Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Leptin-Dependent Toll-Like Receptor Expression and Responsiveness in Preadipocytes and Adipocytes

Arvind Batra, Jeannette Pietsch, Inka Fedke,
Rainer Glauben, Besir Okur, Thorsten Stroh,
Martin Zeitz, and Britta Siegmund

From the Department of Medicine I, Charité Campus Benjamin
Franklin, Berlin, Germany

Leptin, an adipokine mainly produced by adipocytes, has been well characterized with regard to its regulatory function on immune cells. Thus the question occurred of how adipocytes and preadipocytes interact with the immune system and whether or not this communication is regulated by leptin. With the present study we evaluated the Toll-like receptor (TLR) expression and TLR ligand-specific activation of murine preadipocytes and adipocytes in the presence [wild type (WT), 3T3L1] or absence of leptin (*ob/ob*) or leptin signaling (*db/db*). The *ob/ob* as well as *db/db* adipocytes and preadipocytes were characterized by a significant up-regulation of TLR1 to -9 expression when compared with WT cells. In WT preadipocytes the TLR responsiveness increased during maturation to adipocytes; however, stimulation of *ob/ob* and *db/db* cells resulted in a 10- to 20-fold higher interleukin-6 production. Signaling studies revealed, in addition to the increased TLR expression, alterations in the phosphoinositide 3 kinase signaling cascade in *ob/ob* and *db/db* cells as an explanation for this increased responsiveness. In conclusion, the present study indicates the expression and responsiveness of TLR1 to -9 in murine preadipocytes as well as adipocytes, both of which are strongly regulated by the adipokine leptin. In summary, these data further emphasize the role of fat tissue in the immune system. (Am J Pathol 2007, 170:1931–1941; DOI: 10.2353/ajpath.2007.060699)

Adipocytes are the key players in energy storage and metabolism. However, recent reports suggest that adipocytes as well as their precursors interact with the immune system. Lymphatic tissue is always in close proximity to the adipose tissue, and the latter supports the former during activation of the immune system by supplying fatty acids and other nutrients.¹ Furthermore, adipocytes are

not only potent producers of adipokines including adiponectin, adipsin, leptin, and visfatin but respond to and produce various cytokines including interleukin (IL)-6, tumor necrosis factor (TNF)- α , and IL-10.^{2–5}

Recent data from the literature indicate that adipokines can actively participate in the regulation of the immune system. The adipokine characterized best in this regard is leptin, a hormone that can structurally and functionally be classified as a cytokine.^{6,7} Leptin-deficient *ob/ob* mice are protected in multiple models of inflammation such as autoimmune encephalomyelitis, collagen-induced arthritis, delayed-type hypersensitivity, and dextran sulfate sodium- as well as trinitrobenzene sulfonic acid-induced colitis.^{8–12} Additional data from our group indicate that the stimulation of naïve T cells represents the critical step in the leptin-dependent induction of a proinflammatory response.¹³ In contrast, *ob/ob* mice are more susceptible to endotoxin-induced shock, *Klebsiella pneumoniae*-induced pneumonia as well as viral myocarditis and show a delay in the resolution of acute zymosan-induced inflammation.^{9,14–16} At this point there is no conclusive explanation for this controversy. The above data point to a yet incompletely defined position of the fat tissue in the immune system and suggest a regulatory role for leptin.

Besides these adipokine-mediated modulatory effects from the adipose tissue on the immune system, preadipocytes have been shown to be able to convert into macrophage-like cells, which express macrophage surface markers and phagocytose bacterial antigens.¹⁷ Recognition of a variety of evolutionary highly conserved antigens, so called pathogen-associated molecular patterns, is mediated by pattern-recognition receptors including the family of Toll-like receptors (TLRs).¹⁸ In humans TLR1 to -11 and in mice TLR1 to -9 and TLR11 have been identified, respectively.¹⁹ Although the founding

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Address reprint requests to Britta Siegmund, M.D., Charité, Campus Benjamin Franklin, Department of Medicine I, Hindenburgdamm 30, 12200 Berlin, Germany. E-mail: britta.siegmund@charite.de.

member of this family, the *Drosophila* Toll protein, plays a major role in embryogenesis,²⁰ TLR in mammals act as receptors for a variety of bacterial as well as viral products.¹⁸ For instance, TLR3 recognizes double-stranded RNA,²¹ whereas TLR4 interacts with lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria.²² TLR5 recognizes bacterial flagellin,²³ and TLR7 serves as a receptor for single-stranded RNA (ss-RNA) and in addition binds a variety of guanosine derivatives including R-848 as well as loxoribine.^{24–26} TLR8 does not seem to be functional in mice. TLR2 heterodimerizes with either TLR1 or TLR6. The resulting complexes act as pattern-recognition receptors for a variety of bacterial and fungal ligands including zymosan, peptidoglycans, or bacterial lipoproteins.²⁷

With the present study we focused on two aspects. First, we aimed to define the role of preadipocytes as well as adipocytes in the innate immune system by characterizing the cell-specific TLR expression profile as well as the TLR-specific response. Second, to characterize leptin-dependent regulation of TLR expression as well as responsiveness, leptin-deficient (*ob/ob*), leptin receptor-deficient (*db/db*), and wild-type (WT, 3T3L1) preadipocytes were included. Thus the present study explored the possibility of whether preadipocytes and adipocytes are not solely supporting cells of the innate and adaptive immunity via production of cytokines and adipokines but participate independently in the innate immune system.

Materials and Methods

Reagents

Hanks' balanced salt solution, Dulbecco's modified Eagle's medium, Dulbecco's modified Eagle's medium/HAMS F-12, bovine serum albumin, penicillin, as well as streptomycin were purchased from PAA Laboratories GmbH (Cölbe, Germany); fetal calf serum was obtained from Linaris Biologische Produkte GmbH (Wertheim-Bettingen, Germany). Tween 20 as well as Triton X-100 were from Merck KGaA (Darmstadt, Germany). Collagenase II, sodium dodecyl sulfate, ethylenediaminetetraacetic acid, glucose, wortmannin, and 3-isobutyl-1-methylxanthine were obtained from Sigma-Aldrich GmbH (Taufkirchen, Germany), and Ly294002 was purchased from Calbiochem (Schwalbach, Germany). Insulin was from Aventis Pharma Deutschland GmbH (Frankfurt, Germany) and hydrocortisone from Pharmacia GmbH (Karlsruhe, Germany). Recombinant murine leptin was purchased from Peprotech (Hamburg, Germany). The biological activity of leptin was first evaluated in *ob/ob* mice in which intraperitoneal administration of 500 ng leptin/g body weight twice daily resulted in the expected loss of body weight (1 g in 2 days).

Mice

Animal protocols were approved by the regional animal study committee of Berlin, Germany. Six- to 8-week-old female C57BL/6J (WT), C57BLKS/J (WT), B6.V-Lep^{ob}/J

(*ob/ob*), and BKS.Cg-m+/+Lpr^{db}/J (*db/db*) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or Harlan Winkelmann (Borchen, Germany). The animals were housed at controlled temperature with light-dark cycles, fed standard mice chow pellets, had access to tap water from bottles, and were acclimatized before being studied. On the end of an experimental period, mice were sacrificed by cervical dislocation under CO₂ anesthesia.

Isolation of Mouse Preadipocytes

Mice were sacrificed, and mesenteric adipose tissue was excised, minced, and washed in Hanks' balanced salt solution. Tissue was digested during a 25-minute incubation period at 37°C in Hanks' balanced salt solution containing 1.5 mg/ml collagenase II, 3.5% bovine serum albumin, and 0.55 mmol/L glucose. The suspension was subsequently mashed through a 100-μm nylon net (BD Pharmingen GmbH, Heidelberg, Germany) and centrifuged at 200 × *g* for 10 minutes. Pelleted cells were washed twice in culture medium and seeded into 48-well plates (5 × 10⁵ cells/well). The following day nonadherent cells were discarded, and adherent cells propagated.

Cell Culture

3T3L1 murine preadipocytes were propagated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and penicillin/streptomycin (100 U/ml each). The *ob/ob*, *db/db*, and WT preadipocytes were grown in Dulbecco's modified Eagle's medium/HAMS F-12 medium containing 10% fetal calf serum and penicillin/streptomycin (100 U/ml each). Differentiation was induced according to the protocol described previously.²⁸ In brief, the cells were allowed to reach confluence, and in the next step the appropriate medium (see above) was supplemented with 100 nmol/L insulin, 250 nmol/L dexamethasone, and 0.5 mmol/L 3-isobutyl-1-methylxanthine (this supplemented medium is henceforth referred to as differentiation medium I or DMI). Four days later the medium was switched to the appropriate medium supplemented with 1 mmol/L insulin (this supplemented medium is from now on referred to as differentiation medium II; DMII), and the cells were propagated therein until differentiation to adipocytes was detectable. The commitment of each established cell line to the adipocyte lineage was confirmed by the conversion from preadipocytes to adipocytes after culture in the respective differentiation media as described above.

Oil Red O Staining

Accumulation of lipids in cells in which differentiation to adipocytes had been induced was visualized by staining with oil red O (Sigma-Aldrich GmbH). Briefly, 6 ml of stock solution of 0.5% (w/v) oil red O in isopropanol was mixed with 4 ml of water as working solution. Cells were washed with phosphate-buffered saline (PBS), fixed with 4% phosphate-buffered formaldehyde for 10 minutes at room temperature, washed with PBS, stained with oil red

O working solution for 1 hour, and finally washed with PBS. The triglyceride accumulation was assessed using an inverted microscope (Olympus Deutschland GmbH, Hamburg, Germany).

Stimulants

The following stimulants were purchased as indicated: flagellin (Alexis Corp., Lausen, Switzerland); poly(I:C), zymosan, and loxoribine (Invivogen, San Diego, CA); and LPS (from *Escherichia coli*, 055:B5; Sigma-Aldrich GmbH). The oligodeoxynucleotide (ODN) 1668 (5'-TCCATGAC-GTTCCTGATGCT-3') and ODN 2088 (5'-CCTGGCGGG-GAAGT-3') each had a phosphorothioate backbone and were obtained from TIB MolBioL GmbH (Berlin, Germany). For all stimulants except for LPS, endotoxin contamination was excluded by using the Limulus amebocyte lysate assay (Hemochrom Diagnostica GmbH, Essen, Germany).

Cell Stimulation

Cells were incubated for various time periods with different concentrations of TLR-specific ligands as indicated. At the end of stimulation, supernatants were collected, and concentrations of TNF- α , IL-6, as well as IL-10 were determined by enzyme-linked immunosorbent assay (ELISA) as described below. Cells were lysed in Tri Fast Peq Gold for RNA isolation (Peqlab GmbH, Erlangen, Germany), according to standard protocols, or lysed in protein lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate, and 0.1% Triton X-100) for Western blot analysis.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Real-Time RT-PCR

Total RNA was extracted from the lysed cells. Aliquots of each RNA extraction were reverse-transcribed simultaneously into cDNA, using M-MLV reverse transcriptase according to the manufacturer's protocol (Invitrogen GmbH, Karlsruhe, Germany). Semiquantitative RT-PCR was performed with the following primers using standard conditions: TLR1 forward 5'-GGATGTGTCCGTCAGCAC-TA-3'; TLR1 reverse 5'-TGTAACCTTGGGGGAAGCTG-3'; TLR2 forward 5'-AAAATGTCGTTCAAGGAG-3'; TLR2 reverse 5'-TTGCTGAAGAGGACTGTT-3'; TLR3 forward 5'-ACTTGCTATCTTGATGC-3', TLR3 reverse 5'-AGTT-CTTCACTTCGCAAC-3'; TLR4 forward 5'-CCTGATGAC-ATTCTTCT-3'; TLR4 reverse 5'-AGCCACCAGATTCTC-TAA-3'; TLR5 forward 5'-GCTTTGTTTTCTTCGCTTCG-3'; TLR5 reverse 5'-ACACCAGCTTCTGGATGGTC-3'; TLR6 forward 5'-GCAACATGAGCCAAGACAGA-3'; TLR6 reverse 5'-GTTTTGCAACCGATTGTGTG-3'; TLR7 forward 5'-TGACTCTCTCTCTCCA-3', TLR7 reverse 5'-GCTT-CCAGGTCTAATCTG-3'; TLR8 forward 5'-TCCTGGGGA-TCAAAAATCAA-3'; TLR8 reverse 5'-AAGGTGGTAGCC-CAGTTCAT-3'; TLR9 forward 5'-ACCCTGGTGTGGAAC-ATCAT-3', TLR9 reverse 5'-GTTGGACAGGTGGACGAA-GT-3'; and GAPDH forward 5'-ACCACAGTCCATGCCA-

TCAC-3', GAPDH reverse 5'-TCCACCACCCTGTTGCT-GGTA-3'. Primers were chosen according to the literature.^{29,30} All primers were manufactured by TIB MolBioL GmbH. The thermal cycling conditions comprised a 5-minute step at 95°C, followed by 40 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes, and a final step of 72°C for 10 minutes. Results of the GAPDH PCR served as control that equal amounts of cDNA were used for the TLR-specific PCR. The intensity of TLR-specific signals as detected on the agarose gels by density scanning was evaluated as follows. First the density was normalized with regard to GAPDH scanning. Highest density was graded as 1.0 and the rest was expressed as relative expression. Each single TLR bar for each cell line examined represents $n = 10$ different cell samples.

For quantitative real-time PCR (qPCR) the following sets of primers, which were designed using Primer Quest software, were applied: GAPDH forward: 5'-TCAACAG-CAACTCCCACTCTTCCA-3'; GAPDH reverse: 5'-ACCC-TGTTGCTGTAGCCGTATTCA-3'; TLR2 forward: 5'-TCC-CTTGACATCAGCAGGAACACT-3'; TLR2 reverse: 5'-A-CTAACATCCAACACCTCCAGCGT-3'; TLR4 forward: AACCAGCTGTATTCCCTCAGCACT-3'; TLR4 reverse: ACTGCTTCTGTTCCCTTGACCCACT-3'; TLR5 forward: 5'-AAGACTGCGATGAAGAGGAAGCCA-3'; TLR5 reverse: 5'-TGTCCTTGAACACCAGCTTCTGGA; TLR7 forward: 5'-CCACAGGCTCACCCATACTTC-3'; TLR7 reverse: 5'-GGGATGTCCTAGGTGGTGACA-3'; and TLR9 forward: 5'-TGGGCCATTGTGATGAAC-3'; TLR9 reverse: 5'-TTG-GTCTGCACCTCCAACAGT-3'. Each real-time PCR was performed in duplicate on a LightCycler (Roche, Mannheim, Germany) in a total volume of 20 μ L. Briefly, the mix included 2 μ L of template, Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 5 pmol of sense and anti-sense primers, ROX reference dye, bovine serum albumin, and H₂O. The thermal cycling conditions comprised a 2-minute step at 95°C, followed by 40 cycles with denaturation at 94°C for 5 seconds, annealing at 60°C (TLRs) or 62°C (GAPDH) for 20 seconds, and extension at 72°C for 10 seconds. Data were analyzed according to a standard curve generated from a pool of all samples tested. For normalization of differences in RNA amounts, the housekeeping gene GAPDH was co-amplified. Results were calculated as arbitrary units of gene of interest per arbitrary units of GAPDH. GAPDH was chosen as housekeeping gene because mRNA levels of this gene are reported to remain unaltered during the maturation of preadipocytes to adipocytes, an observation confirmed by us when the GAPDH content of samples from preadipocytes and adipocytes, which had been adjusted to identical cDNA concentration based on photometric measurement, was compared.³¹

Antibodies

The antibodies directed against murine TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 were all purchased from BioMol (Hamburg, Germany). Antibodies against Akt, phospho-Akt (Ser473), STAT-3, as well as STAT-3P

(Tyr705) were purchased from Cell Signaling (Beverly, MA). The anti- β -actin antibody was obtained from Sigma-Aldrich GmbH. All antibodies were used at concentrations ranging from 1 to 2 μ g/ml diluted in PBS with 0.05% Tween 20.

Immunoprecipitation

Cell lysates (1×10^6 cells/ml protein lysing solution) were diluted 1:3 in immunoprecipitation buffer (10.56 mmol Na_2HPO_4 , 1.28 mmol NaH_2PO_4 , 93 mmol NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate) and precleared by the addition of Protein A-Sepharose beads (Amersham Bioscience, Uppsala, Sweden) and incubation for 1 hour at 4°C. Subsequently the beads were discarded and the supernatants incubated overnight at 4°C with 0.01 μ g/ μ l of TLR-specific antibody. The following day, Sepharose IgA-beads were added and the samples incubated for another 2 hours at 4°C. The beads were subsequently pelleted by centrifugation and washed four times with immunoprecipitation buffer. Finally, the beads were resuspended in 80 μ l of gel-loading buffer and boiled to release bound antigens.

Western Blot Analysis

For immunoblotting, total cell lysates or the samples from the TLR-specific immunoprecipitation from either preadipocytes or adipocytes were applied. Western blot analysis was performed as described previously³² using the antibodies indicated above. An anti- β -actin antibody was used as control for protein loading.

Densitometric Analysis

To allow for comparison of the band intensity, blots were scanned, and density was compared using the ImageJ 1.34s software (National Institutes of Health, Bethesda, MD). The intensity of the specific bands was normalized according to the density of the β -actin bands.

Cytokine Measurement

Murine IL-6, TNF- α , and IL-10 were determined by using the EIA kit (BD Pharmingen GmbH). The range of quantification for IL-6 and TNF- α is 15 to 1000 pg/ml and for IL-10 is 31 to 2000 pg/ml.

Statistical Analysis

The data are expressed as mean $\pm$ SEM. Statistical significance of differences between treatment and control groups were determined by the Kruskal-Wallis non-parametric test using JMP Software (JMP Version 3.2; SAS Institute Inc., Cary, NC).

Figure 1. Differentiation of preadipocytes to adipocytes. WT, *ob/ob*, or *db/db* preadipocytes were isolated from murine mesenteric fat tissue as described in the Materials and Methods section, and transient cell lines were established. Differentiation to adipocytes was induced by standard protocols as described in Materials and Methods. The picture shown represents differentiation of preadipocytes (left; **1**) throughout time into adipocytes (middle and right; **2** and **3**) after culture in differentiation medium I (DMI) for 4 days (middle; **2**) and differentiation medium II (DMI2) for an additional 4 days (right; **3**) as indicated. Adipocytes are characterized by accumulation of lipid droplets as visualized by oil red O staining as described in the Materials and Methods section. Because the differentiation process was similar in all cell lines tested, only WT cells are shown.

Results

Generation of WT, Leptin-Deficient, and Leptin Receptor-Deficient Preadipocyte Cell Lines

Commercial availability of murine preadipocyte cell lines is limited to 3T3L1 cells. To study WT, leptin-deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) preadipocytes, these cells were isolated from the mesenteric fat of the respective mice as described in the Materials and Methods section. The purity of the preadipocyte cell lines was confirmed by demonstrating that all preadipocyte cell lines matured into lipid droplet-containing adipocytes when differentiation was induced (Figure 1). Differentiation from preadipocytes to adipocytes occurred independently from the presence or absence of leptin signaling. Because in the following studies no significant differences could be observed when comparing C57BL/6J cells, the respective WT control for the *ob/ob* cells, and C57KS/J cells, the respective WT control for *db/db* cells, the results for these cells were summarized under WT cells in the entire study.

Leptin-Dependent Regulation of TLR mRNA Expression in Preadipocytes and Adipocytes

To characterize leptin-dependent TLR expression, mRNA was isolated from 3T3L1, WT, *ob/ob*, and *db/db* preadipocytes as well as adipocytes, and semiquantitative RT-PCR was performed for mouse TLR1 to TLR9. In WT preadipocytes moderate levels of mRNA for TLR1 to TLR7 were detected. The panel of TLR was most restricted in 3T3L1 cells, where only TLR1- to TLR4- as well as TLR6-specific mRNA was present at marginal levels. In contrast, in *ob/ob* as well as in *db/db* preadipocytes, mRNA for all nine TLRs tested could be demonstrated. In *ob/ob* preadipocytes prominent signals for TLR1 to -7 were detected, whereas those for TLR8 and -9 were low. Highest expression of TLR8 and

Figure 2. TLR mRNA expression pattern of preadipocytes and adipocytes. RNA from either preadipocytes or differentiated adipocytes from all cell lines was isolated and RT-PCR performed to analyze TLR1–9 expression. **a:** Results of the semiquantitative RT-PCR as described in detail in the Materials and Methods section. To assess further the differences in TLR mRNA expression with a limited set of samples, quantitative real-time PCR was performed using the LightCycler system as described in the Materials and Methods section. The y axis represents the arbitrary units of the gene of interest per arbitrary units of the housekeeping gene (GAPDH, **b**). Data are shown as mean signal intensity $\pm$ SEM of $n = 10$ (**a**) or $n = 3$ to 5 (**b**); * $P \leq 0.05$, ** $P \leq 0.01$; preadipocytes or adipocytes, respectively, from all cell lines were compared with the corresponding *ob/ob* cells.

TLR9 was found in *db/db* cells. Differentiation to adipocytes influenced the TLR panels in the various cell lines only marginally. In parallel to the preadipocyte cell lines, *ob/ob* as well as *db/db* adipocytes expressed the entire panel of tested TLR, and signal intensity was usually brighter than in WT or 3T3L1 cells (Figure 2a).

Figure 2. Continued

To specify further the observed differences in TLR mRNA expression, quantitative real-time PCR (qPCR) was performed for TLR2, -4, -5, -7, and -9, the respective TLR for which functional differences could be detected in the subsequent studies. Confirming the results described above, the expression of all TLRs analyzed was increased in cells in the absence of leptin or leptin signaling when compared with WT and 3T3L1 cells. In contrast to the results above, using qPCR both TLR7- and TLR9-specific mRNA were now detectable even in 3T3L1 and WT preadipocytes and adipocytes (Figure 2b).

Figure 3. TLR protein expression of preadipocytes and adipocytes. Protein was extracted from either preadipocytes (A) or differentiated adipocytes (B) and Western blot analysis for the indicated TLRs was performed. The blots shown are representative of at least five independent blots performed per condition. β -Actin staining served as control for equal protein loading. For TLR2 and TLR7, Western blot analysis was performed after immunoprecipitation.

Presence of TLR Protein in Preadipocytes and Adipocytes

To confirm the expression of the various TLRs on the protein level, Western blot analysis either using whole cell lysates or, if this produced unspecific bands, after immune precipitation was performed for TLR2, -3, -4, -5, -7, and -9. For the protein detection and subsequent stimulation studies, TLR1 and TLR6 were excluded because they depend functionally on heterodimerization with TLR2. In addition, TLR8 was excluded because it is non-functional in mice.³³ No protein data could be achieved for TLR4 because of unspecific binding of the antibody used, whereas the presence of TLR3, -5, and -9 protein could be confirmed using whole cell lysates and for TLR2 and -7 after immunoprecipitation (Figure 3). Equal loading was confirmed by sequential staining for β -actin. For TLR3, -5, and -9, a tendency toward higher expression in *ob/ob* and *db/db* cells was observed. Because of limited blot quality no further quantitative analysis on the protein level was performed, but functional analysis for the TLRs expressed were conducted.

Leptin-Dependent Regulation of TLR Responsiveness of Preadipocytes and Adipocytes

To evaluate functionality of the TLR receptors expressed by preadipocytes and adipocytes, cells were stimulated with TLR-specific ligands. Supernatants were collected at various time points as indicated and analyzed by ELISA for the presence of IL-6, IL-10, and TNF- α , all known to be released by the adipose tissue.

Kinetic Studies of TLR4-Specific Induction of Cytokines

First, kinetic studies were performed to determine the appropriate time point for cytokine measurement. As an example, the results of the TLR4 ligand LPS are shown;

Figure 4. Kinetic studies for LPS-stimulated preadipocytes. The established cell lines from WT, *ob/ob*, and *db/db* mice or 3T3L1 cells were stimulated with LPS (1 μ g/ml) for the time periods indicated and IL-6 (A) as well as TNF- α (B) were determined in the supernatant by ELISA. Bars represent mean $\pm$ SEM, $n = 7$; * $P \leq 0.05$, ** $P \leq 0.01$ stimulated versus unstimulated cells.

however, no significant differences were observed using any of the other TLR ligands. Preadipocytes and adipocytes were cultured in the presence of 1 μ g/ml LPS and supernatants were collected after 8 hours, 1 day, or 3 days of stimulation. Because the highest concentrations of IL-6 for all cell lines were detected after a 3-day stimulation period, this incubation period was chosen for the following experiments (Figure 4A). No time-dependent increase could be detected for TNF- α , which was only marginally expressed by most cell lines with the exception of *db/db* cells (Figure 4B). IL-10 was below detection limit in all samples analyzed (not shown).

Leptin-Dependent TLR Responsiveness

To evaluate further the functionality of the expressed TLR, the panel of TLR-specific ligands was broadened. The respective cells were stimulated for 3 days, and IL-6 was measured in the supernatant. The IL-6 response to TLR-specific stimulation resembled the mRNA expression profile (Figure 2, a and b, and Figure 5, respectively). Thus stimulation of preadipocytes with the TLR2 ligand zymosan (10 μ g/ml) resulted in a significant increase of cytokine production in *db/db* cells, but not in any of the other cell lines. Stimulation of preadipocytes with poly(I:C) (25 μ mol/L) induced solely in *db/db* and *ob/ob* cells a significant increase in IL-6 synthesis, whereas 3T3L1 as well as WT cells were unresponsive. TLR4 stimulation via LPS (1 μ g/ml) induced the highest production of IL-6 in all preadipocyte cell lines. Additional dose-response studies revealed a significant increase in IL-6 production even after stimulation with 10 ng/ml LPS

Figure 5. TLR-specific stimulation of preadipocytes as well as adipocytes and subsequent IL-6 release. Preadipocytes (**A**) or adipocytes (**B**) were stimulated with the following stimuli for a total of 3 days: zymosan (10 μ g/ml), poly(I:C) (25 μ mol/L), LPS (1 μ g/ml), flagellin (10 ng/ml), loxoribine (10 μ mol/L), or ODN 1668 (0.5 μ mol/L). IL-6 was measured at the end of stimulation in the supernatant by ELISA. To assess influence of leptin substitution on cytokine production, WT and *ob/ob* cells were preincubated with 500 ng/ml leptin followed by LPS stimulation (**C**). Bars represent means $\pm$ SEM, $n = 8$; * $P \leq 0.05$, ** $P \leq 0.01$ versus the unstimulated control.

for all cell lines studied (data not shown). Flagellin (10 ng/ml) as TLR5-specific ligand was a potent inducer of IL-6 production in *ob/ob* cells only, whereas significant

Figure 6. TLR9-specific response in preadipocytes and adipocytes. The *db/db* preadipocytes as well as adipocytes were incubated in the presence or absence of ODN 1668 (0.5 μ mol/L) or a combination of ODN 1668 and 2088 (both 0.5 μ mol/L) for 3 days, and IL-6 was measured in the supernatant by ELISA. Bars represent means $\pm$ SEM, $n = 5$; * $P \leq 0.05$, ** $P \leq 0.001$ versus unstimulated as well as ODN 1699 plus ODN 2088 co-stimulated cells.

IL-6 production in response to stimulation with either the TLR7 ligand loxoribine or the TLR9 ligand ODN 1668 (0.5 μ mol/L) was restricted to *db/db* cells.

LPS remained the strongest inducer of IL-6 in all cell lines studied. The *ob/ob* adipocytes showed no IL-6 production in response to stimulation with flagellin, whereas the cells gained responsiveness to loxoribine. WT adipocytes produced significant IL-6 levels after stimulation with poly(I:C) (25 μ g/ml), flagellin (10 ng/ml), and ODN 1668 (0.5 μ mol/L). 3T3L1 adipocytes showed a significant increase in IL-6 after poly(I:C) and LPS stimulation, respectively (Figure 5B). Remarkably, after TLR-specific stimulation *ob/ob* and *db/db* preadipocytes and adipocytes revealed a 10- to 20-fold higher IL-6 production than WT or 3T3L1 cells.

Leptin Substitution Influences TLR4-Mediated and Spontaneous IL-6 Production

To characterize further the role of leptin signaling in TLR-induced IL-6 production, either WT or *ob/ob* preadipocytes were stimulated with LPS in the presence or absence of 500 ng/ml leptin. Preadipocytes were preincubated with 500 ng/ml leptin 1 day before TLR-specific stimulation. In parallel to the results described above, preincubation with leptin resulted in a significant decrease of LPS-induced IL-6 production in *ob/ob* preadipocytes (Figure 5C).

TLR9-Specific Stimulation of Preadipocytes and Adipocytes

Stimulation of either *db/db* preadipocytes or adipocytes with the stimulatory TLR9 ligand, the synthetic ODN 1668, resulted in a strong induction of IL-6 synthesis. In WT adipocytes ODN 1668 (0.5 μ mol/L) stimulation was also followed by a significant increase in IL-6. To validate a TLR9-specific effect, ODN 2088 (0.5 μ mol/L) was chosen as an ODN known to exert an inhibitory effect mediated specifically by TLR9.³⁴ As shown in Figure 6, co-incubation of cells with ODN 1668 and ODN 2088 resulted in a suppression of IL-6 synthesis in WT adipocytes as well as

Figure 7. Absence of STAT-3 activation in preadipocytes and adipocytes. The *db/db*, *ob/ob*, WT, or 3T3L1 preadipocytes were incubated in the presence or absence of LPS (1 μ g/ml) for 1 hour. Cells were lysed subsequently, and Western blot analysis for total STAT-3 or the activated STAT-3P was performed. The blot shown is one representative blot of five independent blots performed.

in *db/db* adipocytes and preadipocytes. Stimulation of either WT or *db/db* cells with ODN 2088 alone did not result in an induction of IL-6 synthesis (Figure 6).

Phosphoinositide 3-Kinase-Dependent Increased Responsiveness in the Absence of Leptin or Leptin Signaling

The increased TLR expression in *ob/ob* and *db/db* cells might partially contribute to the increased IL-6 production in these cells. However, to reveal additional mechanisms responsible for the significantly increased cytokine production after TLR-specific stimulation in the absence of leptin or leptin signaling, shared intracellular pathways of leptin and TLR signaling were analyzed. As demonstrated previously, STAT-3 represents a critical signal transduction pathway for leptin-mediated effects on the immune system.¹² Consequently, STAT-3 phosphorylation (STAT-3P) was compared in unstimulated as well as LPS-stimulated cells of the various cell lines (Figure 7). However, independent of LPS stimulation an equal expression of STAT-3 and activated STAT-3P was detected, thus excluding differences in the STAT-3 pathway as explanation for the increased responsiveness.

Next, the phosphoinositide 3 kinase (PI3K) pathway was studied because PI3K can be activated by leptin and was shown to regulate TLR-specific responsiveness.^{35–37} To evaluate the activation of PI3K, the downstream product Akt and its activated form phospho-Akt were analyzed. The *ob/ob* or WT preadipocytes were stimulated with LPS for 30 minutes in the presence or absence of the PI3K inhibitor wortmannin or LY294002, respectively, which were added 1 hour before the addition of LPS. Staining of β -actin is shown for equal loading. Akt was present in all samples at equal concentrations. Interestingly, LPS-stimulated and unstimulated *ob/ob* as well as WT cells presented constitutively activated phospho-Akt. Co-incubation with the PI3K inhibitor wortmannin resulted in *ob/ob* as well as in WT cells in a significant decrease of phospho-Akt (Figure 8, B and C).

Although wortmannin did not influence the IL-6 release after LPS stimulation in WT and 3T3L1 cells, in *ob/ob* and *db/db* cells the presence of this inhibitor resulted in a significant suppression of IL-6 synthesis down to concentrations observed in WT cells (Figure 8A). These functional data suggest that an altered activation state of the PI3K pathway is involved in the increased susceptibility in

Figure 8. Leptin-dependent increased responsiveness is PI3K-mediated. After a 60-minute preincubation with the PI3K inhibitor wortmannin, cells were stimulated with LPS (1 μ g/ml) as indicated and described in the Materials and Methods section. **A:** To test for cytokine production, supernatants were collected on day 3, and IL-6 was measured by ELISA. **B:** For protein detection cells were lysed 30 minutes after the addition of LPS and Western blot analysis for total Akt, activated phosphorylated Akt (p-Akt), and β -actin was performed. The blot demonstrated is representative of a total of five independent blots performed. **C:** Densitometric analysis of p-Akt expression in *ob/ob* and WT after normalization for β -actin is shown as fold increase compared with the expression in untreated WT cells. Bars represent means $\pm$ SEM, $n = 5$; * $P \leq 0.05$, ** $P \leq 0.001$ versus unstimulated cells.

the absence of leptin or leptin signaling even though this is not reflected by the activation of Akt. Comparable results concerning the decrease in cytokine production were obtained with the PI3K inhibitor LY294002 (data not shown). However, because the inhibition of the PI3K pathway resulted only in partial decrease of the exaggerated IL-6 response in *ob/ob* and *db/db* cells, involvement of additional signaling pathways cannot be excluded.

Discussion

In the recent years, the regulatory function of leptin in the immune system has been characterized in detail by several groups. Previous studies from our own group and others demonstrated leptin synthesis by lymphocytes infiltrating at the site of inflammation.^{13,38} However, additional experiments in the transfer model of colitis revealed

that T-cell-derived leptin has no impact on the inflammatory process, thus emphasizing adipocytes as the main source.² Consequently, the question occurred of how adipocytes or preadipocytes can interact with the immune system and whether or not this possible communication is regulated by leptin. In the present study, we focused on the innate immune system. Purified WT, *ob/ob*, or *db/db* preadipocytes as well as adipocytes were characterized for their TLR expression pattern and TLR ligand-specific responsiveness.

The results of the present study reveal the expression of the entire TLR panel on isolated preadipocytes and adipocytes as well as the responsiveness to TLR-specific stimulation. The data obtained from the comparison of WT, 3T3L1, and *ob/ob*, as well as *db/db* preadipocytes and adipocytes indicate a strong regulatory potency for leptin, resulting in an increased TLR expression and activation in the absence of leptin or leptin signaling.

TLR2 as well as TLR4 expression has been demonstrated previously for 3T3L1 preadipocytes, but with the present study, a first complete TLR expression profile for preadipocytes and adipocytes is provided.³⁹ Although the expression profile serves for a descriptive characterization, TLR-specific stimulation is required to evaluate the functional significance. In the literature, stimulation of 3T3L1 adipocytes with either the TLR4 ligand LPS or the TLR2 ligand zymosan or TNF- α resulted in a strong induction of TLR2 expression as well as the release of IL-6.³⁹ In agreement with these results, in the present study stimulation of WT or 3T3L1 preadipocytes or adipocytes with LPS was followed by a significant increase in IL-6 synthesis. Remarkably, 10- to 20-fold higher IL-6 concentrations were detected in *ob/ob* and *db/db* cells. Accordingly, preincubation of preadipocytes with leptin resulted in a decreased IL-6 production after LPS stimulation in *ob/ob* cells. The *ob/ob* mice are known to exert a higher sensitivity to systemic LPS administration.¹⁵ The increased responsiveness of adipocytes and preadipocytes to this stimulus provides a first explanation to this yet not well-characterized phenomenon. Notably, during differentiation to adipocytes the spectrum of TLR responsiveness increased in WT cells from TLR4 in preadipocytes to TLR3, -4, -5, and -9 in WT adipocytes. Although the spectrum did not change in the *ob/ob* or *db/db* cells during differentiation, the increased responsiveness as indicated by a profoundly higher IL-6 production was maintained. Consistent with the significantly higher IL-6 release after TLR3-specific stimulation in the absence of leptin or leptin signaling, Kanda and colleagues¹⁶ could demonstrate an increased susceptibility to viral myocarditis in *ob/ob* mice.

Recognition of bacterial DNA by the vertebrate immune system is based on the presence of unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs).⁴⁰ Synthetic ODNs that contain such CpG motifs mimic bacterial DNA and have been shown to induce a coordinated set of immune responses.^{41,42} In 2000, TLR9 was identified as the specific receptor for CpG motif-mediated immune response.⁴³ In subsequent studies plasmacytoid dendritic cells as well as B cells have been identified as TLR9-expressing and thus CpG-responsive

cell populations.⁴⁴ *Db/db* preadipocytes and adipocytes as well as WT adipocytes were the cell populations responding to the TLR9 stimulus ODN 1668. To verify the ODN 1668-stimulatory effect, a previously described B-cell control was included.³⁴ By co-incubation with the inhibitory ODN 2088, the stimulatory potency of ODN 1668 was strongly abrogated, thus supporting the data obtained before.³⁴ In conclusion, *db/db* adipocytes and preadipocytes as well as WT adipocytes can be included as new members of CpG-responding cells.

The potential of cells from the adipose tissue to produce various mediators including TNF- α , IL-6, macrophage migration inhibitory factor, plasminogen activator inhibitor-1, as well as IL-10 has been demonstrated previously.^{45,46} Cytokine production by fat tissue contributes to total cytokine levels in the plasma. For example, adipocytes account for 15 to 35% of the circulating IL-6. In addition, IL-6 as well as TNF- α serum levels correlate with the body mass index.^{5,47,48} Both cytokines have been described to induce lipolysis in mouse and human adipocytes, thus supporting the increased energy expenditure during inflammation.^{49,50} Consequently, adipocytes comprise two functions in sustaining the inflammatory response after TLR-specific stimulation, first by providing the required energy through lipolysis and second by enhancing the systemic inflammatory response by the release of proinflammatory cytokines into the circulation.

The observed increased responsiveness to TLR-specific stimulation in *ob/ob* and *db/db* cells can be partially explained by the increased TLR expression (Figure 2) in these cells. To reveal further possible mechanisms involved in the up-regulated responsiveness of *ob/ob* and *db/db* cells to TLR stimulation, common signaling pathways were characterized. The STAT-3 pathway was chosen as the first target. However, no phosphorylation of STAT-3 was detectable after stimulation of the TLR4 in any of our cell lines. Thus, although it has been demonstrated that various TLR ligands modulate the STAT-3 pathway mediated by IL-10, an alteration of this signaling cascade in *ob/ob* and *db/db* cells was excluded.⁵¹

Second, the PI3K pathway was chosen because it represents a shared intracellular signaling cascade between leptin and TLR-mediated intracellular signaling.^{52,53} Although the role of TLR-induced activation is not fully understood, PI3K has been described to be activated by several TLR members,³⁵ suggesting the presence of shared signaling pathways for TLR-mediated activation of PI3K. Depending on the cell population of interest, pro- as well as anti-inflammatory PI3K-dependent effects have been observed for the various TLRs investigated so far.³⁵ In WT preadipocytes where PI3K is equally activated in unstimulated and LPS-stimulated cells, inhibition of PI3K did not result in a significant change in IL-6 production. However, in the absence of leptin or leptin signaling, the inhibition of PI3K resulted in a profound suppression of IL-6. Further signaling pathways might be involved in these effects, because blocking of the PI3K pathway by the addition of wortmannin or Ly294002 only partially suppressed the IL-6 production in the *ob/ob* and *db/db* cells. However, these data strongly suggest that an elevated activation of PI3K mediated by

the absence of leptin signaling contributes to the observed differences.

After demonstrating the expression and responsiveness of a broad spectrum of pattern-recognition receptors on adipocytes and preadipocytes the question occurs, how do the pathogen-associated molecular patterns reach these cells? Bacterial translocation during experimental colitis as well as in Crohn's disease has been a known phenomenon for more than a decade.⁵⁴ Recent data support this concept by showing that the mesenteric adipose tissue from patients with Crohn's disease is colonized by bacterial flora.⁵⁵ Previous data from this group demonstrated a quantitative increase of the mesenteric fat tissue accompanied by an up-regulation of proinflammatory mediators.⁵⁶ From a phylogenetic point of view, the idea of the fat tissue being involved in the immune defense has been well described. For instance, in *Drosophila*, the prototypical example of the innate immune response, one mechanism contributing to the strong resistance to microbial infections is built by the transient synthesis of potent antimicrobial peptides, primarily produced by the fat.^{57,58} The novelty of the present study is the focused leptin-dependent characterization of isolated preadipocytes as well as adipocytes and not the fat tissue in total.

In conclusion, with the present study we provide evidence for the expression and responsiveness of TLR1 to -9 in murine preadipocytes and adipocytes, both strongly regulated by the adipokine leptin. Thus, these data further underline the regulatory function of leptin in the immune system and in addition contribute to the characterization of adipocytes and preadipocytes as members of the innate immune system.

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