-Dawley rats (200–250 g) were obtained from the animal facilities of the Max Planck Institute of Psychiatry (Munich, Germany). The animals were kept in an environment of constant temperature, humidity and day-night cycle; food and water were made available *ad libitum*. Glands were obtained within minutes of the animals' death by decapitation and were treated as indicated below. All experimental procedures were approved by the Ethics Committee of the Max Planck Institute.

Cell culture

The pituitary mouse FS cell line TtT/GF and human embryonic kidney cells (HEK-293) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2.2 g/l NaHCO₃, 10 mM Hepes, 2 mM glutamine, 10⁵ U/l penicillin-streptomycin. The cells were maintained at 37 $^{\circ}\text{C}$ in humidified CO₂ atmosphere.

Immunohistochemistry

For immunohistochemistry, 8- μm sections of rat pituitary tissue were thaw-mounted onto Super Frost Plus slides (Menzel-Glaser, Hamburg, Germany). TtT/GF cell line was grown for 48 h in multiwell plates at 37 $^{\circ}\text{C}$ and 5% CO₂. Afterwards, pituitary sections and TtT/GF cultures were fixed in 4% paraformaldehyde in TBS, and stored in 96% ethanol, at 4 $^{\circ}\text{C}$ until use. Slides were first washed thrice for 5 min in PBS and then incubated for 30 min in serum (1:10) of the same animal in which specific biotinylated secondary antibody was raised. Subsequently, the corresponding slides were incubated overnight at 4 $^{\circ}\text{C}$ with goat polyclonal NOD2 antibody (1:200) or with rabbit polyclonal NF κ B (p65)/Rel A (1:300) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as indicated in the figure legends. After washing thrice in TBS, the corresponding biotinylated secondary antibody (1:300) was added at room temperature for 30 min. The slides were again rinsed thrice in TBS and incubated for 30 min with avidin-biotin-peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA) at room temperature. Colour development was performed using 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) with 0.01% hydrogen peroxidase applied for 2 min. Finally, after washing in deionised water, the sections were

counterstained in toluidine blue, fixed in Roti–Histol (Roth, Karlsruhe, Germany) and coverslipped using Roti–Histokit (Roth). Negative controls were performed by omitting the primary antibody where no staining was detected.

Double immunofluorescence

For the detection of NOD2, we used a goat polyclonal NOD2 antibody (Santa Cruz Biotechnology) (1:200). For S100, a marker of FS cells, we used a rabbit anti-S100 serum (1:3000) (Swant, Bellinzona, Switzerland). The slides were incubated with 1% Tris–base saline buffer, pH 7.6/BSA for 30 min at room temperature followed by incubation in a cocktail containing goat anti-NOD2 and rabbit anti-S100 in TBS, pH 7.6 4 °C overnight. After incubation, overnight sections were washed and incubated with Alexa 488-conjugated donkey anti-goat IgG (1:1000) and Alexa 594-conjugated goat anti-rabbit IgG (1:1000) (Molecular probes, Eugene, OR, USA) for 45 min at room temperature. Sections were counterstained with 1 g/ml 4,6-diaminidino-2-phenylidole (Vector Laboratories). Finally, sections were mounted with Vectashield (Vector Laboratories) and investigated using a confocal laser scanning microscope (LSM 510, Carl Zeiss, Jena, Germany). To ensure the specificity of the double-immunostaining procedure, control experiments were performed in which the primary antibodies were omitted. No staining was observed under these conditions.

RT-PCR

Total cellular RNA was prepared from normal pituitaries and TtT/GF cells using TRIzol reagent (Life Technologies Inc.) following the manufacturer's protocol. RNA extraction was followed by a DNase digestion step to remove any contaminating genomic DNA. One microgram RNA from normal pituitaries and cell cultures was reverse transcribed to cDNA using Superscript II reverse transcriptase (Life Technologies, Inc). With the cDNA template obtained, a PCR was performed with the following specific primers: mouse *Nod1* upper primer: 5' CTCTCACTGTA-CAGGTCTGTTTCTTC 3'; mouse *Nod1* lower primer: 5' CAACCTCTGAGGACATGAAGATACTTT 3'; PCR product length of 350 bp; mouse *Nod2* upper primer: 5' GAAAGACAGCTCCATTTTATTTATGAC 3'; mouse *Nod2* lower primer: 5' GCTTTCACCTCTGTGAT-TATTTATAGC 3'; PCR product length of 300 bp; mouse *Tlr4* upper primer: 5' GGG TCAAGGAACAGAAG-CAG 3'; mouse *Tlr4* lower primer: GCTCATTTTC TCACCCAGTCC; PCR product length of 264 bp; human *Nod1* upper primer: 5' GTAAAGGTGCT-AAGCGAAGA 3'; human *NOD1* lower primer: 5' TCTGATTCTGGATAAGCCAT 3'; PCR product length of 470 bp; human *NOD2* upper primer: 5' AACCACTCTCTGTGCGGACT 3'; human *NOD2* lower primer: 5' CCTGTTTCAAGAGCCCTTG 3'; PCR product length of 310 bp. Rat *Nod2* upper

primer: 5' AGTGAAGGCAAATGGACTGG; rat *Nod2* lower primer: TCTGTCCGCAGCTCTAAGGT; PCR product length of 219 bp. D-glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) upper primer: 5' ATGGTGAA-GGTCCGGTGTGAACG 3'; *Gapdh* lower primer: 5' GTTGT CATGGATGACCTTGGC 3'; PCR product length of 495 bp.

RT reactions were carried out in a 20 µl final volume by adding 1 µg total RNA, 1 µl Superscript II, 10 µM dNTP mixture, and 250 ng random hexamer primers. The reaction mixtures were incubated at 45 °C for 1 h. Finally, reactions were terminated by heating at 94 °C for 5 min and cooling on ice. PCR analysis was applied to assess the expression of *NOD1* and *NOD2* in mouse and human pituitary glands, and TtT/GF cell cultures with specific primers under 38 amplification cycle non-saturating conditions using a Thermocycler–Techgene (Labtech International, Ringmer, East Sussex, UK). Amplified samples were visualised on 1.5% agarose gels stained with ethidium bromide and photographed under u.v. light.

Transfections assays

Briefly, TtT/GF cells were seeded on six-well plates and were transfected at 80% density using Lipofectamine 2000 reagent (Invitrogen Corp.) according to the manufacturer's instructions. Following 24 h transfection, the cells were stimulated with MDP or LPS as indicated in the legends to the figures. After stimulation, cells were lysed in cell lysis buffer (Promega Corp.) and luciferase activity was measured with the addition of luciferin detection reagent (Promega Corp). Transfection efficiency was determined using the RSV-β-gal construct as previously described (Páez-Pereda *et al.* 2001). The results are ratios of luciferase to β-gal activity. Results are expressed as percentage respect to control plasmids or basals. Since NOD expression is restricted to the cytoplasm, we delivered MDP and iE-DAP directly into the cytoplasm using Lipofectamine 2000 reagent (Invitrogen Corp). Transient transfections were performed using the following reporter plasmids: NF-κB luciferase (κB-LUC) plasmid provided by Dr Bell (Mayo Clinic, Rochester, MN, USA); pIL6 luciferase (651) containing nucleotides –651 to +1 of the human IL6 promoter upstream of the luciferase gene (Eickelberg *et al.* 1999); and pSTAT3 luciferase obtained from Addgene, Cambridge, MA, USA. *Nod2* expression plasmid (pcDNA3 HA-*Nod2*) and control plasmid (pcDNA3 HA) were from Dr Gabriel Nunez (University of Michigan Medical School, Ann Arbor, MI, USA). IκB super-repressor construct containing the mutated site at Ser 32 and 36 was kindly provided by Dr Altschmidt, Institut für Neurobiochemie, University of Witten/Herdecke, Witten, Germany. *Pias3* expression plasmid clone into PCMVJ-FLAG vector was provided from Dr Sohn, University of California, LA, USA.

RNA interference

SiRNA for targeting endogenous NOD2 and STAT3 were from Santa Cruz Biotechnology, Inc. A non-targeting siRNA sequence (Santa Cruz Biotechnology, Inc.) was used as a control (siRNA control) for all transfection experiments. TtT/GF cells were cultured in six-well plates as described in the section 'Transfection assays', and transfected using 13 nM siRNA. After 18 h transfection, the medium was removed and the cells were cultured in DMEM supplemented with 2% FCS for a further 24 h. Then the cells were stimulated as

indicated in the legends to the figures. At the end of the treatment, the lysates were collected and luciferase activity was measured. Culture supernatants were collected and assayed for cytokine production as indicated in the legends to the figures.

Western blot analysis

Proteins were extracted from TtT/GF cells after stimulation with MDP, LPS each, alone or in combination. The total protein content was determined with Bradford method for

Figure 1 NOD1 and NOD2 expression in the pituitary gland and TtT/GF cells. (a) Detection of NOD1 and NOD2 in the human normal pituitary gland (NP) by transcriptase chain reaction (RT-PCR). (b) Detection of NOD2 in rat pituitary by RT-PCR and immunohistochemistry. The images are depicted at the magnification of $\times 40$ and $\times 100$. (c) Dual immunofluorescence by co-localisation of S100 protein and NOD2 in rat pituitary gland. Scale bar represents 10 μ m. (d) Detection of NOD1 and NOD2 by immunohistochemistry and RT-PCR analysis in mouse TtT/GF cell lines. The images are depicted at the magnification of $\times 40$. Inserts represent a background labelling when the primary antibody was omitted.

Figure 2 NFKB activation and nuclear localisation in TtT/GF cells. (a) 2.5×10^5 TtT/GF cells were transfected with κ B-LUC plasmid in the presence of MDP (0.01 ng/ml to 100 µg/ml). Six hours post-transfection, NFKB luciferase activity was measured. (b) 2.5×10^5 TtT/GF cells were transfected with κ B-LUC plasmid in the presence of active MDP, MDP-LD (10 µg/ml) or inactive MDP, MDP-DD (10 µg/ml). Six hours post-transfection, NFKB luciferase activity was measured. (c) HEK-293 cells (2.5×10^5) were co-transfected with κ B-LUC plasmid in the presence of iE-DAP or MDP at the concentration of 10 µg/ml. After 24 h, the cells were lysed and NFKB activity was analysed. (d) TtT/GF cells were transfected with MDP (10 µg/ml) for 6 h. After, cell cultures were fixed and stained with NFKB (p65/Rel A) antibody. Immunohistochemical analyses indicate the translocation of NFKB (black arrows) to the nucleus of cells after stimulation. Inserts represent a background labelling when the primary antibody was omitted. (e) TtT/GF cells were transiently transfected with control plasmids or *Nod2* expression vector along with κ B-LUC plasmid with or without MDP at the concentration of 5 µg/ml. Twenty-four hours post-transfection, luciferase activity was measured. Values indicate the mean $\pm$ S.E.M. of triplicates from one representative experiment ($n=3$). * $P<0.05$ and *** $P<0.001$ versus control plasmids or basal.

further normalisation. Samples containing equal amounts of protein (10 µg) were separated by SDS-PAGE (10% gels), followed by electrotransfer to nitrocellulose membrane. Membranes were blocked with non-fat milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 2 h at room temperature, and probed with anti-NFKB p65/Rel A or anti-STAT3 (Santa Cruz Biotechnology, Inc.) antibodies diluted in a ratio of 1:500 in 2.5% non-fat milk in

TBST for 2 h at room temperature or overnight at 4 °C. After washing, the membranes were further incubated with peroxidase-conjugated anti-rabbit IgG (1:1000) used as secondary antibody for 1 h 30 min. After washing, the bands were revealed by enhanced chemiluminescence (Amersham Biosciences).

Determination of cytokine production in culture supernatants

Following 6 h stimulation with LPS alone or in combination with MDP, cell culture supernatants were removed and stored at -20 °C. Mouse IL6 was measured using specific ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

All experiments were performed at least three times to confirm the reproducibility of the results. All results show a representative of three experiments. Differences were assessed by Student's *t*-test or by one-way ANOVA in combination with the Bonferroni's test (Figs 3a, 4b and 5d). Differences were considered statistically significant when *P* values were <0.05 . Statistical results are expressed as mean $\pm$ S.E.M.

Results

NOD2 expression in the pituitary gland and TtT/GF cells

NOD1 and NOD2 are expressed in different organs (Rodríguez-Martínez *et al.* 2005, Davey *et al.* 2006, Sterka & Marriott 2006, Uehara & Takada 2007, Uehara *et al.* 2007, Stroh *et al.* 2008), but there is no previous evidence of NOD expression in the pituitary gland. We detected for the first time NOD1 and NOD2 transcripts in human pituitary gland (Fig. 1a). NOD2 transcripts were also detected in rat pituitary (Fig. 1b). Moreover, NOD2 immunoreactivity was detected in cells localised in clusters, exhibiting irregular shape and extruded long cytoplasmic processes typical of FS cells (Fig. 1b). Dual colour immunofluorescence in rat pituitaries using antibodies against NOD2 and the FS cell marker S100 protein confirmed NOD2 expression in FS cells (Fig. 1c). Interestingly, NOD2 was not only expressed in FS cells but also in some S100-negative cells (Fig. 1c). Consistent with these findings, we detected in mouse TtT/GF cell lines the expression of NOD1 and NOD2 receptors (Fig. 1d). These results demonstrate that NOD molecules are expressed in FS cells.

NOD2 induced NFKB activation in TtT/GF cells

To determine the functional significance of NOD2 receptors in the FS cells, we first analysed the effects of NOD2 receptor ligand, MDP, on the activation of NFKB. Different MDP

Figure 3 Synergism between MDP and LPS on NFκB activation in TtT/GF. 2.5×10^5 TtT/GF cells were co-transfected with κB-LUC plasmid in the presence of: (a) MDP (10 μg/ml) and/or LPS (10 μg/ml). Twenty-four hours post-transfection cells were collected and *NfκB* transcriptional activity was determined by luciferase activity. (b) IkB super-repressor (IkBsr) plasmid, MDP and/or LPS for 6 h. (c) TtT/GF cells were co-transfected with κB-LUC plasmid with NOD2 siRNA (black bars) or with control siRNA (white bars). Forty-eight hours post-transfection, cells cultures were treated with LPS (10 μg) for 6 h. (d) MDP and LPS upregulate *NOD2* and *Tlr4* mRNA levels. TtT/GF cells were stimulated with MDP (10 μg/ml) and LPS (10 μg/ml) for 24 h. RNAs extracted from these cells were subjected to RT-PCR analysis. Values indicate the mean $\pm$ S.E.M. of triplicates from one representative experiment ($n=3$). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ versus basal.

concentrations were tested in TtT/GF cells. We found that from 10 μg/ml on, MDP-induced NFκB transcriptional activity by around 2.5-fold (Fig. 2a). The inactive MDP isomer, MDP-DD, which is not recognised by NOD2 was used as a negative control (Girardin *et al.* 2003c, Inohara *et al.* 2003). Using MDP-DD did not enhance NFκB transcriptional activity (Fig. 2b). HEK-293 cells that express NOD1, but do not express detectable levels of NOD2 are accepted as a model to evaluate the specificity of NOD2 ligand (Ogura *et al.* 2001). We observed that NFκB in HEK-293 cells did not respond to MDP treatment but was activated by the NOD1 ligand iE-DAP (Fig. 2c) demonstrating that the MDP treatment is selective for NOD2 in our experiments. Furthermore, we observed by immunohistochemistry that MDP induced NF-κB nuclear translocation indicating that NFκB may modulate gene transcription in the nucleus (Fig. 2d). In addition, NOD2 overexpression in TtT/GF cells increased basal and MDP induced NFκB transcriptional activity (Fig. 2e).

These results demonstrate that TtT/GF cells express functional NOD2 receptors.

MDP potentiated the effects of LPS on NFκB transcriptional activity through NOD2

Several studies show that TLR4 induces NFκB transcriptional activity after binding to LPS and that NOD and TLR4 can synergistically activate NFκB in a variety of cells (Fritz *et al.* 2005, Uehara & Takada 2007, Zhao *et al.* 2007). In addition, silencing TLR4 significantly abolished the LPS-induced NFκB activity as expected (data not shown). To elucidate whether MDP also potentiates the LPS-induced NFκB activation in FS cells, TtT/GF cells were stimulated with MDP in combination with LPS for 6 and 24 h. LPS increased NFκB transcriptional activity, and its action was potentiated by co-treatment with MDP (Fig. 3a). These results demonstrate a synergistic action between NOD2 agonist, MDP and TLR4 agonist, LPS on the activation of NFκB in TtT/GF cell cultures.

We evaluated the transcriptional activation of *NfκB* by MDP or/and LPS in the presence of a super-repressor form of IkB, resistant to phosphorylation and proteolytic degradation. Consistent with the results showed in Fig. 3a,

Figure 4 Induction of IL6 secretion by MDP and/or LPS. (a) Cell cultures were exposed to MDP at different concentrations (0.01 ng/ml to 100 µg/ml) for 6 h. Six hours post-transfection, IL6 luciferase activity was measured. (b) Cell cultures were exposed to MDP and/or LPS for 6 h. IL6 levels in culture supernatants were measured by ELISA. (c) Cell cultures were co-transfected with IL6-LUC plasmid and siRNA NOD2 (black bars) or with control siRNA (white bars). Forty-eight hours post-transfection, cells were treated with LPS (10 µg) for 6 h. IL6 activity was measured. *** $P < 0.001$; Si control plus LPS versus basal, Si NOD2 versus Si control, Si NOD2 plus LPS versus Si control plus LPS, ** $P < 0.01$; Si NOD2 plus LPS versus NOD2. (d) TtT/GF cells cultures were co-transfected with IL6 reporter plasmid along with control plasmid or *Nod2* expression plasmid with and without LPS (10 µg/ml). Twenty-four hours after transfection, IL6 activity was measured. *** $P < 0.001$; LPS versus basal, ** $P < 0.01$; *Nod2* versus control plasmid, *Nod2* plus LPS versus control plasmid plus LPS. (e) Cells were transfected with IL6 reporter and κ B super-repressor in the presence of control plasmid or NOD2 expression plasmid as indicated in the figure. Twenty-four hours after transfection, IL6 was assessed. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus basal. Values indicate the mean $\pm$ S.E.M. of triplicates from one representative experiment ($n = 3$).

the overexpression of κ B super-repressor totally abolished the MDP and LPS-induced NF κ B transcriptional activity (Fig. 3b), showing a specific induction of NF κ B. Furthermore, the synergistic effect of MDP plus LPS was

significantly inhibited by the κ B repressor (Fig. 3b). However, the effect of κ B super-repressor at this particular concentration was not strong enough to block the synergism.

Figure 5 Involvement of NFKB and STAT3 on NOD2 signalling pathway. (a) TtT/GF cells were stimulated with LPS (10 µg/ml) and/or MDP (10 µg/ml) for 24 h. Cell lysates were resolved in 10% SDS gel and subjected to western blotting analyses. Nitrocellulose membranes were immunoblotted with STAT3 antibody. (b) TtT/GF cells cultures were co-transfected with STAT3 along reporter plasmid along with control plasmid or *Nod2* expression plasmid. Twenty-four hours after transfection, luciferase activity was measured. (c) TtT/GF cells cultures were co-transfected with STAT3 reporter plasmid along with control siRNA or siRNA NOD2. Twenty-four hours after transfection, luciferase activity was measured. (d) TtT/GF cells were transfected with *Stat3* and/or *Pias3* expression plasmids. pcDNA3 was used as control plasmid. Twenty-four hours post-transfection, NF-κB activity was measured. (e) Cells were transfected with STAT3 siRNA or control siRNA as indicated. Forty-eight hours post-transfection, the cells were stimulated with LPS (10 µg/ml) and MDP (10 µg/ml) each alone or in combination for 6 h. Total cell lysates were immunoblotted with NFKB (p65Rel A) and β-actin specific antibodies. Values indicate the mean $\pm$ S.E.M. of triplicates from one representative experiment ($n=3$). $P<0.05$, versus control plasmids or basal.

To further clarify the synergistic role of NOD2 and TLR4 on the NFKB transcriptional activity, TtT/GF cells were transfected with NOD2 siRNA. Interestingly, silencing NOD2 receptor impaired LPS-induced NFKB activation (Fig. 3c), suggesting that NOD2 receptors might be necessary for LPS/TLR4-induced activation of FS cells.

In addition, we estimated the effect of LPS and MDP on the expression of TLR4 and NOD2 receptors in TtT/GF cells. Cell cultures were stimulated with LPS (10 µg/ml) or MDP (10 µg/ml) for 24 h. Both ligands seem to upregulate mRNA levels of NOD2 and TLR4 (Fig. 3d), as detected by RT-PCR, a process that could enhance the response of these cells to microbial products.

MDP and LPS synergistically induced IL6 production through NOD2

LPS-induced NFKB activation plays an important role in the regulation of IL6 synthesis in TtT/GF cells (Lohrer *et al.* 2000). Since MDP induces NFKB activation, we examined whether this induction could also enhance IL6 transcriptional activity in TtT/GF cells. Indeed, 10 µg/ml MDP stimulated IL6 activation (Fig. 4a), in accordance with previous studies in immune cells (Tada *et al.* 2005, Uehara *et al.* 2005, Yang & Stark 2008). To determine whether there was a cooperative effect between MDP and LPS on the IL6 synthesis, TtT/GF cells were treated with LPS alone or in combination with MDP. LPS significantly induced IL6 production and MDP potentiated this effect (Fig. 4b). Next, we analysed the role of NOD2 receptor in LPS-induced IL6 transcriptional activity. For this purpose, TtT/GF cells were transfected with NOD2 siRNA and IL6 activity was determined in the presence or absence of LPS. NOD2 silencing impaired the LPS-induced IL6 production in TtT/GF cells (Fig. 4c). This data expanded our previous results on the LPS-induced NFKB activation in the presence of NOD2 siRNA, where the NOD2 receptors appeared to be critical for the TLR4-induced activation of FS cells.

In addition, we found that *Nod2* overexpression induced IL6 promoter activity and potentiates the effect of LPS (Fig. 4d), further indicating the importance of NOD2 on the modulation of IL6 synthesis in TtT/GF cells. To analyse whether NFKB mediates the IL6 production in these cells, IκB super-repressor was co-transfected together with the *Nod2* expression plasmid. IκB super-repressor inhibited the *Nod2*-stimulated IL6 promoter activity (Fig. 4e), demonstrating that *Nod2* induces the production of IL6 in TtT/GF cells via NFKB.

Altogether, these findings indicate that NOD2 modulates basal and LPS-induced IL6 production through the activation of NFKB in TtT/GF cells.

NFKB activation by NOD2 requires STAT3

IL6-activated STAT3 and IL-1-induced NFKB were shown to physically interact and increase the transcriptional activity of κB sites (Zhang & Fuller 1997, Yoshida *et al.* 2004). Therefore, the role of STAT3 in MDP and MDP/LPS-induced NFKB activation in TtT/GF cell lines was investigated. First, we observed that MDP and LPS increased STAT3 protein expression, and that MDP potentiated LPS-induced STAT3 protein expression (Fig. 5a). Next, we observed that the overexpression of *Nod2* in TtT/GF cells induced STAT3 activity (Fig. 5b). In contrast, silencing of NOD2 using siRNA interference resulted in the decrease of STAT3 transcriptional activity (Fig. 5c). Interestingly, *Stat3* overexpression induced NFKB activation in TtT/GF cells cultures, whereas the inhibitor of activated STAT3 (*Pias3*) inhibited basal NFKB and

Figure 6 Involvement of NFKB on STAT3-induced IL6 promoter. Cell cultures were co-transfected with IL6-LUC in the presence of *Stat3* alone or in combination of *IkBsr*. IL6 promoter activity was measured after 24 h.

abolished the effect of *Stat3* overexpression on NFKB transcriptional activity (Fig. 5d). These data indicate that STAT3 modulates the activation of NFKB in TtT/GF cells. Consistently, silencing STAT3 decreased the protein level of the NFKB subunit p65/RelA (Fig. 5e). Moreover, STAT3 downregulation abolished the effect of LPS and MDP on NFKB protein expression (Fig. 5e). Thus, STAT3 is necessary for the NFKB induction in TtT/GF cells and STAT3/NFKB participates in the NOD2 signalling pathway.

These results show that *Nod2* induces STAT3 transcriptional activity in TtT/GF cells. Moreover, the findings indicate that STAT3 is important for the stimulatory action of LPS and MDP on NFKB, suggesting that STAT3 acts downstream to TLR4 and NOD2 signalling pathways.

STAT3-induced IL6 promoter activity is mediated by NFKB in TtT/GF cells

The role of STAT3 and NFKB on the IL6 production in TtT/GF cells was further examined. *Stat3* overexpression slightly increased IL6 under basal conditions, and this enhancement is abolished when NFKB activation is blocked by *IkB* super-repressor (Fig. 6).

Discussion

The aim of this work was to study the expression and function of NOD2 receptors in the FS cell line TtT/GF. We report for the first time that NOD2 receptors significantly modulate the function of TtT/GF cells during stimulation with microbial cell wall components. Moreover, our data demonstrate a synergic action of NOD2 with TLR4 on the induction of IL-6 by NFKB, and that STAT3 acting downstream to the NOD2 receptor participates in this induction.

In the present work, we provide evidence for the constitutive expression of NOD2 in the human and rat pituitary and in TtT/GF cells. Double immunofluorescence

of rat normal pituitary gland revealed that FS cells and some S100-negative cells of the adenohypophysis display a high level of NOD2. Furthermore, we observed that in TtT/GF cells, MDP and LPS seem to increase *Nod2* and *Tlr4* mRNA, consistently with the finding of Takahashi *et al.* (2006). They demonstrated that *Nod2* expression was up-regulated in immune cells stimulated with LPS and NOD agonists. In addition, we found that unliganded *Nod2* has a basal level of activity in TtT/GF cells since the overexpression studies show changes in *Il6* transcriptional activity and STAT3 signalling even in the absence of LPS and MDP agonist. Therefore, it is tempting to speculate that changes in the levels of *Nod2* expression might be enough to induce pathophysiological alterations in the pituitary gland. The NOD2 receptor expression indicates a possible direct MDP action on FS cells in the pituitary gland. Binding of MDP to its receptor dose-dependently induces *Nfkb* transcriptional activity, in agreement with previous report in immune cells (Ogura *et al.* 2001, Chin *et al.* 2002, Inohara *et al.* 2002, Kobayashi *et al.* 2002). This data indicates that the NOD2-signalling pathway is functional in TtT/GF cells. The specificity of the MDP/NOD2 receptor binding was confirmed using NOD2 receptor lacking cells, HEK-293 (Ogura *et al.* 2001). As we expected in these cells, MDP did not induce NFKB activity, whereas the iE-DAP NOD1 receptor ligand did. Moreover, we observed that the overexpression of NOD2 activates basal and MDP-induced *Nfkb* transcriptional activity further confirming the functionality of this receptor in TtT/GF cells. TLR and NOD receptors have been reported to trigger the activation of macrophages and dendritic cells by inducing *Nfkb* transcriptional activity, MAP kinases activation and cytokine production in cells from the immune system (Dumitru *et al.* 2000, McDonald *et al.* 2005, Kim *et al.* 2008). Moreover, NOD and TLR receptors can act in a cooperative manner inducing activation of immune cells (Fritz *et al.* 2005, Uehara *et al.* 2005). Several studies conducted with immune cell lines or using peripheral blood mononuclear cells have reported that MDP together with LPS highly increases NFKB activation and cytokine production (Bertin *et al.* 1999, Inohara *et al.* 1999). Since previous studies showed that TLR4 is expressed on the membrane of TtT/GF cells (Lohrer *et al.* 2000), we investigated whether LPS and MDP act in a synergic manner on these cells. In agreement with the literature on the immune system, our experiment demonstrated that MDP in combination with LPS synergistically induced NFKB activation on TtT/GF cells. In addition, we showed that siRNA targeting NOD2 significantly blocked the NFKB activation induced by LPS as revealed by NFKB reporter gene activation (Fig. 3c). These demonstrate a role of NOD2 receptors in mediating the LPS-induced NFKB activation in TtT/GF cells, suggesting that TLR4 may transduce its signals through NOD2. Thus, in TtT/GF cells a functional NOD2 receptor might be critical not only for the transductions of signals of MDP but also for the response to LPS. Nevertheless, Kobayashi *et al.* (2005) showed in a study of macrophages from

NOD-deficient mouse that responses to LPS were generally equivalent in wild-type and NOD2 knockout mice. Therefore, the involvement of NOD2 in the TLR4 signal transduction might be specific for FS cells, although further studies are needed to test this hypothesis.

Considering that NF κ B has been described to induce the production of IL6, we assessed IL6 protein expression and transcriptional activity in TtT/GF cells treated with MDP. We found that MDP not only affected NF κ B activity but also increased IL6 production in TtT/GF cells in a dose-dependent manner. Additionally, we clearly demonstrated that MDP potentiated the effect of LPS on the induction of IL6 in FS cells. These results are in accordance with the literature that showed that MDP stimulates pro-inflammatory cytokine production in immune cells and that MDP and LPS cooperatively amplified this production (Ogura *et al.* 2001, Chin *et al.* 2002, Kobayashi *et al.* 2002, Fritz *et al.* 2005). Therefore, our results confirm that FS cells are involved in the intrapituitary response to infection.

Interestingly, we found that NOD2 siRNA abolished LPS-induced IL6 production in TtT/GF cells and overexpression of NOD2 induced additional stimulation of cells by LPS, further confirming our data on NF κ B and reflecting once more the importance of NOD2 on the TLR4 pathway. The activity of IL6 promoter was found to be significantly enhanced in TtT/GF cells overexpressing *Nod2*, while NOD2 siRNA inhibited this activation. As in the immune system (Medzhitov & Janeway 1997, Aderem & Ulevitch 2000, Lohrer *et al.* 2000, Gloddek *et al.* 2001) in TtT/GF cells, IL6 synthesis is mediated by the activation of the NF- κ B, since the presence of I κ B repressor inhibited the cytokine production.

We also demonstrate evidence for a novel mechanism that involve the synthesis of STAT3 protein and transcriptional activity after activation of the NOD2 receptor. Thus, MDP stimulation and NOD2 overexpression result in the synthesis and activation of STAT3 in TtT/GF cells. Whether IL6 mediates the stimulation of STAT3 is currently under investigation.

Cross-talk between transcription factors has become a commonly recognised mode of gene regulation (Yoshida *et al.* 2004). *In vivo*, STAT3 can physically interact with NF κ B p65 and p65 homodimers cooperate with STAT3 when bound to specific types of κ B motifs (Yoshida *et al.* 2004). In this report, we demonstrate an interaction between STAT3 and NF κ B, which may contribute to the activation of IL6. Silencing STAT3 either by using siRNA or by overexpressing *Pias3* abolished NF κ B transcriptional activity, indicating the participation of STAT3 in the activation of NF κ B. Whether STAT3 and NF κ B bind each other at the level of IL6 promoter remains to be elucidated.

These findings demonstrate that NOD2 receptor activation significantly modulates the function of TtT/GF cells during bacterial infection and inflammation.

A major concern using siRNAs is the non-specific activation of the immune-signalling pathways (Marques & Williams 2005). In this direction, it was described that siRNAs delivered to the cytoplasm of mammalian cells can be recognised by TLRs such as TLR3, TLR7 and TLR8. Once recognised, these siRNAs may produce off-targets effects inducing the activation of immune signalling. In contrast, in our experiments, the NOD2 and STAT3 siRNAs had no stimulatory effects under basal conditions and did not potentiate the stimulatory effect of LPS. Moreover, basal and LPS effects were blocked in the presence of NOD2 and STAT3 siRNAs. These data demonstrate that we have no off-target stimulatory effects produced by siRNAs. Moreover, we noticed that the luciferase activity levels measured after transfection with control plasmids were comparable with the levels that were obtained after the treatment with silencing control RNA.

The importance of understanding the regulatory events within the pituitary gland during infection or inflammation is exemplified by the role that hormones play in adapting the endocrine system to the activated immune system. Thus, intrapituitary IL6 produced by FS cells may act in a paracrine manner to module endocrine cell function by inducing hormone production (Renner *et al.* 1996, Ray & Melmed 1997), and by affecting pituitary cell growth in response to external stimuli (Renner *et al.* 1998). In turn, the activated HPA axis will prevent an over-reaction of the stimulated immune system (Elenkov *et al.* 1999, Chrousos 2000).

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of the research reported.

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