The central regulatory role of the adipocyte in whole body energy homeostasis is well established. However, recent findings suggest that preadipocytes and adipocytes may play an important physiological role in the regulation of both the innate and adaptive immune response. To systematically characterize the molecular machinery of the adipocyte that mediates the recognition of pathogens, we have focused our analysis on the recently identified Toll-like receptors (TLRs). These receptors have been implicated as mediators of the cellular response to bacterial lipopolysaccharides (LPSs). Here, we report the cloning and functional characterization of mouse TLR-2 from 3T3-L1 adipocytes. TLR-2 synthesis is strongly induced in the adipocyte by LPS, TNFα, and the yeast cell wall extract zymosan. TLR-2 undergoes a lengthy intracellular maturation process with a half-life of exit from the ER of approximately 3 h. Furthermore, LPS treatment of adipocytes results in dramatic changes at the level of gene expression, including the synthesis of a distinct set of secretory proteins such as interleukin-6. Our studies demonstrate the presence of a fully intact pathway of innate immunity in the adipocyte that can be activated by LPS binding to the cell surface and results in the secretion of immunomodulatory molecules.

The recent identification of the Toll-like receptor-4 (TLR4) as the gene responsible for the LPS-hyporesensitive phenotype of the C3H/HeJ mouse (1) has drawn much attention to this newly identified mammalian receptor family that currently consists of at least eight members. TLR1–6 have been described in the literature (2, 3), and Beutler and colleagues recently deposited the sequences for TLR-7 and TLR-8 in GenBank (accession nos. NM_016562 and NM_016610). The founding member of the family, the Drosophila Toll protein, plays an essential role in embryonic development as a receptor for the maternal factor spaetzle and is critically involved in the establishment of dorsoventral polarity (4). Furthermore, it participates in the antifungal response in the adult fly (5). The fat body is the primary organ responsible for this humoral defense in the fly and secretes a battery of antimicrobial peptides in response to infection (reviewed in Ref. 6).

In mammals, we and others have demonstrated the expression of molecules related to the innate immune system in adipose tissue, such as complement factors D (adipsin), B, and C (7–9) as well as acute phase reactant proteins (10). Adipocytes also actively secrete and respond to inflammatory cytokines, such as TNFα, IL-1β, and IL-6 (11–13). A recent report by Marette and colleagues (14) demonstrated that systemic administration of LPS to rats markedly increased inducible nitric-oxide synthase mRNA and protein levels in white and brown adipose tissues. This effect was comparable with or greater than the induction of inducible nitric-oxide synthase in liver, kidney, or skeletal muscle. Loskutoff and colleagues (15) report that LPS induces plasminogen activator inhibitor 1 mRNA in adipose tissue. Many of these observations could be explained with a response to LPS via increased TNFα secretion from macrophages. This macrophage-borne TNFα in turn was thought to be responsible for the downstream effects triggered in the adipocyte. We wanted to test whether adipocytes can directly and acutely respond to systemic or local LPS. We therefore determined whether adipocytes express the two known "pattern recognition receptors" for bacterial and fungal cell wall components, TLR-2 and TLR-4. We found that TLR-4, but not TLR-2, is constitutively present in 3T3-L1 adipocytes. Interestingly, activation of TLR-4 with LPS results in the rapid induction of high levels of TLR-2 in these cells. This allowed us to use the adipocyte system to study the biogenesis of the endogenous TLR-2 receptor and to examine the effects of TLR-4 receptor activation at the gene expression level.

**EXPERIMENTAL PROCEDURES**

Materials—Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Cellgro Inc. EXPRE35S35S protein labeling reagent was purchased from NEN Life Science Products. DMEM lacking methionine was purchased from Cellgro Inc. EXPRE35S35S protein labeling reagent was purchased from NEN Life Science Products. DMEM lacking methionine, cysteine, and glutamate was purchased from ICN. Marine TNFα

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF165189.

1 The abbreviations used are: TNFα, tumor necrosis factor α; LPS, lipopolysaccharide; IL-6, interleukin-6; DMEM, Dulbecco’s modified Eagle’s medium; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; PBS, phosphate-buffered saline; C/EBP, CCAAT/enhancer-binding protein; PNGase F, peptide N-glycanase F.

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The Lipopolysaccharide-activated Toll-like Receptor (TLR)-4 Induces Synthesis of the Closest Related Receptor TLR-2 in Adipocytes*
and IL-6 were purchased from Pharmingen. LPS (from *E. coli*) and zymosan were purchased from Sigma. All other chemicals were purchased from Fisher. Cell wall preparations from *Cryptococcus neoformans* were a kind gift of Rena May and Matthew Scharff (Department of Cell Biology, Albert Einstein College of Medicine).

Cell Culture—Cell cultures were maintained in 75-cm² flasks (generous gift of Dr. Charles Rubin, Department of Molecular Pharmacology, Albert Einstein College of Medicine) were propagated and differentiated according to the protocol described in Ref. 16. In brief, the cells were propagated in FCS (Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (JRH Biosciences) and penicillin/streptomycin (100 units/ml each) for 12 days until they reached confluence (day 2). After 2 days (day 0), the medium was changed to DM1 (containing FCS and 160 nM insulin, 250 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine). Two days later (day 2), the medium was switched to DM2 (FCS containing 160 nM insulin). After another 2 days, the cells were switched back to FCS. NIH-3T3 cells were grown and propagated in DMEM containing 10% donor calf serum and antibiotics. J774 cells were grown in FCS and were a gift from Dr. Matthew Scharff.

Isolation of Mouse Adipocytes and Peritoneal Exudate Cells—Four days before the experiment, mice were injected intraperitoneally with 2 ml of a 4% solution of thioglycollate medium (Difco). Mice were sacrificed, the peritoneum was perfused with DMEM plus 1% bovine serum albumin, and cells were collected by centrifugation at 1000 × g. From the same mice, fat from the peritoneal cavity was isolated and propagated in DMEM. Adipocytes were then isolated as described in Refs. 11 and 17. Both peritoneal exudate cells and adipose tissue were then immediately incubated in DMEM lacking glucose and methionine and used for an in vivo labeling reaction with or without treatment with LPS or TNF.

Cloning of Mouse TLR-2—A cDNA clone for TLR-2 was isolated essentially as described in Ref. 18. In brief, a day 8 3T3-L1 adipocyte library in vector pCDNA1 (19) in MC1961 cells was subcloned in 96 pools, each pool representing 5000 individual clones. Bacteria were grown up overnight in 96-well plates in 200 μl of selective LB medium. Subsequently, 50 μl of culture medium from each well was pooled across each column and each row. The resulting 20 pools were then screened by PCR for the presence of a 400-bp fragment generated with primers based on a mouse EST sequence with strong homology to human TLR-2 (4). The primers used were 5′-CGCTCTTTGTTTTTCGCCAAC-3′ and 5′-ATGTTGAGGCTGATGCTCCT-3′, and were designed to amplify a 400-bp fragment from the mouse TLR-2 cDNA. 

mRNA Isolation, Northern Blot Analysis, and RT-PCR—Isolation of mRNA was as described in Ref. 19, as was analysis of electrophoresis of mRNA and its transfer to nylon membranes. Hybridizations were performed overnight at 42 °C in 50% formamide, 5 mM Denhardt’s solution, 1% SDS, and 0.1 mg/ml poly(A); the 32P-labeled DNA probes were used at concentrations of 2 × 106 cpm/ml. The filters were subsequently washed in 2× SSC plus 0.1% SDS and 0.1× SSC plus 0.1% SDS at 55°C before autoradiography. RT-PCR was performed on mRNA using a RT-PCR kit from Life Technologies, Inc. Primers used for PCR comprised the 5′ and 3′ regions of the open reading frame of mouse Cab45 (1.1-kilobase fragment) and mouse caveolin-2 (0.5-kilobase fragment) and a portion of the IL-6 coding region (5′ primer, CTT CCA TTC AGT TGG CTT CTT; 3′ primer, GCT TGG TCT TCT TTG TTA TCT). 

TLR-2 Expression Plasmids and Transient Transfections in 293-T Cells—TLR-2 was subcloned into vector pEF6/V5-His-Topo (Invitrogen) and used for transient transfection assays in 293-T cells. Plasmids were transiently transfected into 293-T cells (10-cm dishes) by the Effectene reagent (QIAGEN). 

Immunoprecipitation—Cells were lysed in TNET-OG buffer. When indicated, total cpm of protein-precipitated[35S]cys/Met were adjusted using labeled trichloroacetic acid-precipitable counts by mixing 50 μl of lysate with an equal volume of 80% acetone, 20% trichloroacetic acid solution and incubation on ice for 10 min followed by centrifugation at 15,000 × g for 5 min. Pellets were washed with 1 ml of acetone (−20 °C), and the centrifugation was repeated. Pellets were resuspended in 50 μl of 2% SDS, 100 μm Tris, pH 8.0, and cpm were determined by liquid scintillation counting. Lysates were preincubated by the addition of 50 μl of a 1:1 slurry of protein A-Sepharose (Amersham Pharmacia Biotech) in TNET buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 μM Tris, pH 8.0) containing 1 mg/ml bovine serum albumin. After 30 min at 4 °C, samples were centrifuged for 5 s at 15,000 × g, the supernatants were transferred to a fresh tube, and 50 μl of protein A-Sepharose was added together with the corresponding antiserum. Samples were then incubated for 3 h at 4 °C. Immunoprecipitates were washed six times in TNET buffer and analyzed by SDS-PAGE.

Deglycosylation of TLR-2—For Endo H digestions, immunoprecipitated TLR-2 was incubated in PBS and then resuspended in Endo H buffer (0.1 mM sodium citrate, pH 6.0, 1% SDS) and boiled for 5 min. For peptide N-glycans F (PNGase F) reactions, samples were resuspended in 0.5% SDS, 1% β-mercaptoethanol, and boiled for 5 min; and then supplemented with Nonidet P-40 (1% final concentration) and sodium phosphate buffer (50 mM final concentration; pH 7.0); samples were incubated for 2 h in the presence of 500 units of Endo H (New England Biolabs) or 1 unit of PNGase F (Roche Molecular Biochemicals) at 37 °C. Reactions were stopped by boiling in 2× sample buffer (250 mM Tris, pH 6.8, 4 mM EDTA, 4% SDS, 20% sucrose) and analyzed by SDS-PAGE.

Velocity Gradient Centrifugation—Cells were lysed in 300 μl of TNT buffer (TNET lacking EDTA) containing 60 mM octyl glucoside and 1% phenylmethylsulfonyl fluoride. Solubilized material was then layered on a 40-ml, 5–20% linear sucrose gradient (4.0 mM sodium citrate, 0.5% deoxycholate, and 1 mg/ml bovine serum albumin), and centrifuged at 60,000 rpm (−340,000 × g) for 7 h in a SW 60 rotor (Beckman Instruments, Palo Alto, CA). Note that the gradient was prepared with TNT buffer containing the appropriate amount of sucrose and LPS where indicated. After centrifugation, 13 80-μl gradient fractions were collected from the top of the gradient.

Antibodies—The antibodies to GDP dissociation inhibitor were generous gifts from Dr. Perry Bickel (Washington University, St. Louis). A fusion protein construct was generated in pMALc (New England Biolabs) comprising the cytoplasmic tail of TLR-2. Antibodies were prepared in rabbits using the facility at Covance Inc. (Denver, PA). Anti-FLAG M2 antibodies were purchased from Sigma.

Immunoblotting—After SDS-PAGE, proteins were transferred to BA83 nitrocellulose (Schleicher & Schuell). Nitrocellulose membranes were blocked in PBS or Tris-buffered saline with 0.1% Tween 20 and 5% nonfat dry milk. Primary and secondary antibodies were diluted in PBS or Tris-buffered saline with 0.1% Tween 20 and 1% bovine serum albumin. Bound antibodies were detected by enhanced chemiluminescence according to the manufacturer’s instructions (NEN Life Science Products).

Two-dimensional Gel Analysis—Isoelectric focusing followed by SDS-PAGE was performed as described previously (20) on a Hoefer GT-1 tube gel apparatus.

RESULTS

Cloning and Expression of Mouse TLR-2 from 3T3-L1 Adipocytes—Since the sequence for mouse TLR-2 was not available at the beginning of this project, we decided to obtain a full-length version of TLR-2 from a 3T3-L1 adipocyte library. We employed a PCR-screening strategy described by Takumi and Lodish (18) to isolate a mouse cDNA clone. The sequence of TLR-2 is 70% conserved with its human homolog and has been deposited in GenBank™ with accession no. AF165189. While this manuscript was in preparation, the sequence for mouse TLR-2 was also reported by Heine and colleagues (22).

Fig. 1A shows the tissue distribution of TLR-2 mRNA. Poly(A)+ RNA was isolated from healthy mice. Northern analysis was performed using a probe comprising the open reading frame of the mouse TLR-2 cDNA. A number of tissue, including spleen, smooth muscle, and lung and to a lesser extent ing spleen, smooth muscle, and lung and to a lesser extent
Northern blot of mRNA isolated from the cell lines and tissues

The reactions were subsequently chased for 1 h in the presence of cycloheximide and excess unlabeled Cys/Met. Cells were lysed and immunoprecipitated with anti-FLAG antibodies. Immunoprecipitates were either treated with Endo H or PNGase F and pulse-labeled as indicated in A. However, the chase time was extended to 14 h in absence of cycloheximide but in presence of excess unlabeled Cys/Met. Cells were then lysed and immunoprecipitated with either nonimmune rabbit polyclonal antiserum, the rabbit polyclonal anti-FLAG antibodies. Where indicated, immunoprecipitates were treated with Endo H.

Expression of TLR-2 in 293 Cells: Analysis of N-Linked Carbohydrate Modifications—To determine whether the TLR-2 clone isolated from 3T3-L1 adipocytes encodes a full-length protein with a functional signal sequence capable of translocating the protein into the luminal portion of the secretory pathway, the cDNA for TLR-2 was carboxyl-terminally tagged with the FLAG epitope and subcloned into vector pEF6/V5-His toxin (10). To test whether TLR-2 expression is inducible or not, we treated 3T3-L1 adipocytes for various times with TNFα. After the TNFα treatment, cells were briefly pulsed with [35S]Cys/Met, and cells were lysed and immunoprecipitated with anti-FLAG antibodies. The reactions were subsequently chased for 1 h in the presence of cycloheximide and excess unlabeled amino acids. Cells were then lysed and immunoprecipitated with anti-FLAG antibodies. To determine whether TLR-2 undergoes post-translational modifications with N-linked carbohydrates, immunoprecipitates were divided and treated with either endoglycosidase H (Endo H) or PNGase F and analyzed by SDS-PAGE and autoradiography. Fig. 2A shows that 293-T cells indeed produce a protein of the estimated molecular weight that is completely sensitive to Endo H and PNGase F, suggesting that the bulk of the transiently expressed protein resides in a pre-Golgi compartment.

To be able to study endogenous TLR-2, we generated polyclonal antibodies against the cytoplasmic tail of the receptor. Fig. 2B shows an experiment similar to the one shown in Fig. 2A, except that the chase period was extended to 14 h to allow the TLR-2 to reach its final intracellular destination. Another labeling reaction was performed in parallel with 3T3-L1 adipocytes. Lysates were then immunoprecipitated with anti-FLAG or anti-TLR-2 antibodies and, when indicated, treated with Endo H. Both anti-FLAG and anti-TLR-2 antibodies immunoprecipitate a protein of similar electrophoretic mobility from 293-T cells as from 3T3-L1 adipocytes. Surprisingly, even after 14 h of chase, the vast majority of TLR-2 remains in a pre-Golgi compartment in 293-T cells.

TNFα Induces TLR-2 Expression in 3T3-L1 Adipocytes—3T3-L1 adipocytes are highly responsive to TNFα. Many groups have demonstrated the effects of TNFα on the insulin signal transduction cascade in which TNFα acts as an insulin desensitizer (23–25). At the gene expression level, we have previously demonstrated that TNFα induces a host of secretory proteins while repressing the expression of others (10). To test whether TNFα has an effect on TLR-2 expression, we treated 3T3-L1 adipocytes for various times with TNFα. After the TNFα treatment, cells were briefly pulsed with [35S]Cys/Met, and cells were lysed and immunoprecipitated with anti-FLAG-2 (Fig. 3A). 3T3-L1 adipocytes induce TLR-2 with maximal induction observed after approximately 3 h. These observations were confirmed by Western blot analysis on uninduced and TNFα-induced cells. This confirmed that 3T3-L1 adipocytes have very low levels of TLR-2 present in the uninduced state that cannot be detected by Western blot analysis. This is in contrast to adipose tissue (Fig. 1) that expresses detectable mRNA levels for TLR-2 even in the uninduced state.

Endogenous TLR-2 Has an Intracellular Half-life of Approximately 3 h before Reaching the Cell Surface in 3T3-L1 Adipocytes—Our initial observations in 293-T cells with a transiently transfected TLR-2 construct suggested that TLR-2 is post-translationally modified by N-linked carbohydrates. Additionally, using Endo H resistance, we determined that the bulk of TLR-2 remains in a pre-Golgi compartment in 293-T cells. To determine the fate of endogenously expressed TLR-2 in 3T3-L1
adipocytes, cells were pretreated with TNFα for 1.5 h and then pulse-labeled with [35S]Cys/Met for 30 min (Fig. 4). The cells were subsequently chased in presence of cycloheximide for various lengths of time and analyzed for resistance to Endo H treatment. Similar to the situation in 293-T cells, endogenous TLR-2 is N-glycosylated in adipocytes as well. TLR-2 undergoes a lengthy intracellular maturation process as judged by the kinetics with which it acquires resistance to Endo H, with a half-life of maturation of approximately 3 h. Note that a doublet is observed at later chase points in the untreated samples with the lower band corresponding to the Endo H-resistant form.

Comparison of TLR-2 Induction by TNFα in 3T3-L1 Fibroblasts and Adipocytes, NIH-3T3 Fibroblasts, and J774 Macrophages—Northern blot analysis suggested significant expression of TLR-2 in adipose tissue. While it is difficult to precisely gauge the significance of the systemic contributions of TLR-2 expressed in adipose tissue, we have undertaken a qualitative assessment of TLR-2 induction in response to TNFα in various cell lines. NIH-3T3 fibroblasts (an uncommitted, undifferentiated cell line) were compared with 3T3-L1 fibroblasts in both the pre- and postdifferentiation state as well as J774 cells that represent a widely used model macrophage cell line. All cells were pretreated for the indicated lengths of time with TNFα and subsequently pulse-labeled for 1 h with [35S]Cys/Met.

**Fig. 3**. TNFα induces the expression of TLR-2 in 3T3-L1 adipocytes. A, 3T3-L1 adipocytes were exposed for the indicated length of time to 5 nM TNFα in standard growth medium. Cells were subsequently labeled for 30 min with [35S]Cys/Met and then immunoprecipitated with anti-TLR-2 antibodies. Immunoprecipitates were analyzed by SDS-10% PAGE. B, 3T3-L1 adipocytes were maintained in standard growth medium. To one sample, TNFα was added for 3 h at a final concentration of 5 nM. At the end of the incubation period, cells were washed in PBS, lysed in 5× sample buffer, and analyzed by SDS-10% PAGE and Western blotting with anti-TLR-2 antibodies.

**Fig. 4**. Maturation of TLR-2 in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pretreated for 2 h with 5 nM TNFα in DMEM lacking Cys/Met to induce TLR-2 production. The cells were then labeled with [35S]Cys/Met for 15 min and then chased in presence of excess unlabeled Cys/Met and 300 μg cycloheximide. Cells were lysed at the indicated time points and immunoprecipitated with anti-TLR-2 antibodies. Immunoprecipitates were digested with Endo H where indicated and analyzed by SDS-10% PAGE. Two different exposures are shown.
cytes and J774 macrophages in response to LPS shows a more rapid up-regulation of TLR-2 in the adipocyte compared with the macrophage, suggesting functional differences in the response to LPS in the two cell types (Fig. 6B). Additionally, the fact that NIH 3T3 cells fail to induce TLR-2 despite significant levels of TLR-4 mRNA (Fig. 1B) is surprising. Possibly, mRNA levels for TLR-4 may not reflect protein levels in the various cell types. Alternatively, additional cell type-specific factors may be needed for the downstream response that are present in macrophages and (pre)adipocytes but are not available in NIH 3T3 fibroblasts.

In our pulse-labeling assay, we are looking only at de novo synthesized TLR-2. To test whether LPS stimulation of TLR-2 synthesis also requires de novo transcription, we included the transcriptional inhibitor actinomycin D during incubation of 3T3-L1 adipocytes with LPS. Fig. 6C shows that the presence of actinomycin D inhibits the induction of TLR-2, suggesting that TLR-4 activation triggers a transcriptional event that leads to TLR-2 induction. In addition, we tested whether a known activator of TLR-2, zymosan (a yeast cell wall preparation), or GXM, (a cell wall preparation from *C. neoformans*) was able to trigger TLR-2 induction within a similar time frame. Treatment at this short time scale with either of these preparations did not result in induction of TLR-2. This is in agreement with our observation that in unstimulated cells TLR-2 is not present at significant levels. Fig. 6D demonstrates that the LPS-triggered TLR-2 induction occurs even at very low LPS concentrations in 3T3-L1 adipocytes, with changes in TLR-2 expression triggered between 1 and 10 ng/ml LPS, reaching a plateau at concentrations higher than 100 ng/ml under the conditions chosen.

Up-regulation of TLR-2 in 3T3-L1 Adipocytes Can Also Be Achieved by TLR-2 Activation, Albeit with Slower Kinetics—Fig. 6C has demonstrated that acute treatment of 3T3-L1 adipocytes with TLR-2 ligand zymosan does not lead to the up-regulation of TLR-2 expression. We wanted to test whether prolonged incubation with zymosan could trigger up-regulation of TLR-2. The rationale is that low basal level expression of TLR-2 present in the unstimulated state may up-regulate TLR-2 expression at low levels. Newly synthesized TLR-2 that reaches the cell surface may in turn cause a burst of TLR-2 induction in the continued presence of zymosan. Since we have shown that newly synthesized TLR-2 requires more than 3 h to appear on the cell surface, we would expect a delayed but sudden burst of TLR-2 induction concomitant with the rate of maturation of the *de novo* synthesized TLR-2. This should be followed by rapid down-regulation of *de novo* synthesis similar to the phenomenon observed for TLR-4-triggered TLR-2 up-regulation. Fig. 7 shows that this indeed the case. We have assayed TLR-2 production in this case by immunoprecipitation of lysates from treated cells with anti-TLR-2 antibodies followed by SDS-PAGE and Western blot analysis with anti-

![Fig. 6. LPS-induced expression of TLR-2 in fibroblast, adipocyte and macrophage cell lines. A, 3T3-L1 adipocytes and NIH 3T3 fibroblasts were treated as described in the legend to Fig. 5 except that 10 ng/ml LPS was used as a stimulus instead of TNFα. B, comparison of induction kinetics of LPS-triggered TLR-2 induction between 3T3-L1 adipocytes and J774 macrophages. Note that TLR-2 is more rapidly induced in 3T3-L1 adipocytes. C, induction of TLR-2 in 3T3-L1 adipocytes by various ligands. Cells were stimulated for 1 h in standard growth medium containing 100 ng/ml LPS, 10 nm TNFα, 30 μg/ml zymosan, or 8 μg/ml GXM. When indicated, cells were pretreated for 30 min with actinomycin D at 2.5 μg/ml. Cells were subsequently pulse-labeled with [35S]Cys/Met for 1 h, lysed, and immunoprecipitated with anti-TLR-2 antibody. D, dose response to LPS. 3T3-L1 adipocytes were pretreated for 1 h with the indicated concentrations of LPS in standard growth medium. Cells were then pulse-labeled for 30 min, lysed, and immunoprecipitated with anti-TLR-2 antibodies.](image)
TLR-2. Therefore, in contrast to the experiments involving metabolic labeling with \[^{35}\text{S}]\text{Cys/Met}\) that reflect the rate of synthesis, we are measuring the total accumulation of TLR-2 in this case. The top panel shows the Western blot of TLR-2, and the bottom panel shows a quantitation of the experiment. LPS triggers a much more linear response for TLR-2 induction, whereas zymosan treatment causes a delayed response with an acute burst of TLR-2 induction between 3 and 6 h. By 12 h, the LPS-treated cells start to clear TLR-2 as judged by the decreased overall levels of TLR-2 at later time points.

**Induction of TLR-2 Is Not Limited to 3T3-L1 Adipocytes in Tissue Culture but Can Also Be Detected in Adipose Tissue**—The Northern blot data shown in Fig. 1 demonstrates that TLR-2 is constitutively expressed in adipose tissue. To determine whether TLR-2 expression can be induced in \textit{bona fide} mouse adipocytes, we treated adipocytes isolated from a visceral fat pad for 3 h with LPS and TNF\(\alpha\) in the presence of \[^{35}\text{S}]\text{Cys/Met}\). As a control, peritoneal exudate cells were isolated from the same animals and treated in parallel. After the labeling reaction, cells were lysed and immunoprecipitated with anti-TLR-2 antibodies. Both adipocytes and peritoneal exudate cells induced the expression of TLR-2 in response to either LPS or TNF\(\alpha\) (Fig. 8). Surprisingly, freshly isolated adipocytes expressed TLR-2 even in the unstimulated state at levels much higher than observed for uninduced 3T3-L1 adipocytes. Since the TLR-2 induction is not limited to the 3T3-L1 tissue culture system, these experiments suggest that TLR-2 induction in adipose tissue may play an important physiological role \textit{in vivo}.

**LPS Binding Induces Complex Formation of TLR-2**—To test whether binding of LPS induces changes in the oligomerization state of TLR-2, the size of the TLR-2 complex was estimated by velocity sedimentation analysis. Cells were labeled for several hours with \[^{35}\text{S}]\text{Cys/Met}\) in the presence of TNF\(\alpha\) to achieve high level expression of receptor on the cell surface. Subsequently, the cells were incubated in 10% fetal calf serum, either in the presence or in the absence of LPS for 10 min. Extracts were prepared and immediately subjected to velocity sedimentation analysis (Fig. 9). The addition of LPS induces a significant shift in the size distribution of TLR-2 complex toward a larger molecular weight complex, suggesting that activation of

**FIG. 7.** LPS and zymosan trigger induction of TLR-2 in 3T3-L1 adipocytes with different kinetics. 3T3-L1 adipocytes were incubated in growth medium with either 100 \(\text{ng/ml}\) LPS or 30 \(\mu\text{g/ml}\) zymosan. At the indicated times, cells were lysed, immunoprecipitated with anti-TLR-2 antibodies, and analyzed by SDS-PAGE and Western blotting with anti-TLR-2 antibodies (top panel). The signal for TLR-2 was integrated on a Molecular Dynamics ChemiImager and plotted against the time (in hours) after the addition of LPS and zymosan, respectively.

**FIG. 8.** TNF\(\alpha\)- and LPS triggered induction of TLR-2 adipocytes and peritoneal exudate cells. Primary adipocytes and peritoneal exudate cells were harvested as indicated under “Experimental Procedures.” Cells were labeled for 3 h with \[^{35}\text{S}]\text{Cys/Met}\), lysed, and immunoprecipitated with anti-TLR-2 antibodies. Note that unlike 3T3-L1 adipocytes, primary adipocytes express a significant level of TLR-2 in the unstimulated state.
TLR-4 may recruit TLR-2 into a complex independent of the TLR-2 activation state.

Changes in Gene Expression Associated with Prolonged LPS and TNF Treatment of 3T3-L1 Adipocytes—In order to test whether chronic treatment of adipocytes with LPS triggers changes in gene expression patterns beyond the induction of TLR-2, we examined the effects of LPS on the total complement of secretory proteins released from adipocytes. As a comparison, we also examined the effects of other inflammatory cytokines, such as TNFα and IL-6, on adipocytes as well (Fig. 10). Cells were subjected for 12 h to treatment with LPS or the respective cytokine and were then pulse-labeled with [35S]Cys/Met for 30 min. The cells were subsequently chased for 2 h in the presence of excess unlabeled amino acids. The supernatants were collected and analyzed by isoelectric focusing followed by SDS-PAGE. LPS, TNFα, and IL-6 all dramatically influence the pattern of secretory proteins released from adipocytes, causing the induction of a number of overlapping proteins as well as factor-specific gene products.

In order to determine whether LPS treatment of adipocytes leads to the induction of specific inflammatory cytokines such as IL-6, we performed RT-PCR assays on mRNA isolated from 3T3-L1 cells treated with LPS and, as positive controls, TNFα and IL-6 (26) (Fig. 11). LPS, TNFα, and IL-6 all trigger the synthesis of IL-6 with kinetics similar to the induction observed for TLR-2. As internal controls, we have used a set of primers specifically amplifying Cab45 (27) within the same reaction. Unlike IL-6, Cab45 mRNA is expressed constitutively and is down-regulated upon prolonged LPS incubation. Using the same cDNA sample, we have also amplified the cDNA for caveolin-2 (28) in a separate reaction with no significant effect observed after LPS, TNFα, or IL-6 treatment. Combined, these results suggest that IL-6 mRNA expression is specifically up-regulated in response to LPS in the mature 3T3-L1 adipocyte.

**Discussion**

**Drosophila** Toll and the mammalian Toll-like receptors share a high level of sequence homology in both their extracellular and their intracellular domains. The extracellular domains contain a number of leucine-rich repeats and have a cysteine-rich cluster in the juxtamembrane region. The cytoplasmic domains are not always conserved within the Toll family but also share homology with the cytoplasmic domain of the IL-1 receptor as well as MyD88, a cytoplasmic adaptor molecule that is essential for IL-1 receptor signaling (29, 30).

By now, it is well established that TLR-4 is the receptor for LPS. Poltorak et al. (1) initially identified a mutation in TLR-4 that leads to decreased sensitivity to LPS. This has been substantiated by Hoshino et al. (31), who looked at mice carrying a complete deletion of TLR-4. Additionally, two recent reports (32, 33) demonstrated that TLR-2-deficient mice respond normally to LPS, further underlining the concept that TLR-4 is the primary receptor for LPS. TLR-2, in contrast, has been mainly implicated as the receptor for Gram-positive bacterial cell wall components and fungal cell wall components (34, 35). There is a general consensus in the recent literature that this model for the “division of labor” between these two receptors with respect to the ligands responsible for their activation is indeed correct, at least at the level of physiological concentrations of the ligands.

We have chosen to study Toll-like receptors in the adipocyte for several reasons. First and foremost, it permits the further characterization of the adipocyte beyond its role in energy homeostasis and allows us to focus on its contribution toward the innate immune response. Second, we can take advantage of the observation that endotoxin stimulation of adipocytes induces high levels of TLR-2. This allows us for the first time to follow the intracellular fate of endogenous TLR-2. We demonstrate that TLR-2 undergoes N-linked glycosylation and use this modification to determine the life span of newly synthesized receptor in a pre-Golgi environment, using resistance to endoglycosidase H as a criterion for exit from the endoplasmic reticulum. We find that it takes 3–3.5 h for half of the newly made endogenous TLR-2 receptors in 3T3-L1 cells to travel beyond the cis-Golgi compartment. This is an indication that the receptor may undergo a lengthy assembly and/or modification process. Even more strikingly, transient transfection of 293T cells with TLR-2 results in extremely poor transport of receptor to the cell surface even after extended chase times. Since transiently transfected 293T cells have been used extensively in the characterization of these receptors, such overexpression artifacts have to be taken into account when studying these receptors and the corresponding signal transduction pathways in heterologous systems.

Since TLR-2 is not the LPS-CD14 receptor, our finding that TLR-2 shifts to a higher molecular weight complex upon stimulation with LPS is surprising. One possible interpretation is that TLR-4 recruits TLR-2 into a complex. Alternatively, TLR-4 activation could result in the activation of intracellular effectors, which could, in turn, associate with TLR-2. Future studies will have to elucidate the mechanism of signal transduction and possible cross-talk between these two receptors. However, our findings that the TLR-4 receptor can induce expression of TLR-2 and that TLR-2 activation can also induce further TLR-2 synthesis suggest that the expression of these receptors is governed by common regulatory factors. The kinetic differences we observe for TLR-2 induction depending on whether we use a TLR-4 ligand (LPS) or a TLR-2 ligand (zymosan) are fully consistent with the proposed “division of labor” for these two receptors.

Our observation that LPS but not zymosan induces TLR-2 expression acutely is consistent with the notion that TLR-4 but not TLR-2 is constitutively present on the cell surface of the 3T3-L1 adipocyte. TLR-4 activation then results in induction of TLR-2, and this newly synthesized TLR-2 reaches the cell surface, where it may be used to further increase signaling. Interestingly, a recent report by Fenton and colleagues (36) demonstrates that CD14 can activate both TLR-2 and TLR-4, depending on the CD14-associated ligand; LPS bound to CD14 employs the TLR-4 pathway, while another mycobacterial cell wall component that also binds CD14, lipooligosaccharides, activates TLR-2.

We also observe a relatively rapid desensitization of TLR-2 induction upon LPS stimulation. Peak levels for LPS-triggered
TLR-2 induction are reached within 1 h and subsequently decrease, suggesting the existence of a mechanism for negative feedback inhibition. In macrophages, the key mediator for the proinflammatory response, NF-κB, is activated by TLR-2, TLR-4, and TNFα. Activation of NF-κB also leads to the transcriptional activation of IkB, which in turn leads to the cytoplasmic sequestration of NF-κB. It will be interesting to see whether in the case of the adipocyte, the transcriptional events that lead to TLR-2 induction are also mediated by NF-κB. Clearly, induction of TLR-2 is not limited to TLR-4 activation via LPS. TNFα treatment results in a similar level of activation, suggesting that multiple signal transduction pathways converge at the level of TLR-2 induction. In this context, it is important to bear in mind that adipocytes are sensitive to LPS at the level of nanograms per milliliter. This surprising sensitivity of the adipocyte may have important consequences for the interpretation of results obtained in the context of bacterially produced ligands that have not been tested for the presence of endotoxin, since LPS triggers the activation of a large number of intracellular signaling cascades in 3T3-L1 adipocytes.2

What is the physiological role of TLR-2 and TLR-4 expression in the adipocyte? In many respects, the adipocyte has features of an immune cell. It expresses high levels of both TNFα receptors and secretes TNFα. Beyond its function as an immune modulator, there is increasing evidence that implicates TNFα as one of the key factors involved in obesity-induced insulin resistance (11, 37, 38). Adipocytes secrete high levels of complement factors B, C3, and D (7, 8). Much like TNFα, these factors serve a dual role in the immune system as well as in energy homeostasis; while they are part of the complement fixation cascade, they are also part of an autocrine activity termed “acylation stimulation protein” that stimulates triacylglycerol synthesis in adipocytes (39, 40). Adipocytes also express significant levels of C/EBPb and C/EBPd. These factors play a crucial role in adipogenesis (41). In the liver, C/EBPb was originally known as NF-IL-6 because it was identified as a transcription factor that bound to the interleukin-1-responsive element in the promoter of the IL-6 gene. NF-IL-6/C/EBPb as well as C/EBPd are critically involved in the acute phase response, an evolutionarily conserved reaction to a wide range of inflammation stimuli (42, 43). Cytokines and signaling molecules are produced and secreted by macrophages, fibroblasts, and epithelial cells at the site of trauma. Interleukin-1 and -6 are two of the primary cytokine mediators of the acute phase response. Adipocytes have significant levels of IL-1 and -6 receptors. Combined with the high C/EBPβ levels found in the adipocyte, it is not surprising that these cells are capable of producing high levels of acute phase reactant proteins (10). Mattacks et al. (44) concluded that the adipocytes surrounding lymph nodes are actively involved in local, transient immune responses.

There are many reports in the literature describing the relationship of LPS and leptin during infection. Leptin is a key cytokine predominantly expressed in adipose tissue and involved in energy homeostasis (45). After administration of LPS, leptin gene expression and leptin protein levels are increased.

2 Y. Lin, H. Lee, A. H. Berg, M. P. Lisanti, L. Shapiro, and P. E. Scherer, unpublished observations.
Induction of leptin during the host response to infection may therefore contribute to the anorexia of infection (46, 47). On the other hand, Diehl and colleagues (48) have shown that leptin up-regulates both phagocytosis and the production of proinflammatory cytokines, thereby up-regulating the inflammatory immune responses. All of these results are consistent with the emerging view that leptin is a key hormone coupling immune system activity to energy balance.

It is apparent that many secretory products of the adipocyte, including TNFα, various complement factors, and leptin, all serve dual roles in energy homeostasis and the immune response. The observation that adipocytes express the receptor for LPS and induce the receptor for fungal and Gram-positive cell wall components in response to Gram-negative components at levels comparable with macrophages fills an important gap. It is apparent that adipocytes are involved even at the earliest stages of the immune response, capable of directly sensing the presence of bacterial cell wall components. Upon stimulation, adipocytes react with a general change in the pattern of secretory cell wall components to levels comparable with macrophages, thereby contributing to the anorexia of infection (46, 47). On the other hand, Diehl and colleagues (48) have shown that leptin therefore contribute to the anorexia of infection (46, 47).

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