CD1d-mediated Presentation of Endogenous Lipid Antigens by Adipocytes Requires Microsomal Triglyceride Transfer Protein*

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Background: Natural killer T (NKT) cells in adipose tissue (AT) contribute to whole body energy homeostasis.

Results: Lipid antigen presentation genes, including microsomal triglyceride transfer protein (MTP), are switched on during adipocyte differentiation, and affect iNKT cell activity.

Conclusion: Adipocytes can communicate with iNKT cells by presenting endogenous and exogenous lipid antigens.

Significance: Unraveling adipocyte-iNKT cell communication may help to fight obesity-induced AT dysfunction.

Obesity-induced adipose tissue (AT) dysfunction results in a chronic low-grade inflammation that predisposes to the development of insulin resistance and type 2 diabetes. During the development of obesity, the AT-resident immune cell profile alters to create a pro-inflammatory state. Very recently, CD1d-restricted invariant (i) natural killer T (NKT) cells, a unique subset of lymphocytes that are reactive to so called lipid antigens, were implicated in AT homeostasis. Interestingly, recent data also suggest that human and mouse adipocytes can present such lipid antigens to iNKT cells in a CD1d-dependent fashion, but little is known about the lipid antigen presentation machinery in adipocytes. Here we show that CD1d, as well as the lipid antigen loading machinery genes pro-saposin (Psap), Niemann Pick type C2 (Npc2), α-galactosidase (Gla), are up-regulated in early adipogenesis, and are transcriptionally controlled by CCAAT/enhancer-binding protein (C/EBP)-β and -δ. Moreover, adipocyte-induced Th1 and Th2 cytokine release by iNKT cells also occurred in the absence of exogenous ligands, suggesting the display of endogenous lipid antigen-D1d complexes by 3T3-L1 adipocytes. Furthermore, we identified microsomal triglyceride transfer protein, which we show is also under the transcriptional regulation of C/EBPβ and -δ, as a novel player in the presentation of endogenous lipid antigens by adipocytes. Overall, our findings indicate that adipocytes can function as non-professional lipid antigen presenting cells, which may present an important aspect of adipocyte-immune cell communication in the regulation of whole body energy metabolism and immune homeostasis.

Although previously regarded as a neutral lipid storage organ, adipose tissue (AT) is now recognized as a site where the immune system and metabolic cascades intersect (1–3). For example, obesity-induced adipocyte dysfunction has been shown to alter the AT-resident immune cell profile (i.e. more pro-inflammatory) and promote the release of inflammatory cytokines, which impair insulin receptor signaling and result in metabolic derangements (4–7). These findings underscore the importance of the complex interplay between adipocytes and immune cells in local and systemic regulation of energy homeostasis and prevention of inflammation.

AT-resident immune cells include macrophages, neutrophils, mast cells, B-lymphocytes, CD8+ and CD4+ Th1 cells, dendritic cells, regulatory T cells (Treg), and eosinophils. More recently, natural killer T (NKT) cells were demonstrated in AT (8). NKT cells are a T lymphocyte lineage with both innate and adaptive properties, as they can rapidly produce effector cytokines, yet are stimulated via the T cell receptor (9–11). A considerable fraction of NKT cells express an invariant αβ T cell receptor (in mouse harboring the Vα14 and in human the Vα24 chain) that responds to glycolipid antigens when displayed in complex with the non-classical MHC-like molecule CD1d (12–14). These cells are named invariant NKT (iNKT) cells and are enriched in liver, and present in considerable numbers in other tissues such as thymus, spleen, bone marrow, peripheral blood, and gastrointestinal mucosa. iNKT cells are also present in significant numbers in mouse and human AT, where they represent a significant proportion of resident T lymphocytes (10–20%) (15–19). Upon antigen stimulation, using the synthetic CD1d ligand α galactosylceramide (αGalCer), iNKT cells within hours secrete both Th1 cytokines, including interferon γ (IFNγ) and interleukin (IL)-2, and Th2 cytokines, including IL-4, IL-10, and IL-13 (8).

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3 The abbreviations used are: AT, adipose tissue; NKT, natural killer T cells; iNKT, invariant NKT; MTP, microsomal triglyceride transfer protein; PPAR, peroxisome proliferator activated receptor; RXR, retinoid X receptor; APC, antigen-presenting cell; FABP, fatty acid-binding protein.
αGalCer is the most potent iNKT cell exogenous ligand that was originally identified from a screening in extracts of a marine sponge (20–22). Importantly, iNKT cells also recognize endogenous lipid self-antigens (β-linked glycolipids), among which several β-glucosylceramides were recently identified (23). In the absence of external stimuli, AT-resident iNKT cells exhibit a Th2-biased cytokine profile (e.g. high IL-4 production) as compared with spleen iNKT cells (19). AT-resident iNKT cells are depleted with increased adiposity (15–19). Recent studies showed the ability of iNKT cells to exert pathogenic and protective effects on AT function and metabolism (15–19, 24–28). Interestingly, recent evidence suggests that adipocytes are able to present lipid antigens to iNKTs in a CD1d-dependent manner, and can therefore be considered non-professional lipid APCs (19, 29). It is, however, not understood which proteins in adipocytes facilitate the CD1d lipidation and presentation pathway, and how regulation is established to produce and present lipid self-antigens in an immunogenic manner that stimulates T cell responses. In addition, information on the regulation of the CD1d gene (and genes involved in CD1d lipidation) in adipocytes is limited.

A candidate protein to be involved in CD1d-mediated presentation of endogenous lipid antigens in adipocytes is microsomal triglyceride transfer protein (MTP). MTP has two isoforms: a canonical isoform, referred to as MTP-A, and a second isoform referred to as MTP-B or MTPv1, which contains a unique first exon located ~2.7 kilobases upstream of MTP-A exon 1 (30, 31). As a consequence, MTP-A and -B contain 20 or 35 unique amino acids at their N terminus, respectively. MTP-A is the predominant isoform in mouse liver and intestine (>95%) and is known for its role in transferring phospholipids, triglycerides, and cholesterol to apoB in hepatocytes and other cells involved in apolipoprotein homeostasis (32). MTP-B is found in professional APCs, where it is required for loading of endogenous and exogenous (e.g. αGalCer) lipids onto CD1d molecules in the endoplasmic reticulum and endosomes (31, 33). In addition, MTP-B-mediated lipid transfer in thymocytes is essential for NKT cell development (31). When over-expressed, both MTP isoforms behave similarly in triglyceride transfer and apoB-containing lipoprotein assembly (31). Similar to professional APCs, mouse adipocytes predominantly express MTP isoform B (>90%), which localizes primarily to the Golgi complex (30). Its function in adipocytes has not been addressed experimentally, but it has been proposed that MTP plays a role in lipid droplet formation upon maturation (34). The co-localization of MTP-B with the glucosylceramide synthesis pathway (which provides candidate iNKT cell lipid self-antigens) in adipocytes, together with the involvement of the MTP in CD1d lipidation in professional APCs, prompted us to investigate whether MTP-B may be involved in generation of antigenic lipid-CD1d complexes in adipocytes.

Here, we show that mouse 3T3-L1 adipocytes harbor a functional machinery for CD1d-mediated lipid presentation. CD1d, as well as the antigen loading machinery genes Psap, Npc2, and Gla are up-regulated in early adipogenesis, and are transcriptionally controlled by C/EBPβ and -δ. Interestingly, adipocyte-induced cytokine release by iNKT cells occurred in the absence of exogenous ligand, suggesting the production of endogenous lipid antigens by 3T3-L1 adipocytes. Furthermore, we identified MTP-B, which is also under the transcriptional regulation of C/EBPβ and -δ, as an essential player in the presentation of such endogenous lipid antigens. Overall, our findings support the view that adipocytes, which are engaged in dynamic lipid metabolism, can function as non-professional lipid APCs, which may present an important aspect of adipocyte-immune cell communication in the regulation of whole body energy metabolism and maintenance of immune homeostasis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dexamethasone and isobutylmethylxanthine were from Sigma. αGalCer was from Avanti Polar Lipids (Instruchemie). Anti-C/EBPβ (sc-150) and anti-C/EBPδ (sc-636) from Santa Cruz Biotechnology were used for ChIP assays and Western blotting. The following antibodies were used: anti-FABP4 (sc-18661) from Santa Cruz Biotechnology, anti-MTP (612022) from BD Transduction Laboratories, anti-CD1d mAbs from DBA2 (DB12022) from BD Transduction Laboratories, anti-tubulin (T9026) from Sigma. RNAI Max was purchased from Invitrogen. IFNγ ELISA kit (BD551866) was from BD Biosciences and IL4 ELISA kit (807044) was from eBioscience (Vienna, Austria). The Vα14 self-ligand and αGalCer-reactive DN32.D3 and DN29.3E iNKT hybridomas were a kind gift of Prof. A. Bendelac (35). BMS212122 was kindly provided by Dr. J. Edwards, Bristol-Myers Squibb.

**Cell Culture and Differentiation**—The murine 3T3-L1 cell line (ATCC, Manassas, VA) was cultured in DMEM Glutamax (Dulbecco) containing 10% bovine serum (Invitrogen), penicillin and streptomycin (both 100 μg/ml; Invitrogen). For differentiation, 3T3-L1 cells were grown to confluence and after 2 days incubated with culture medium containing dexamethasone (250 nM), 3-isobutyl-1-methylxanthine (500 μM), and insulin (170 nM) for 3 days. On day 3, medium was changed for culture medium supplemented with insulin (170 nM) and left for 4 days. Primary adipocytes were prepared by the fractionation of mouse epididymal AT as described before (19). For Western blot analyses, differentiated 3T3-L1 cells were lysed in RIPA lysis buffer (200 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% Triton X-100, 10 mM EDTA, 150 mM NaCl, 1% sodium deoxycholate containing protease inhibitors). Cell lysates were subjected to SDS-PAGE, and transferred to Immobilon membranes (Millipore). ECL Plus (PerkinElmer Life Sciences) was used for detection on an ImageQuant LAS 4000 (GE Healthcare).

**Chromatin Immunoprecipitation (ChIP)**—For chromatin preparation, 10⁸ 3T3-L1 cells (days 0, 2, and 4 of differentiation) were cross-linked with 12.5 mM disuccinimidyl glutarate (Symchньt UG & Co. KG) and 1% formaldehyde as previously described (36). The cross-linked 3T3L1 cells were washed twice with cold PBS and removed from the plate in lysis buffer (0.1% SDS, 1% Triton X-100, 0.15 M sodium chloride, 1 mM EDTA, 20 mM Tris, pH 8.0) containing protease inhibitors (Complete; Roche Applied Science).

Chromatin was sheared using a Bioruptor (Diagenode, Denville, NJ), and chromatin was precipitated with 1 μg of anti-C/EBPβ antibody (sc-150), 1 μg of anti-C/EBPδ (sc-636) and protein A-Sepharose beads (Sigma). De-cross-linked and precipitated chromatin was dissolved in 200 μl of...
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**TABLE 1**

| Primer sequences for quantitative RT-PCRs | Forward primer | Reverse primer |
|------------------------------------------|----------------|----------------|
| mC/EBPβ                                  | GCTGCCGACCAGTACAAGA | CAGTCCGACCTCTGAG |
| mC/EBPδ                                  | CCTTTGATGCTGTCGAGAG | GCAACGAGATACAGTCCA |
| mC/CD1d                                  | CTCTGCTGCGGCTGTAATT | TCCCGATCTCTGGAGCAATTT |
| mMTPA                                    | GGCAGCTTTCTCTGCTCT | TGGAGGCGGATTCTGAG |
| mMTPB                                    | CTCGCGTCAGAGAAATAA | AGCTGCGCTCCACTGATTAC |
| mpPPARγ                                  | CGCCTGATGACTTCTCTTGA | AGAAGTCCACAGACGTATTCC |
| mtTfIB                                   | GTTCTGGTGCGGCTTGGCTT | TGGTGAGCTGGCTGACCTTT |
| m36B4                                    | AGGCCGCTTCTGGGACATTGG | GGGCCAGATGTTGGACAGAG |

water, and 5 μl of DNA solution was used for quantitative PCR analysis. A 10% aliquot of the fragmented chromatin was applied as input. The primers used for quantitative PCR were CD1d forward primer, 5′-CTGCGCTGAGATTTCTCTCT-3′ and reverse primer, 5′-ATGCTGCAACTTCTCCACTC-3′; MTP-B forward primer, 5′-CTCCTGATACGGGCAAGAG-3′ and reverse primer 5′-TCCCTAAGGGGCAACCAGA-3′; intergenic region on mouse chromosome 15 forward primer, 5′-TGGTAGCCTCAGGACT-3′ and reverse primer, 5′-ATCCAAGATGGGGCAACGCTG-3′; Psap forward primer, 5′-TGGCAACAGGGTCTCTAAC-3′ and reverse primer, 5′-AGCAGAGGGAAAAACTCT-3′; Npc2 forward primer, 5′-AATGGGCAACTTATTAC-3′ and reverse primer, 5′-GCTCCTGAGATTCACCAGAA-3′; Glα forward primer, 5′-CAGGGACGATGGTGATTTCT-3′ and reverse primer, 5′-GAAGCCAAACCTTCTCAACCA-3′.

siRNA Transfection—Undifferentiated 3T3-L1 cells were transfected with siRNA oligonucleotides as described above (37) using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s protocol. The siRNA oligonucleotides were provided by Dharmacon and are described in Table 1.

**RESULTS**

C/EBPβ and -δ Regulate Expression of CD1d and Its Loading Machinery in Adipocytes—To characterize CD1d and its loading machinery in adipocytes we first examined the expression of CD1d itself and a number of genes implicated in its lipidation (MTP, Psap, Npc2, and Gla) in 3T3-L1 (pre)adipocytes by quantitative RT-PCR. CD1d itself, as well as the antigen loading machinery genes MTP, Psap, Npc2, and Gla were expressed at low levels in undifferentiated pre-adipocytes, but were clearly expressed in mature 3T3-L1 adipocytes (Fig. 1A), indicating that these cells may be able to present lipid antigens to iNKT cells. Of note, the expression of CD1d in primary mouse adipocytes was even higher than in mature 3T3-L1 adipocytes (Fig. 1A). To identify the molecular mechanisms underlying the transcriptional regulation of CD1d during adipogenesis, we first studied its mRNA expression pattern during differentiation of 3T3-L1 cells into mature adipocytes. As shown in Fig. 1B, the expression level of CD1d increased early in adipogenesis, within the first 2 days, after which the levels decreased to some extent. Interestingly, the mRNA expression pattern of CD1d followed the expression pattern of two transcriptional regulators of early adipocyte differentiation, C/EBPα and -δ. These transcription factors are induced in early adipogenesis, which further leads to C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) transcription and terminal differ-
The relevance of C/EBPβ and -δ in adipocyte differentiation has also been clearly demonstrated in knockout mouse models, which show severe impairment in adipogenesis (40). Very recently, a genome-wide analysis of C/EBPβ and -δ binding during 3T3-L1 differentiation by ChIP sequencing technology was reported (41). Detailed analysis of the chromosomal region surrounding the CD1d gene revealed clear C/EBPβ and -δ binding 4 h after induction of differentiation, with increased binding at some C/EBPβ peaks on day 2 (Fig. 1C). The recruitment of C/EBPβ and -δ to the candidate-binding site in exon 2 of the CD1d gene in differentiating 3T3-L1 cells and mature adipocytes (day 2 and 4, respectively) was confirmed by ChIP-PCR (Fig. 1D). In addition, the recruitment in pre-adipocytes (day 0), when C/EBPβ and -δ expression is low, was negligible and neither C/EBPβ nor -δ were detected on an arbitrary intergenic region on chromosome 15, which served as a control.

**FIGURE 1. CD1d expression is regulated by C/EBPβ and -δ in adipogenesis.** A, mouse 3T3-L1 pre-adipocytes, mature adipocytes, and primary adipocytes were subjected to quantitative RT-PCRs for analysis of CD1d and its lipid loading machinery genes (MTP, Psap, Npc2, and Gla) mRNA expression. Mean expression in 3T3-L1 pre-adipocytes was set at 1. Data are presented as mean ± S.E. (n = 3). B, mouse 3T3-L1 pre-adipocytes were differentiated into mature adipocytes, and RNA samples were taken at different time points during differentiation. CD1d, C/EBPβ and -δ, PPARγ mRNA levels were analyzed by quantitative RT-PCRs. Data are presented as mean ± S.E. (n = 3). C, ChIP-Seq data of C/EBPβ and -δ in the vicinity of the CD1d gene according to Siersbæk et al. (41) viewed in the UCSC genome browser (57). D, ChIP-PCRs for C/EBPβ and -δ were performed in 3T3-L1 cells (days 0, 2, and 4 of differentiation) with primers encompassing the candidate-binding site in exon 2 of the CD1d gene. As a negative control, an intergenic region on chromosome 15 was used. Results are indicated as % of immunoprecipitated chromatin compared with the input. E, 3T3-L1 cells were subjected to siRNA-mediated knockdown of C/EBPβ and -δ from the start of differentiation. C/EBPβ and -δ, CD1d mRNA expression were analyzed on day 4 of the differentiation. Relative mRNA expression levels were related to control siRNA-treated cells. Data are presented as mean ± S.E. (n = 2–3). F, 3T3-L1 cells, transfected with either control or C/EBPβ-δ siRNA oligonucleotides, were subjected to differentiation conditions. On the day 4 of differentiation, cells were lysed. Cell lysates were subjected to Western blot analysis using antibodies against C/EBPβ and -δ and tubulin. Fold-inductions/relative expressions were normalized for housekeeping genes TFIIIB or 36B4.
a negative control (Fig. 1D). To further address the relevance of these findings, we investigated whether combined lowering of endogenous C/EBPβ and -δ protein levels affected CD1d expression. Combined siRNA-mediated knockdown of C/EBPβ and -δ, as assessed on the mRNA and protein level (Fig. 1, E and F, respectively), resulted in a significant reduction of CD1d mRNA expression in mature adipocytes (day 4 of adipogenesis) (Fig. 1E).

No prominent binding sites for the adipogenic transcription factor PPARγ (Fig. 2a) or its heterodimer partner RXR (data not shown) were observed in the CD1d locus in two independent ChIP-Seq runs during 3T3-L1 differentiation experiments (41, 42) or upon 1 h treatment with the PPARγ ligand rosiglitazone (43), whereas PPARγ binding to the Cidec locus was clearly observed (Fig. 2A). In agreement with this, knockdown of PPARγ did not affect CD1d expression (Fig. 2B).

Interestingly, C/EBPβ and -δ binding sites were also present in the chromosomal regions surrounding the CD1d machinery genes Npc2, Psap, and Gla, and C/EBPβ binding was confirmed by ChIP-PCR (Fig. 3, A and B, respectively). Taken together, these data suggest that C/EBPβ and -δ are direct transcriptional regulators of CD1d and its machinery in early adipogenesis.

The CD1d-mediated Lipid Antigen Presentation Machinery in Adipocytes Is Functional—Having established that CD1d as well as the antigen loading machinery genes MTP, Psap, Npc2, and Gla are expressed in mature 3T3-L1 adipocytes (Figs. 1 and 3), we next investigated the functionality of the CD1d-mediated lipid antigen presentation machinery. For this, 3T3-L1 (pre) adipocytes were co-cultured with the mouse iNKT DN32D3 hybridoma cell line for 24 h, and the production of IFN-γ and IL-4 by the iNKT cells was assessed. To establish the relevance of CD1d in these assays, 3T3-L1 cells were generated with reduced CD1d expression (shRNA-mediated knockdown) and with CD1d overexpression (Fig. 4A) by lentiviral transduction. Of note, CD1d depletion or overexpression did not influence the differentiation potential of the pre-adipocytes, as assessed by expression of the differentiation marker fatty acid-binding protein 4 (FABP4) (Fig. 4A).

Cytokine measurements in the supernatant showed induction of IFN-γ and IL-4 release by iNKTs co-cultured with mature adipocytes in the presence of αGalCer. Interestingly, also in the absence of αGalCer, IFN-γ release, and to a lesser extent IL4 release, was reproducibly increased when compared with pre-adipocytes, suggesting the presence of lipid self-antigens in mature adipocytes (Fig. 4B). Similar results were obtained with a second iNKT cell clone (DN29.3E; data not shown). Please note that whereas IL2 is often used to boost cytokine production (44, 45), we purposely choose not to do this to avoid effects potentially mediated by such co-stimuli. IL2 release by iNKT DN32.D3 cells was also assessed, as this is a reported activity read-out. IL2 levels were very low without αGalCer stimulation in our co-culture set-up (data not shown), as also reported by Huh et al. (29), and therefore not analyzed in our subsequent experiments focusing on endogenous lipid antigen presentation. As a control, cytokine measurements were performed in the absence of iNKT cells, and only low concentrations were observed, which were not modulated by the addition of αGalCer (Fig. 4C). Furthermore, cytokine release by iNKT cells was reduced significantly by CD1d knockdown and was the highest when stimulated by CD1d overexpressing adipocytes (Fig. 4B), indicating that modulation of iNKT cytokine production by adipocytes requires CD1d. Anti-CD1d antibody blocking confirmed the CD1d dependence of lipid presentation (data not shown).
Taken together, these data show that mature 3T3-L1 adipocytes express a functional CD1d-mediated lipid antigen presentation machinery, and can thereby act as lipid APCs to modulate iNKT cell function. Furthermore, the stimulation of cytokine production observed in the absence of the exogeneous ligand αGalCer suggests that mature adipocytes are capable of producing endogenous lipid self-antigens.

**MTP Is Regulated by C/EBPα and -δ Early in Adipocyte Differentiation**—To identify proteins potentially involved in CD1d-mediated presentation of endogenous lipid antigens, we focused on MTP, a protein previously implicated in lipid antigen loading on to CD1d in professional APCs but with no clear function in adipocytes (31, 33). To investigate the potential role of MTP in adipocytes, we examined mRNA expression of the A and B isoforms of MTP in mature adipocytes and during adipogenesis. In line with previous findings (30), we detected both MTP isoforms in 3T3-L1 adipocytes with MTP-B as the dominantly expressed isoform (Fig. 5A). Similar to the CD1d mRNA expression pattern (Fig. 1B), MTP-B mRNA levels increased significantly within the first 2 days of differentiation and...
decreased slightly afterward (Fig. 5B). Given the regulation of the CD1d gene by C/EBPβ and -δ (Fig. 1), we examined binding of these transcription factors in the chromosomal region surrounding the MTP gene. Several binding sites for C/EBPβ and -δ were observed at 4 h and on day 2 of adipogenesis (Fig. 5C), and binding of C/EBPβ to the region upstream of the MTP-B-specific exon 1 was confirmed by ChIP-PCR (Fig. 5D). Combined siRNA-mediated knockdown of C/EBPβ and -δ resulted in a significant reduction of MTP-B mRNA expression in mature adipocytes (Fig. 5E). Together, these data show that
MTP-B expression is up-regulated by C/EBPβ and -δ during 3T3-L1 adipogenesis.

**MTP Is Involved in Antigen Presentation in Adipocytes**—Because MTP-B is significantly expressed in mature adipocytes with an expression pattern similar to CD1d (Fig. 5) and given that MTP is known to have a role in CD1d antigen loading in professional APCs (31, 33), we hypothesized that MTP-B may be involved in CD1d-mediated antigen presentation to iNKT cells by mature adipocytes. To investigate this, we generated 3T3-L1 knockdown cells with stable shRNA-mediated knockdown of MTP (both isoforms). As depicted in Fig. 6, these cells displayed significantly reduced MTP-B mRNA levels.

**FIGURE 4.** *Endogenous and exogenous CD1d-mediated lipid presentation by 3T3-L1 adipocytes.* A, 3T3-L1 pre-adipocytes were subjected to shRNA-mediated knockdown or overexpression of mouse CD1d. The quantitative RT-PCRs (left panel) shows the CD1d mRNA expression in differentiated adipocytes compared with scramble shRNA-treated cells. Data are presented as mean ± S.E. (n = 3). Fold-inductions are normalized for the housekeeping gene TfIIB. The right panel shows Western blot analysis of shRNA CD1d transduced adipocytes and mCD1d overexpressing adipocytes compared with scramble (pre)adipocytes using antibodies against Fabp4 and tubulin. B, INKT cells were co-cultured with scramble 3T3-L1 (pre)adipocytes, and transduced lines (24 h). The levels of IFN-γ and IL-4 in the supernatant were measured by ELISA. Mature adipocytes were incubated with vehicle or αGalCer (100 ng/ml) 24 h before adding the iNKT cells. A representative experiment is shown with data presented as mean ± S.E. (n = 8). C, IFN-γ and IL-4 levels were measured in the supernatants of iNKT cells alone, and undifferentiated 3T3L1 pre-adipocytes and mature adipocytes alone, with and without loading of the (pre)adipocytes with αGalCer (24 h). Cytokine levels were measured by ELISA kits. A representative experiment is shown with data presented as mean ± S.E. (n = 8).
els, whereas MTP protein levels were also reduced (please note that the MTP antibody recognized both isoforms). MTP deple-
tion did not influence the differentiation capacity of 3T3-L1
cells, as assessed by expression of the differentiation marker
Fabp4 (Fig. 6C). To address the relevance of MTP in lipid anti-
gen presentation, we co-cultured these shMTP mature adi-
pocytes with iNKT cells. As shown in Fig. 6D, MTP knockdown resulted in a significant reduction of IFNγ and IL4 cytokine
release of iNKT cells. Treatment of adipocytes with the MTP inhibitor BMS212122 also resulted in significant reduction of IFNγ release by iNKT cells. Importantly, this inhibition was also observed when BMS212122 was applied for short term (24 h) on mature adipocytes (Fig. 6E). MTP inhibition by BMS212122 did not influence the differentiation potential of the pre-adipocytes, as assessed by expression of the Fabp4 (Fig. 6F). Next, we tested primary iNKT cells, purified from mouse spleens, in co-culture with shRNA CD1d and shRNA MTP-transduced adipocytes. The results confirmed the antigen presenting capacity of mature adipocytes and the role of CD1d and MTP as presentation machinery (Fig. 6G). Taken together, these data qualify MTP as part of the CD1d machinery in mature adipocytes and show its critical role in the presentation of endogenous lipid antigens by adipocytes.

**DISCUSSION**

In the present study we show that mature 3T3-L1 adipocytes contain a functional machinery for the presentation of endogenous lipid antigens to iNKT cells. The CD1d gene, as well as the antigen loading machinery genes Psap, Npc2, and Gla, are up-regulated in early 3T3-L1 adipogenesis, and transcriptionally controlled by C/EBPβ and -δ. Interestingly, adipocyte-induced cytokine release by iNKT cells occurred in the absence of exogenous ligand, suggesting the production of endogenous lipid antigens by 3T3-L1 adipocytes. Furthermore, we identified MTP-B as an essential player in the presentation of these endogenous lipid antigens. Taken together, these findings underscore the qualification of mature adipocytes, which are engaged in dynamic lipid metabolism, as non-professional lipid APCs, which may present an important aspect of adipocyte-immune cell communication in the regulation of whole body energy metabolism.

Interestingly, the qualification of adipocytes as non-professional lipid APC is not limited to a functional CD1d machinery and extensive lipid handling capacity: adipocytes also express a number of proteins that have been implicated in the presentation of (endogenous) lipid antigens more indirectly in professional APC. For example, adipocytes express several Toll-like receptors, pattern recognition receptors by which APCs can sense danger signals (46). Toll-like receptor activation can modulate the lipid biosynthetic pathway, induce essential cytokine production, and induce the specific up-regulation of lipid-handling proteins, and a pivotal role of the low-density lipoprotein receptor-mediated uptake has been proposed in directing antigens toward CD1d presentation (52). Also other scavenger receptors (e.g. CD36, SRA, and SRB1) are expressed by adipocytes and have been shown to be involved in directing antigens toward CD1d presentation and iNKT cell activation (53). In summary, adipocytes therefore contain many components of the presentation machinery and appear to be well equipped for lipid antigen presentation.

We observed that the up-regulation of CD1d expression occurs relatively early in adipogenesis. Our data indicates that C/EBPβ and -δ are important transcriptional regulators of CD1d expression, in line with a central role for C/EBPβ in CD1d expression in keratinocytes (54). In addition, C/EBPβ and -δ most likely also regulate the lipid loading machinery genes Psap, Npc2, and Gla. Furthermore, MTP-B, which we implicated in lipid antigen loading of CD1d in adipocytes in the present study, is controlled by the same 2 transcription factors. This type of control ensures simultaneous expression of the key players required for endogenous lipid antigen presentation. Previously, several other transcription factors have been implicated in the regulation of CD1d expression in different cell types, including Ets family members (in hematopoietic cells) (55), retinoic acid receptor (in dendritic cells and mature adipocytes) (56), and PPARγ (in mature adipocytes) (29). However, we consider it unlikely that CD1d is under direct control of PPARγ during adipocyte differentiation as (i) we could not detect prominent PPARγ binding sites in a 200-kb chromosomal region surrounding the CD1d gene in mature adipocytes (41, 42) or after short term stimulation (1 h) of mature 3T3-L1 adipocytes with the PPARγ ligand rosiglitazone (43) (Fig. 2A), (ii) siRNA-mediated knockdown of PPARγ did not affect CD1d expression (Fig. 2B), and (iii) PPARγ activated the CD1d promoter only modestly in reporter assays (1.5-fold) (29). Taken together, this suggests that the increase in CD1d expression observed upon 24 h of treatment with the PPARγ ligand rosiglitazone (29) occurs through indirect mechanisms. Therefore, we would argue that C/EBPβ and -δ are required for the initial induction of CD1d expression during adipocyte differentiation, whereas the role of PPARγ is more likely to be indirect, analogous to the situation in dendritic cells (29, 56).

MTP proteins, located in the ER and endosomal pathway, can lipidate CD1d and are involved in CD1d antigen presentation in hepatocytes, intestinal epithelial cells, and professional APCs (31, 33). The exact function of MTP in adipocytes is unclear; it has been proposed to play a role in lipid droplet formation, but this has not been proven experimentally (34). Based on knockdown and MTP inhibition experiments we con-

**FIGURE 6. MTP-B is required for iNKT cell activation by 3T3-L1 adipocytes.** A, 3T3-L1 pre-adipocytes were subjected to shRNA-mediated knockdown of MTP by lentiviral transduction. The quantitative RT-PCR shows the MTP-B mRNA expression in differentiated shRNA MTP-transduced adipocytes compared with cells transfected with scrambled shRNA. Data are presented as mean ± S.E. (n = 3). Fold-induction is normalized for the housekeeping gene TFIIB. B and C, cell lysates of differentiated shRNA MTP-transduced cells and scramble cells were subjected to Western blot analysis using antibodies against MTP, Fabp4, and tubulin. D, iNKT cells were co-cultured with scramble 3T3-L1 (pre)adipocytes and shRNA MTP transduced adipocytes (24 h). The levels of IFNγ-γ and IL4 in the supernatant were measured by ELISA. A representative experiment is shown with data presented as mean ± S.E. (n = 8). E, MTP inhibitor (BMS212122) (13 μmol) or dimethyl sulfoxide was added to the 3T3-L1 cells on day 0 of the differentiation. On day 7 inhibitor was washed away and 3T3-L1 (pre)adipocytes were co-cultured with DN32.D3 hybridoma cells (24 h). The levels of IFNγ and IL4 in the supernatant were measured by ELISA. A representative experiment is shown with data presented as mean ± S.E. (n = 8). F, cell lysates of 3T3-L1 (pre)adipocytes treated with BMS212122 (13 μmol) were subjected to Western blot analysis using antibodies against Fabp4 and tubulin. G, primary iNKT cells were co-cultured with scramble 3T3-L1 (pre)adipocytes, shRNA CD1d and shRNA MTP transduced adipocytes (24 h). The levels of IFNγ in the supernatant were measured by ELISA. A representative experiment is shown with data presented as mean ± S.E. (n = 5).
clude that CD1d-mediated presentation of endogenous lipid antigens requires MTP. In adipocytes this function is most likely performed by the MTP-B protein, given the high expression levels of this isoform compared with MTP-A in adipocytes (Ref. 30, and current study). Interestingly, MTP-B localizes primarily to the Golgi in adipocytes (30, 34), suggesting that its primary role may be to load newly synthesized CD1d with glucosylceramide lipid self-antigens. Whether or not MTP-B influences the trafficking of CD1d to the adipocyte cell membrane needs further research.

Interestingly, in the absence of the exogenous antigen αGalCer we observed increased cytokine release in the co-culture experiments with mature adipocytes and iNKT cells. These findings suggest presentation of lipid self-antigens (or other co-stimulatory signals) by mature adipocytes that are able to modulate iNKT cell activity. Adipocytes seem ideally suited for CD1d-mediated endogenous lipid presentation: (i) they express CD1d and CD1d lipidation genes (including MTP-B), (ii) they are able to handle and metabolize lipids, and the iNKT self-antigens identified so far are lipid derivatives (e.g. β-glucosyloceramides (23)), (iii) they express various other receptors (e.g. Toll-like receptors, scavenger receptors) that have been implicated more indirectly in CD1d lipidation. These characteristics also suggest that CD1d-mediated lipid antigen presentation by adipocytes may be subject to different levels of regulation, thereby affecting the response of the iNKT target cells. Identification of endogenous lipid antigens and unraveling the intracellular pathways of CD1d-mediated lipid self-antigen presentation in adipocytes are therefore important challenges for the future.

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