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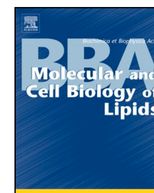
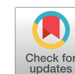


Adipocytes harbor a glucosylceramide biosynthesis pathway involved in iNKT cell activation



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ABSTRACT

Background: Natural killer T (NKT) cells in adipose tissue (AT) contribute to whole body energy homeostasis.
Results: Inhibition of the glucosylceramide synthesis in adipocytes impairs iNKT cell activity.
Conclusion: Glucosylceramide biosynthesis pathway is important for endogenous lipid antigen activation of iNKT cells in adipocytes.
Significance: Unraveling adipocyte-iNKT cell communication may help to fight obesity-induced AT dysfunction.

Overproduction and/or accumulation of ceramide and ceramide metabolites, including glucosylceramides, can lead to insulin resistance. However, glucosylceramides also fulfill important physiological functions. They are presented by antigen presenting cells (APC) as endogenous lipid antigens via CD1d to activate a unique lymphocyte subspecies, the CD1d-restricted invariant (i) natural killer T (NKT) cells. Recently, adipocytes have emerged as lipid APC that can activate adipose tissue-resident iNKT cells and thereby contribute to whole body energy homeostasis. Here we investigate the role of the glucosylceramide biosynthesis pathway in the activation of iNKT cells by adipocytes.

UDP-glucose ceramide glucosyltransferase (*Ugcg*), the first rate limiting step in the glucosylceramide biosynthesis pathway, was inhibited via chemical compounds and shRNA knockdown in vivo and in vitro. β-1,4-Galactosyltransferase (B4Galt) 5 and 6, enzymes that convert glucosylceramides into potentially inactive lactosylceramides, were subjected to shRNA knock down. Subsequently, (pre)adipocyte cell lines were tested in coculture experiments with iNKT cells (IFNγ and IL4 secretion).

Inhibition of Ugcg activity shows that it regulates presentation of a considerable fraction of lipid self-antigens in adipocytes. Furthermore, reduced expression levels of either B4Galt5 or -6, indicate that B4Galt5 is dominant in the production of cellular lactosylceramides, but that inhibition of either enzyme results in increased iNKT cell activation. Additionally, in vivo inhibition of Ugcg by the aminosugar AMP-DNM results in decreased iNKT cell effector function in adipose tissue.

Inhibition of endogenous glucosylceramide production results in decreased iNKT cells activity and cytokine production, underscoring the role of this biosynthetic pathway in lipid self-antigen presentation by adipocytes.

Abbreviations: AMP-DNM, *N*-(5′-adamantane-1′-yl-methoxy)-pentyl-1-deoxynojirimycin; GM2/3, monosialic ganglioside 2/3; Ugcg, UDP-glucose ceramide glucosyltransferase

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1. Introduction

Adipose tissue (AT), a site where the immune system and metabolic pathways intersect, undergoes marked changes during the progression of obesity [1,2]. Adipocyte hypertrophy and hyperplasia contribute to adipocyte dysfunction, which in turn promotes an infiltration of proinflammatory immune cells. The pro-inflammatory state of AT is characterized by inflammasome activation, increased release of free fatty acids (FFAs) and cytokines [3,4]. In addition, lipid overloading of the adipocytes results in accumulation of ceramide and ceramide metabolites, including glucosylceramides, which, together with the pro-inflammatory state of AT result in impaired insulin receptor signaling and metabolic derangements [5–7].

While clearly implicated in the pathophysiological consequences of obesity, ceramide metabolites also serve physiological functions. For example, exogenous and endogenous glycosylceramides including glucosylceramides (GluCer) and galactosylceramides (GalCer), can be displayed by APC in an immunogenic fashion that can activate a unique subset of lymphocytes called invariant Natural Killer T (iNKT) cells [8,9]. iNKT cells are a lineage of T lymphocytes that have both innate and adaptive characteristics and mediate a range of immune responses [10,11]. NKT cells have a long established role in various disease conditions such as autoimmunity, cancer, and infectious diseases [12–16]. iNKT cells express a semi-invariant T cell receptor (TCR) that responds to glycolipid antigens presented via the non-classical MHC-like antigen-presenting molecule CD1d [10]. Exogenous lipid antigens all harbor a sugar headgroup with α stereochemistry, with αGalCer, originally identified from a screening in extracts of a marine sponge, as the most potent exogenous ligand [17–19]. Using ceramide as a starting point, mammalian cells can produce > 200 different glycosylceramides. Until recently, mammals were thought to produce glycosylceramides only as β-anomers, but recent studies have challenged this notion, as the original preparations of β-glycosylceramides contained minor but highly active fractions of α-glycosylceramides. Several α-glycosylceramides (α-GalCer, α-GluCer) were detected in mammalian antigen-presenting cells (APCs), including dendritic cells (DC) and primary thymocytes [20–23].

iNKT cells are present in high numbers in human and mouse lean AT whereas obese adipose tissue shows a decrease in iNKT cell numbers [24–32]. In the absence of external stimuli, AT-resident iNKT cells exhibit a Th2-biased cytokine profile (e.g. high IL-4 production) as compared to spleen iNKT cells [29,31]. Upon stimulation by lipid antigens presented by the MHC1-like CD1d protein, AT-resident iNKT cells can secrete both Th1 cytokines (e.g. IFNγ) and Th2 cytokines (IL-4, IL-13, IL-10) [2,24–26,29,31,33–35].

Interestingly, recent studies showed that human and mouse adipocytes express CD1d and its loading machinery, and regulate iNKT cell function by acting as lipid antigen displaying APC [26,29,36]. We and others have shown that iNKT cell activation by adipocytes is dependent on CD1d [26,34,36]. When cultured in the presence of adipocytes that overexpress CD1d, iNKT cells show higher cytokine secretion, whereas CD1d knockdown in adipocytes results in decreased activation of iNKT cells. Additionally, these co-culture experiments indicate that adipocytes can produce endogenous lipid antigens [36], but the cellular pathways for lipid antigen presentation in adipocytes remain to be defined and endogenous adipocyte lipid antigens have not yet been identified. Such identification would be highly relevant, as endogenous adipocyte lipid antigens may help to prevent the development of insulin resistance by preserving AT-resident iNKT cell numbers and activity [24,25,29,31]. Candidate endogenous adipocyte lipid antigens however include glucosylceramides, which originate from ceramide, a lipid species associated with insulin resistance [5].

Here, we address the role of endogenous lipid presentation by adipocytes through examination of the ceramide pathway consisting of glucosylceramide synthase, which catalyzes the conversion from ceramide to glucosylceramide, and lactosylceramide synthases, which catalyze the conversion from glucosylceramide to lactosylceramide. Glucosylceramide synthase is encoded by a single gene called UDP-glucose ceramide glucosyltransferase (*Ugcg*). Therefore, we hypothesize that Ugcg is a rate limiting step in the synthesis of endogenous lipid antigens [37]. This notion is supported by *Ugcg*$^{-/-}$ mice, which die in utero (around E8) [38]. Lactosyl synthase is encoded by two genes, β-1,4-Galactosyltransferase (B4Galt) 5 and 6, which are part of the B4Galt family consisting of seven members all of which are responsible for the production of different oligosaccharides and glycoconjugates [39]. B4Galt5 reportedly being the dominant enzyme in the conversion of glucosylceramide to lactosylceramide [40], which is substantiated by the in utero death of B4Galt5$^{-/-}$ mice (around E10.5) whereas B4Galt6$^{-/-}$ mice show no apparent phenotype [40–42]. By reducing Ugcg expression levels and inhibiting its activity, we show that this enzyme regulates lipid self-antigen presentation by adipocytes. Furthermore, by reducing B4Galt5 expression, we show that iNKT cell activating capacities of adipocytes increase, suggesting a prominent role for glucosylceramide as a lipid antigen. Ugcg controls endogenous lipid antigen processing in adipocytes in vivo, inhibition of its function results in decreased iNKT cell effector function in AT. Taken together, our data suggest that adipocyte-derived glucosylceramides, an important class of ceramide metabolites, supporting iNKT cell function in AT.

2. Materials & methods

2.1. Materials

Dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma Aldrich. The following antibodies were used: Anti-Ugcg (SAB2104830) and anti-tubulin (T9026) from Sigma-Aldrich (St Louis, MO, USA), anti-Fabp4 (sc-18661) from Santa Cruz Biotechnology, anti-B4Galt6 (SAB2106738), anti-B4Galt5 (SAB2106739) from Sigma Aldrich. IFNγ ELISA kit was from BD biosciences and IL4 ELISA kit was from eBiosciences. The Vα14- self-lipid and αGalCer -reactive DN32.D3 iNKT hybridoma was a kind gift of Prof. A. Bendelac [43]. Iminosugar AMP-DNM was synthesized as described previously [44,45].

2.2. Cell culture and differentiation

The murine 3 T3-L1 cell line (ATCC, Manassas, VA) was cultured in DMEM Glutamax (Dulbecco) containing 10% bovine serum (Life Technologies), penicillin and streptomycin (both 100 μg/ml; Life Technologies). For differentiation, 3 T3-L1 cells were grown to confluence and after 2 days incubated with culture medium containing dexamethasone (250 nM), IBMX (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. For Western blot analyses, differentiated 3 T3-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 Lifesciences).

2.3. Lentiviral knockdown of Ugcg

The shRNA constructs for mouse Ugcg, (clone nm_011673.2-374S1C1) B4Galt5. (clone NM_019835.1-733s1c1), and B4Galt6 (clone NM_019737.1-3486s1c1) were all provided in pLKO-1 lentiviral vectors (Sigma-Aldrich) Lentiviral particles were produced in 293 T cells. After lentiviral infection, 3 T3-L1 pre-adipocytes were maintained on 3 μg/ml puromycin. Stably transduced cells were used for the adipocyte-iNKT interaction studies.

2.4. Sphingolipid quantification of 3 T3-L1 adipocytes by HPLC

Lipids were extracted with the Bligh-Dyer method [46]. A water lysate was prepared of adipocytes previously grown in a 6-well plate (200 µl of MQ). Lipids were extracted from 75 to 150 µl of lysate, and samples were divided in two. One part was subjected to deacylation for ceramide quantification and the other part was directly derivatized for free sphingosine base quantification. For plasma 50 µl was analyzed. Deacylation was performed by use of microwave-assisted hydrolysis in methanolic NaOH. Next, samples were subjected to derivatization of the sphingoid bases with o-phthaldialdehyde, and separated by HPLC on a C18 reverse-phase column with methanol/water phase (84.5% methanol, 15.5% water) and quantified with a fluorescence detector (λ(ex) 340 nm and λ(em) 435 nm). One nmol of C17-sphinganine, a sphingolipid species that is not naturally generated in the mammalian cells, was used for internal calibration.

2.5. RNA isolation and quantitative PCR

One µg of RNA, extracted using TRIzol reagent (Invitrogen), was used for cDNA synthesis with the superscript first strand synthesis system (Invitrogen) according to manufacturer's protocol. Gene expression levels were determined by quantitative real time PCR with the MyIq cycler (Bio-Rad) using SYBR-green (Bio-Rad) and normalized to TFIIb or 36B4 expression. Primers for quantitative RT-PCR were designed with free Primer3 software or taken from Harvard primer bank and are described in Table 1.

2.6. (Pre)adipocyte-iNKT interaction

Scramble shRNA, Ugcg shRNA transduced mouse 3 T3-L1 (pre)adipocytes were co-cultured with 50.000 DN32.D3 hybridoma cells per well in a 96 wells format. After 24 h of co-culture, the supernatant was stored at −80 °C until analysis of IFNγ and IL4 cytokine levels. For Ugcg inhibition assay, 10 µM AMP-DNM, a specific chemical inhibitor of Ugcg [44,45], was added to the 3T3L-L1 cells on day 2 of the differentiation. On day 7 inhibitor was washed away and (pre)adipocytes were co-cultured with 50.000 DN32.D3 hybridoma cells per well in a 96 wells format (24 h). Cytokine levels were measured with commercially available ELISA kits.

2.7. Animal study/in vivo inhibition of Ugcg

WT C57BL/6 J mice (8 weeks; Charles River Laboratories) were fed standard chow until age 9 weeks, and subsequently fed LFD (10 kcal% fat, Research Diet D12450B) for 9 weeks. In the third week of LFD feeding, weight-matched groups were fed a LFD with or without 25 mg AMP-DNM/kg bodyweight per day until the end of the study (Research Diet Services). All mouse study protocols were approved by the Utrecht University Ethical Committee for Animal Experimentation (protocol 2013.III.06.046) and were in accordance with current Dutch laws on animal experimentation.

Table 1
Primers for quantitative RT-PCR.

Primer	Sequence
mUgcg_fw	AGGAAGGATGTGCTAGATCAGG
mUgcg_rev	TTTGCATGGCAACTTGAGTAGA
m36B4_ fw	ATGGGTACAAGCGCGTCCTG
m36B4_ rev	GCCTTGACCTTTTCAGTAAG
mTFIIb_ fw	GTTCTGCTCCAACCTTTGCCT
mTFIIb rev	TGTGTAGCTGCCATCTGCACTT
mMCP1_fw	CTTCTGGGCCTGCTGTTCA
mMCP1_rev	CCAGCCTACTCATTGGGATCA
mIL6_fw	CATCCAGTTGCCTTCTTGGG
mIL6_rev	CCAGTTTGGTAGCATCCATC
mIL6_fw	CTTCCATCCAGTTGCCTTCTTG
mIL6_rev	AATTAAGCCTCCGACTTGTGAAG
mTNFα_Fw	CAACCTCCTCTCTGCCGTCAA
mTNFα_rev	TGACTCCAAAGTAGACCTGCCC
mF4/80_Fw	CTTTGGCTATGGGCTTCCAGTC
mF4/80_rev	GCAAGGAGGACAGAGTTTATCGTG
mB4Galt5_fw	ACTTGGATTGGGATTGTCTGAT
mB4Galt5_rev	CGCAGAGTAGTTCAGGTTGTTG
mB4Galt6_fw	TATGTCATCGAACAGACCGGCACA
mB4Galt6_rev	AGGCTCTGTCTTTCATGGCCTCTT

2.8. Isolation of mouse leukocytes and (intracellular) flow cytometry staining

Murine visceral (epididymal) AT was collected, washed in PBS, and digested for 45 min with collagenase type II (Sigma-Aldrich) and DNAse I (Roche). Stromal vascular cells (SVCs) were pelleted by centrifugation, incubated for 20 min with NH_4Cl erythrocyte lysis buffer, and passed through a 100-µm cup filter (BD). Simultaneously, spleens were minced through a 70 µm mesh filter (BD) and collected in NH_4Cl lysis buffer. Subsequently, AT SVCs and spleen cells were washed in FACS buffer (2% fetal calf serum and 0.1% NaN_3 in PBS); preincubated with 10% rat serum in FACS buffer; and stained with mAbs specific for TCRβ, CD3, CD8, CD4, CD25, and for iNKT cell selection αGalCer-loaded CD1d tetramers (NIH) were used. Part of the cells were then fixed and permeabilized using the FixPerm (BD) followed by intracellular staining for IL-4 (BD), IFNγ (BD), IL-13 (BioLegend) and IL-17 (Affimetrix/eBioscience). As a negative control, the non -iNKT fraction of SVCs (low αGalCer-loaded CD1d tetramer and TCRβ staining) was analyzed and CD25 and intracellular cytokine signals were below those observed in the untreated iNKT cell population (Supplemental Fig. S1). Cells were analyzed by flow cytometry with a FacsCanto II (BD) flow cytometer and FACSDiva (BD) and FlowJo (Tree Star Inc.) software.

2.9. Statistical analyses

Data are routinely presented as means ± s.e.m. Statistical significance between two groups was determined using Student's *t*-tests. Values of $p < 0.05$ were considered significant and are indicated by *.

3. Results

3.1. Ugcg regulates lipid self-antigen presentation in adipocytes

Glucosylceramides have been identified as potent iNKT cell self-antigens in both mouse and human professional antigen presenting cells [20–22]. The rate-limiting enzyme for the synthesis of all glucosylceramides from ceramide is glucosylceramide synthase, which is encoded by the gene UDP-glucose ceramide glucosyltransferase (*Ugcg*; Fig. 1a). Therefore, we hypothesized that Ugcg may be involved in adipocyte self-antigen presentation to iNKT cells. To characterize the role of Ugcg in antigen presentation by adipocytes, we first studied its mRNA expression pattern during the differentiation of 3 T3-L1 pre-adipocytes to mature adipocytes. Expression of Ugcg was detected in pre-adipocytes and did not change significantly during adipogenesis, as analyzed by quantitative RT-PCR (Fig. 1b).

To investigate the role of Ugcg in lipid self-antigen presentation, we generated 3 T3-L1 cells with stable shRNA-mediated knockdown of Ugcg. As depicted in Fig. 1c and d, these cells displayed significantly reduced Ugcg mRNA and protein levels. Ugcg reduction did not critically impair the differentiation capacity of 3 T3-L1 cells, as assessed by expression of the differentiation marker Fabp4 (Fig. 1e). We next quantified glucosylceramide content of 3 T3-L1 (pre)adipocytes by HPLC [47]. Ugcg knock down reduced intracellular glucosylceramide levels significantly when compared to control cells, both in pre-adipocytes and in mature 3 T3-L1 adipocytes (Fig. 1f). The levels of ceramide, the precursor for all glucosylceramides, were increased moderately by Ugcg knock down (Fig. 1f). Lactosylceramide concentration was reduced in Ugcg knockdown cells, most likely due to the decreased

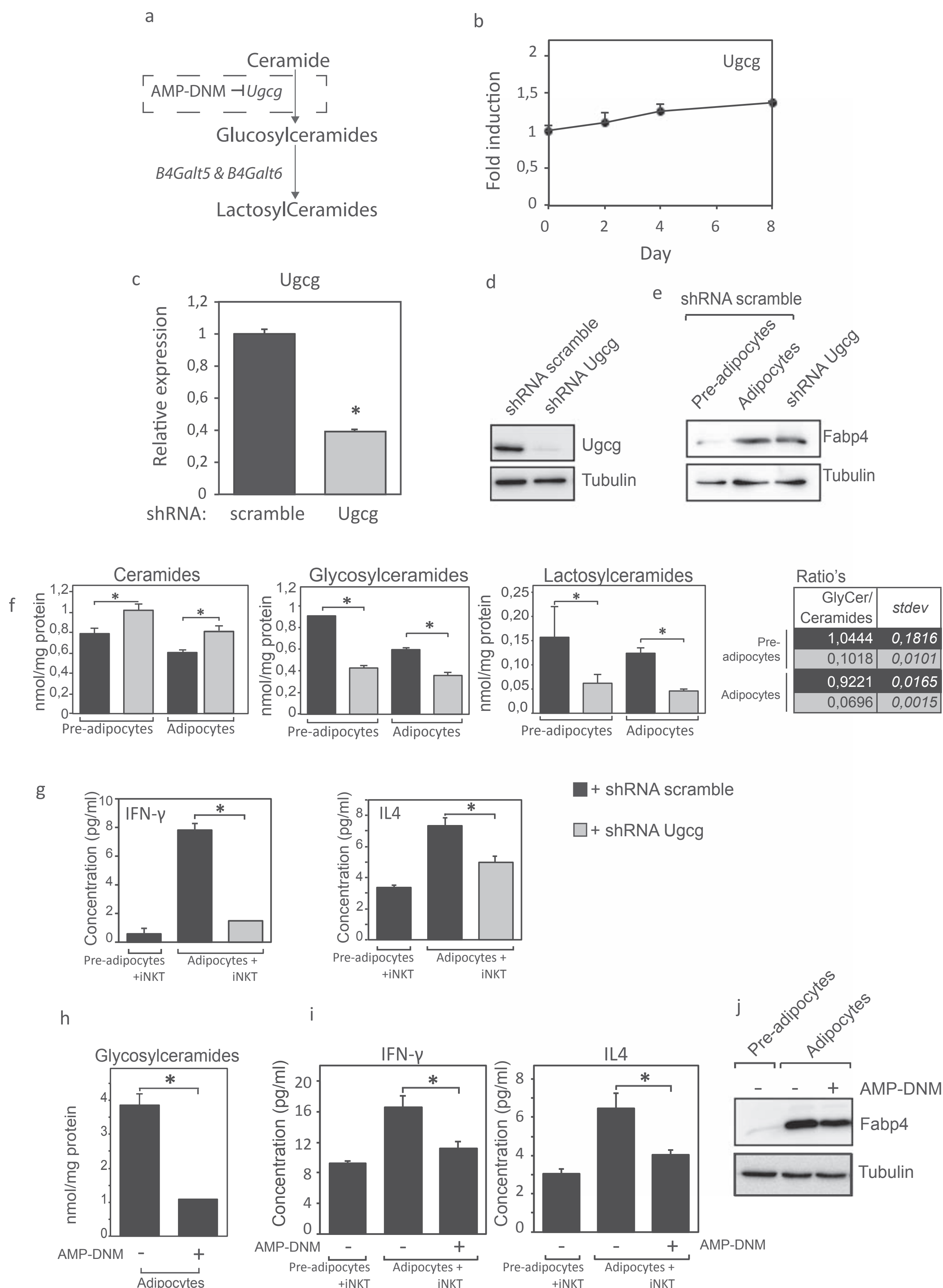

Fig. 1. Ugcg regulates lipid self-antigen presentation in adipocytes. (a) Schematic representation of glucosylceramide and lactosyl ceramide synthesis and its inhibition by the Ugcg inhibitor AMP-DNM. The focus of this figure is boxed. (b) Mouse 3 T3-L1 preadipocytes were differentiated into mature adipocytes and RNA

samples were taken at different time points during differentiation. *Ugcg* mRNA levels were analyzed by quantitative RT-PCR. Data are presented as mean ± S.E. (n = 3). Expression levels were normalized for housekeeping gene TFIIB, mean expression at Day 0 was set at 1. (c) 3 T3-L1 preadipocytes were subjected to shRNA-mediated knockdown of Ugcg by lentiviral transduction and Ugcg mRNA expression was analyzed by quantitative RT-PCR. Data are presented as mean ± S.E. (n = 3). Expression levels were normalized for housekeeping gene TFIIB, mean expression in scramble shRNA 3 T3-L1 adipocytes was set at 1. (d, e) Cell lysates of differentiated shRNA Ugcg transduced cells and scramble cells were subjected to Western blot analysis using antibodies against Ugcg, FABP4 and tubulin. (f) Lactosylceramides, glucosylceramides and ceramide levels in shUgcg transduced and scramble 3T3L1 (pre)adipocytes was determined by HPLC analysis of orthophtaldehyde-conjugated lipids. Additionally glucosylceramide ceramide ratios in scramble and shUgcg are depicted. (g) iNKT cells were co-cultured with scramble 3 T3-L1 (pre)adipocytes and shRNA Ugcg transduced adipocytes (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 (n = 8). (h) Ugcg inhibitor (AMP-DNM; 10 μM) or DMSO was added to the 3T3L-L1 cells on day 2 of differentiation. On day 7 inhibitor was washed away and 3 T3-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). (i) Cell lysates of 3 T3-L1 (pre)adipocytes treated with AMP-DNM (10 μM) or DMSO were subjected to Western blot analysis using antibodies against FABP4 and tubulin. (j) glucosylceramides – ceramide ratio in mature 3 t3-L1 adipocytes with and without treatment of AMP-DNM.

concentration of glycosylceramides (Fig. 1f). Aditionally, the ratio between glycosylceramide and ceramide was greatly reduced in the Ugcg knockdown (pre)adipocytes (Fig. 1f).

To address the relevance of Ugcg in lipid antigen presentation, we made use of a recently developed co-culture system in which 3 T3-L1 (pre)adipocytes are cultured with the mouse iNKT DN32D3 hybridoma cell line for 24 h, and the production of IFNγ and IL-4 by the iNKT cells is assessed [36]. This setting has been used previously to show that iNKT cell activation is CD1d dependent [26,36]. In this setting, cytokine release is observed in the absence of exogenously added lipid antigens, suggesting CD1d mediated presentation of lipid self-antigens by mature 3 T3-L1 adipocytes [36]. As shown in Fig. 1g, Ugcg knockdown resulted in a significant reduction of IFNγ and IL4 cytokine release by DN32D3 iNKT cells. Treatment of adipocytes with the synthetic Ugcg inhibitor AMP-DNM [44] also significantly decreases the glucosylceramide concentration (Fig. 1h). Inhibiting Ugcg with AMP-DNM resulted in a significant reduction of IFNγ and IL4 release by iNKT cells (Fig. 1i). This inhibition of iNKT cell activity required functional lipid antigen presentation machinery, as AMP-DNM treatment of 3 T3-L1 pre-adipocytes, which are CD1d negative and incapable of lipid antigen presentation [26,36], had no effect (data not shown). In agreement with the Ugcg knockdown experiments (Fig. 1e), Ugcg inhibition by AMP-DNM did not influence the differentiation potential of the pre-adipocytes, as assessed by Fabp4 expression (Fig. 1j). Taken together, these data qualify glucosylceramide synthesis by Ugcg as a critical step in adipocyte lipid self-antigen presentation to iNKT cells.

3.2. Inhibition of B4Galt5 and -6 results in higher iNKT cell activation

To further address the importance of Ugcg in iNKT cell activation by adipocytes, we focused on the next enzymatic step that converts glucosylceramides into lactosylceramides (Fig. 2a). Two enzymes are reported to be responsible for this conversion: β-1,4-Galactosyltransferase (B4Galt) 5 and B4Galt6 [41,42]. We hypothesize that the inhibition of B4Galt5 and 6 increases the DN32D3 iNKT cell activating capacity of 3 T3-L1 adipocytes due to a higher availability of glucosylceramides.

To investigate the consequences of decreased B4Galt5 and 6 expression we used a similar approach to the Ugcg experiments described above. B4Galt6 was assessed first due to the non-lethal effects in knockout mice [40]. We checked the mRNA expression of B4Galt6 during differentiation of 3 T3-L1 pre-adipocytes to mature adipocytes. B4Galt6 expression shows a slight increase during differentiation, as analyzed by RT-PCR (Fig. 2b). An shRNA mediated B4Galt6 knockdown 3 T3-L1 cell line was created and was confirmed by Western blot (Fig. 2c). The reduced expression of B4Galt6 also has no effect on adipogenesis as shown by the expression differentiation marker Fabp4 (Fig. 2d).

The knockdown of B4Galt6 did not result in a significant decrease of lactosylceramides in mature adipocytes (Fig. 2e). Also, the ratio between lactosylceramides and glycosylceramides was not decreased in B4Galt6 knockdown cells (Fig. 2e). This indicates that B4Galt6 does not play a dominant role in determining total cellular lactosylceramide levels. Additionally, ceramide and glycosylceramide levels and ratios were not significantly altered (Fig. 2e). Nevertheless, B4Galt6 knock down did result in reduced secretion of IL-4 and IFNγ by DN32D3 iNKT cells (Fig. 2f), suggesting that B4Galt6 has a role in iNKT cell activation by adipocytes.

To assess if reducing B4Galt5 expression has a similar effect, as reducing B4Galt6, we knocked down B4Galt5 using shRNA in 3 T3-L1 adipocytes. Firstly, natural B4Galt5 mRNA expression detected in pre-adipocytes by RT-PCR shows a slight increase during differentiation (Fig. 2g). The shRNA mediated B4Galt5 knockdown 3 T3-L1 cell line supported reduced expression of B4Galt5 by Western blot (Fig. 2h). As observed with the B4Galt6 knockdown, adipogenesis is not altered by the reduced expression of B4Galt5 as shown by the expression of differentiation marker Fabp4 (Fig. 2i). Next, we analyzed the lactosylceramide concentration in B4Galt5 knockdown cells by HPLC. In contrast to B4Galt6 knock down (Fig. 2f), a significant decrease of lactosylceramide production was detected in (pre)adipocytes lacking B4Galt5 compared to a shRNA scrambled cell line (Fig. 2j). Also, the ratio between lactosylceramides and glycosylceramides in the B4Galt5 knockdown cells was significantly decreased. This difference was absent in the B4Galt6 knockdown cells. Ceramide and glycosylceramide content in B4Galt5 knockdown cells also differed compared to shRNA scrambled cells (Fig. 2j). Additionally, co-culture experiments with DN32D3 iNKT hybridoma's and 3 T3-L1 mature adipocytes showed that cytokine secretion measured by ELISA (Il-4 and IFNγ) increases when B4Galt5 expression is decreased (Fig. 2k).

Taken together, these data suggest a dominant role for B4Galt5 compared to B4Galt6 in the conversion of glucosylceramide to lactocylceramide in adipocytes. However, inhibition of either enzyme resulted in increased cytokine production, suggesting that they play non-redundant roles in iNKT cell activation. These data stress the importance of the ceramide pathway enzymes in CD1d-dependent activation of iNKT cells.

3.3. Ugcg inhibition by AMP-DNM in vivo

To address the role of Ugcg on AT iNKT cell numbers and cytokine production in vivo, mice were treated with the synthetic inhibitor AMP-DNM for 6 weeks. The inhibitory effect of AMP-DNM on glucosylceramide synthesis was confirmed by blood plasma analysis, showing significantly reduced levels of glucosylceramide and increased levels of ceramide (Fig. 3a). Regrettably, glycosylceramide measurements in AT could not be performed due to high concentrations of TG causing interference in the analytical procedure [45]. Weight gain and food intake were not affected by the AMP-DNM treatment (Fig. 3b and c, respectively). In addition, no significant changes were observed in the expression of several inflammatory genes and the macrophage markers F4/80 in adipose tissue (Fig. 3d).

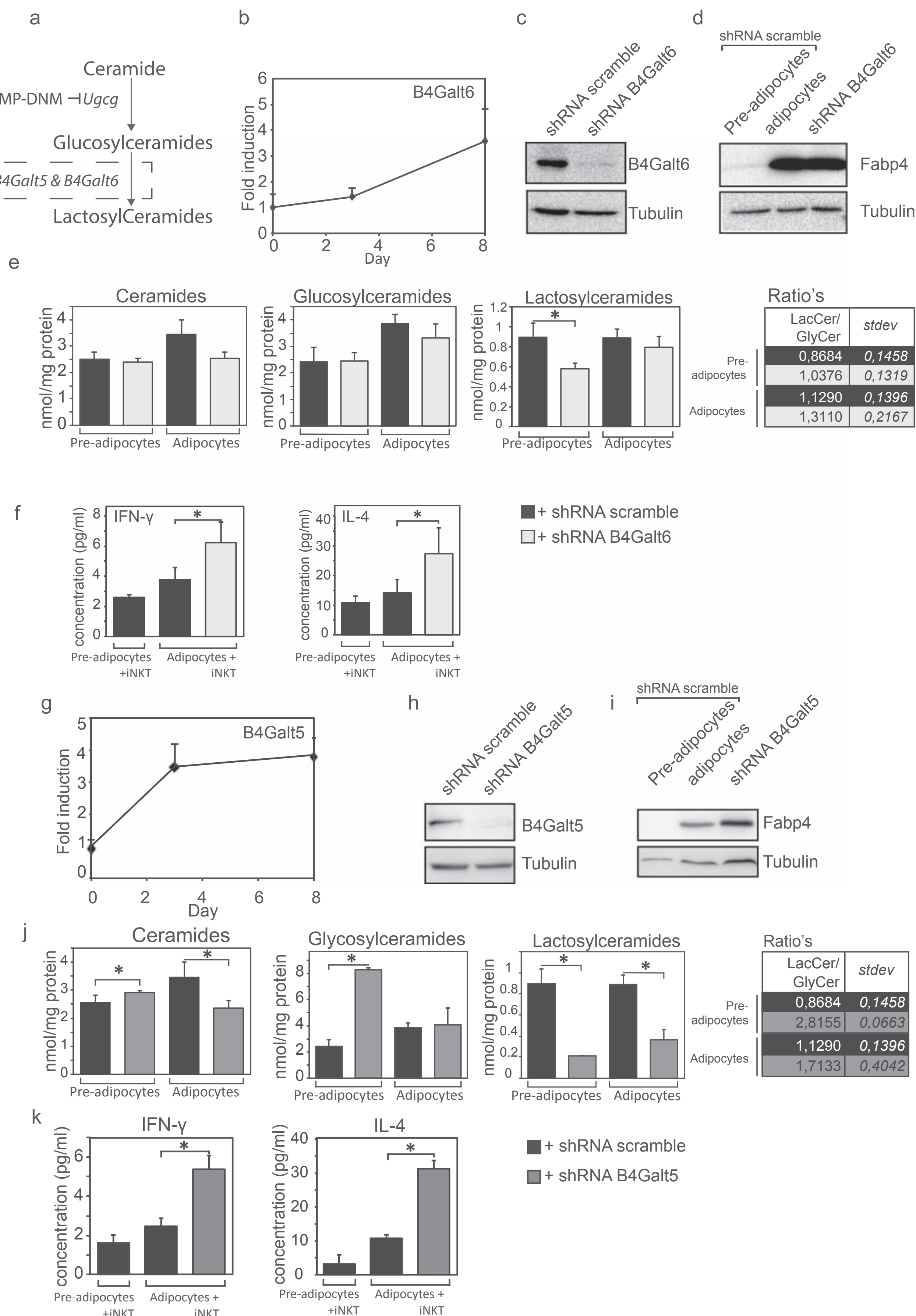

Fig. 2. Inhibition of B4Galt5 and B4Galt6, results in higher iNKT cell activating capacities of adipocytes (a) Schematic representation of glucosylceramide and

lactosyl ceramide synthesis and its inhibition by the Ugcg inhibitor AMP-DNM. The focus of this figure is boxed. (b) Mouse 3 T3-L1 preadipocytes were differentiated into mature adipocytes, and RNA samples were taken at different time points during differentiation, *B4Galt6* mRNA levels were analyzed by quantitative RT-PCR. Data are presented as mean ± S.E. (n = 3). Expression levels were normalized for housekeeping gene TFIIB, mean expression at Day 0 was set at 1. (c, d) Cell lysates of differentiated shRNA B4Galt6 transduced cells and scramble cells were subjected to Western blot analysis using antibodies against B4Galt6, FABP4 and tubulin. (e) Lactosylceramide, glucosylceramide and ceramide concentration in shRNA B4Galt6 and scramble 3 T3-L1 (pre)adipocytes was determined by HPLC analysis of orthophtaldehyde-conjugated lipids. Additionally lactosylceramide/glucosylceramide ratio in shRNA B4Galt6 and scramble 3 T3-L1 (pre)adipocytes. (f) iNKT cells were co-cultured with scramble 3 T3-L1 (pre)adipocytes and shRNA B4Galt6 transduced adipocytes (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 (n = 4). (g) Mouse 3 T3-L1 preadipocytes were differentiated into mature adipocytes, and RNA samples were taken at different time points during differentiation, *B4Galt6* mRNA levels were analyzed by quantitative RT-PCR. Data are presented as mean ± S.E. (n = 3). Expression levels were normalized for housekeeping gene TFIIB, mean expression at Day 0 was set at 1. (h, i) Cell lysates of differentiated shRNA Ugcg transduced cells and scramble cells were subjected to Western blot analysis using antibodies against B4Galt5, FABP4 and tubulin. (j) Lactosylceramide, glucosylceramide and ceramide concentration in shRNA B4Galt5 and scramble 3 T3-L1 (pre)adipocytes was determined by HPLC analysis of orthophtaldehyde-conjugated lipids. Additionally lactosylceramide/glucosylceramide ratio in shRNA B4Galt5 and scramble 3 T3-L1 (pre)adipocytes. (k) iNKT cells were co-cultured with scramble 3 T3-L1 (pre)adipocytes and shRNA B4Galt5 transduced adipocytes (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 (n = 4).

As systemic AMP-DNM treatment may not only inhibit endogenous lipid antigen presentation in adipose tissue, but also by APCs elsewhere in the body, we examined the percentage of iNKT cells within the TCRβ positive population in the spleen. No significant changes were observed upon AMP-DNM treatment (Fig. 3e), suggesting that there are no general systemic effects on iNKT cells after Ugcg inhibition.

3.4. Inhibition of endogenous glucosylceramide production results in decreased iNKT cell effector function in adipose tissue

Having established that Ugcg inhibition by AMP-DNM effectively reduced glycosylceramide levels (Fig. 3a) without affecting splenic iNKT cell numbers (Fig. 3e), the effect of Ugcg inhibition on the relative numbers and activity of iNKT cells in adipose tissue was addressed. No significant changes in number of iNKT cells were detected between the AMP-DNM treated and untreated mice (Fig. 4a and b). However, after a 6-week diet containing AMP-DNM mice displayed markedly reduced numbers of $CD25^+$ iNKT cells, indicating decreased iNKT cell activity in AT (Fig. 4a and b). To characterize the phenotype of the AT-resident iNKT cells upon Ugcg inhibition in more detail, cytokine production by AT-resident iNKT cells was analyzed. As shown in Fig. 4 c and d, AT derived iNKT cells from AMP-DNM treated animals produced significantly less pro-inflammatory (IFNγ, IL-17) and anti-inflammatory cytokines (IL-4, IL-13) compared to iNKT cells from control animals. iNKT cell activity and proliferation can also (co)regulated by other signals, including inhibition by the adipokine leptin [48]. Previously, Van Eijk et al. reported improved insulin sensitivity upon AMP-DNM treatment in leptin-deficient *ob/ob* mice [47], indicating that inhibition of Ugcg activity can act independent of leptin. Here, we observed lower leptin levels upon AMP-DNM treatment, which makes it unlikely that the drug-induced lowering of iNKT cell activity occurs through leptin (data not shown). Taken together, these findings indicate that inhibition of endogenous glucosylceramide production results in decreased iNKT cells activity and overall cytokine production and underscores the major role of this enzymatic pathway in lipid self-antigens presentation by adipocytes.

4. Discussion

Multiple studies in various cellular and animal models have shown that accumulation of ceramide and ceramide metabolites, including glucosylceramides, in obesity results in insulin resistance [5,49,50]. For example, inhibition of Ugcg, the enzyme that catalyzes the first step in the biosynthesis of glucosylceramides, results in reduced ceramide metabolite levels (e.g. GM2 and GM3), improved insulin signaling in adipocytes, and improved whole body insulin sensitivity in several rodent models of obesity [45,47]. Here, we show that the glucosylceramide synthesis pathway is also involved in a different aspect of adipose tissue function. Inhibition of Ugcg in adipocytes results in decreased iNKT cell action. Both in vitro and in vivo iNKT cell cytokine production is reduced upon Ugcg inhibition (Figs. 1 and 4). As glucosylceramides have been implicated as endogenous iNKT cell ligands [51], Ugcg inhibition may well reduce ligand availability and thereby decrease iNKT cell action. Co-culture studies with CD1d-proficient adipocytes and iNKT cells support this hypothesis, as Ugcg inhibition in adipocytes decreased iNKT cell cytokine production, while Ugcg inhibition of CD1d-deficient pre-adipocytes did not affect iNKT cell function. (Fig. 1 and data not shown). As lipid rafts contain multiple glycosylated lipids [52] and lipid rafts have been associated with CD1d functioning [53,50], it seems possible that inhibition of CD1d-mediated lipid antigen presentation occurs indirectly by interfering with lipid raft integrity. However, Lu et al. recentlty showed that lipid raft disruption in 3 T3-L1 (pre)adipocytes results in increased MCP-1 production [55], while we observed no changes in MCP-1 levels upon AMP-DNM treatment in AT (Fig. 3d). Therefore, the decrease of iNKT cell activity upon Ugcg inhibition is most likely due to impaired lipid antigen production and unrelated to lipid raft disruption.

Furthermore, assessment of the next step in the biosynthesis pathway – the conversion of glucosylceramide into lactocylceramide – shows that iNKT cell cytokine secretion increases upon tinhibition of B4Galt5 and -6 (Fig. 2). Interestingly, while both enzymes can catalyze the same reaction, our data suggest that their activities in adipocytes are not identical: i) B4Galt5 plays a larger role in determining total cellular lactocylceramide levels than B4Galt6, and ii) inhibition of either enzyme resulted in increased iNKT cell activation, indicating that their activities are not redundant. While the biochemical basis for these differences remains to be established, our data support a role for glucosylceramide-to-lactocylceramide conversion in iNKT cell activation. The blockade of lactosylceramide production may possibly improve ligand availability increasing the iNKT cell activating capacity of adipocytes. These data underscore the role of adipocytes as lipid antigen-presenting cells, driving adipose tissue immune homeostasis through modulating iNKT cell responses [26,29,34–36].

As we, and others have suggested a role for iNKT cells in the prevention of insulin resistance [24,25,29,31,34], inhibition of the glucosylceramide biosynthesis pathway, which we show in the present study to result in altered iNKT cell activity, may be predicted to impair insulin sensitivity. We did not observe a significant impact on insulin sensitivity in our lean animals upon AMP-DNM treatment ([47] and data not shown). An important difference between the previous and the present study lies in the experimental set-up. Data showing the protective role of iNKT cells was largely obtained from experiments in genetic $CD1d^{-/-}$ and $J\alpha18^{-/-}$ knockout models, while a short term (6 weeks) partial inhibition of a key enzyme in adult mice was performed in the present study. It is therefore possible that more effective inhibition of Ugcg (i.e. higher dose or prolonged treatment) would not only inhibit iNKT cell activity as observed here but would eventually also lead to reduced AT-resident iNKT cell numbers and result in insulin resistance. It should

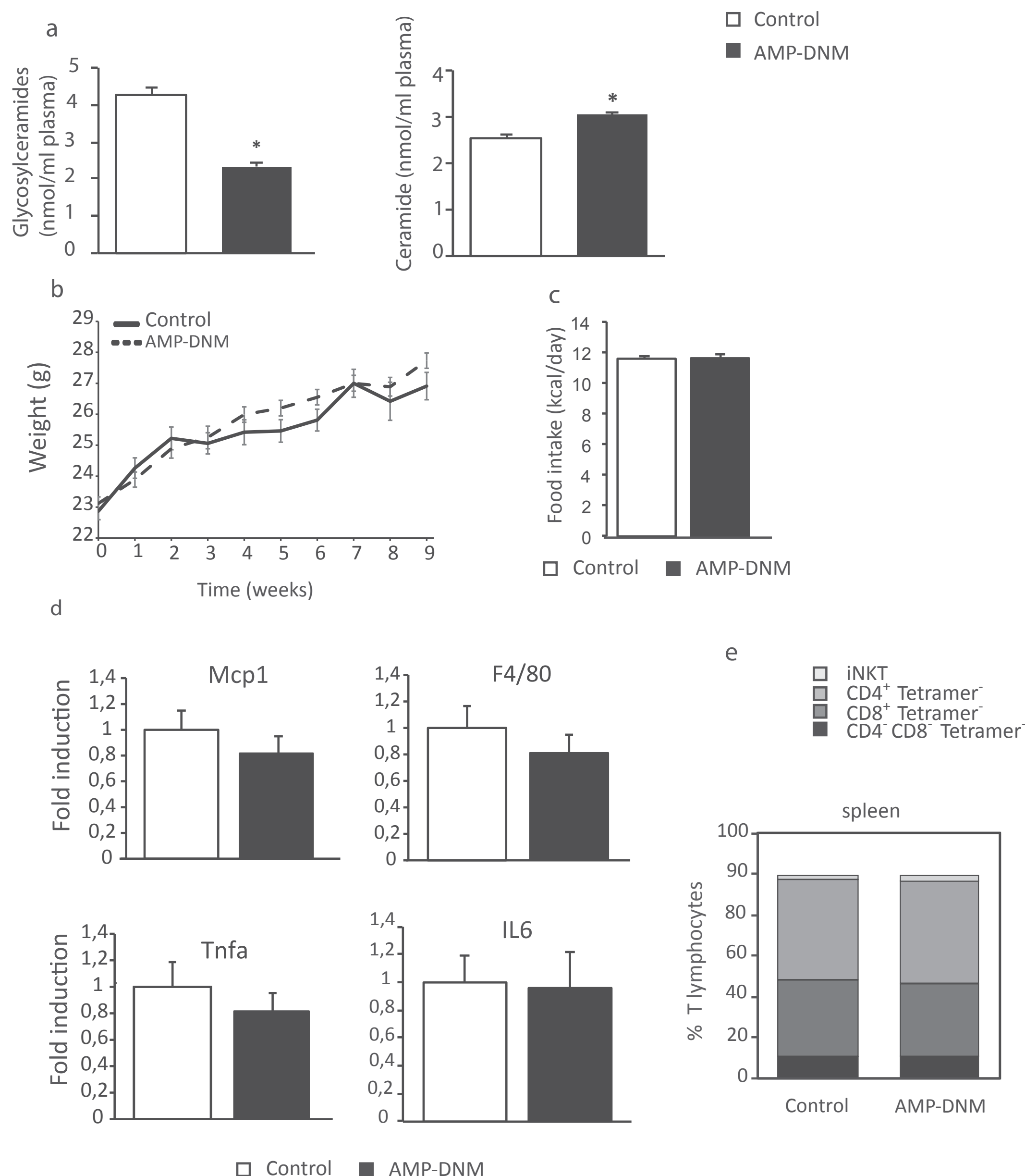

Fig. 3. Ugcg inhibition by AMP-DNM in vivo (a) Plasma and adipose tissue ceramide and glucosylceramide concentrations of mice on control and AMP-DNM diet by HPLC analysis of orthophtaldehyde-conjugated lipids. N = 10 mice per group, total 20 mice. (b) Weight gain of the mice on control and AMP-DNM diet. Mice were weighed weekly. (c) Weekly caloric intake of the mice on control and AMP-DNM diet. n = 10 mice per group, total 20 mice. (d) Adipose tissue mRNA expression of *Mcp1*, *F4/80*, *Tnfa* and *Il6*, analyzed by quantitative RT-PCR. Data are presented as mean ± S.E. (n = 3). Expression levels were normalized for housekeeping gene TFIIB, mean expression in control diet animals was set at 1. (f) Distribution of different of TCRβ positive immune cells in the spleen upon AMP-DNM (10 μM) treatment.

however be noted that inhibition of glucosylceramide biosynthesis can improve insulin sensitivity by decreasing adipose tissue inflammation and improving adipocyte function, at least in obese animals [45,47]. Moreover, ceramide metabolites are key players in multiple immunometabolic pathways, which makes it difficult to predict their net effect on whole body insulin sensitivity [6,56]. For example, ceramide itself may play a dual role, antagonizing insulin action in the short-term, but in the long-term its anti-anabolic effects may contribute to improved glucose tolerance [49]. It is therefore possible that potential insulin-desensitizing effects of glucosylceramide biosynthesis inhibition (e.g. impaired iNKT cell activity) are counterbalanced by insulin-sensitizing effects (e.g. improved adipocyte function), resulting in a minor net effect on whole body insulin sensitivity under our experimental conditions.

Unraveling Ugcg function may be key to untangling the metabolic effects of iNKT cells, ceramide and glucosylceramides, which appear to be intertwined. Ugcg is considered the only genomic glucosylceramide synthase producing glucosylceramides, and as such is a pivotal enzyme in glycosphingolipid metabolism [6,37]. While Ugcg exclusively functions as an inverting glucosyltransferase, i.e. transferring glucose to ceramide in a β-anomeric linkage, α-glucosylceramides are naturally occurring in mammalian milk and serum as well [20,21]. Recent studies suggest that these α-linked glycolipids, although present in low concentrations, serve as the main endogenous lipid antigens for iNKT cells in thymus and periphery [20]. The essential role of α-linked glycolipids is supported by the NKT cell hyperstimulation phenotype observed in patients with α-galactosidase (GLA) deficiency, also known as Fabry disease. The catabolic enzyme GLA controls degradation of α-linked

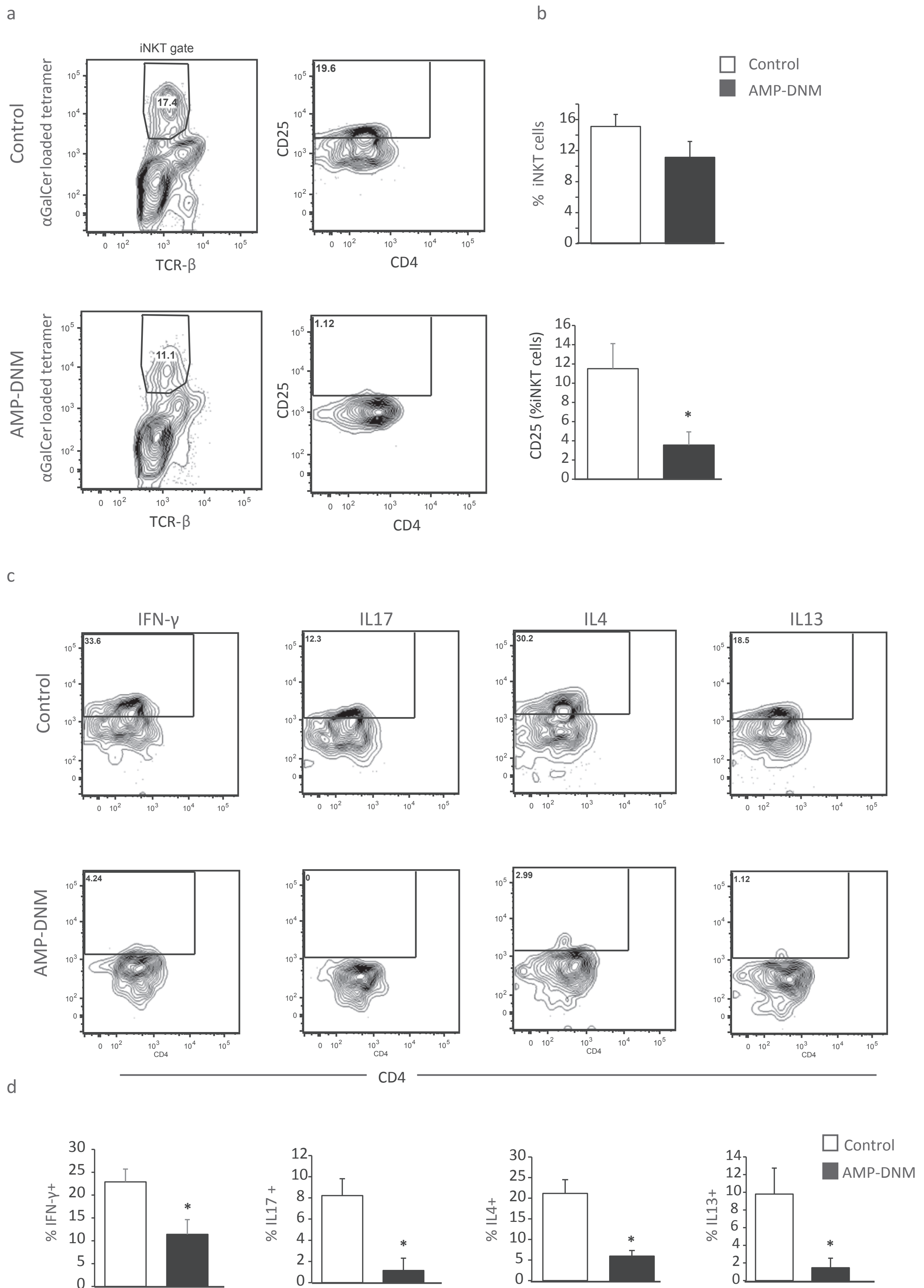

Fig. 4. Inhibition of endogenous glycosphingolipid production results in decreased iNKT cell effector function in adipose tissue. (a) Left panel, number of iNKT cells

(gated on TCRβ and CD1d/αGC-loaded tetramer) as % of T cells in adipose tissue of mice fed control or AMP-DNM diet. Right panel, CD25 staining of adipose tissue extracted iNKTs. Shown are representative flow cytometric plots. Numbers in graphs indicate the percentage of cells in that gate. (b) Number of iNKT cells and $CD25^+$ iNKT cells in adipose tissue of mice on control and AMP-DNM diet. Graphs show averages of n = 4 mice per group. (c) Representative histograms of intracellular cytokine staining of AT-extracted iNKTs in control and AMP-DNM diet groups. Numbers in graphs indicate the percentage of iNKT cells in that gate. (d) Percentage of cytokine-positive iNKT cells (gated on TCRβ and CD1d/αGC-loaded tetramer) in adipose tissue of mice fed control or AMP-DNM diet.
(n = 5–6, mice per group).

glycolipids, and its absence in Fabry disease is associated with increased amounts of self-ligands at the surface of antigen-presenting cells in thymus and periphery [57], resulting in an altered iNKT phenotype and ultimately reduced iNKT cell numbers [57,58]. These findings indicate that the delicate balance between the various enzymes in the biosynthetic pathway needs to be maintained for proper iNKT cell development, proliferation and survival. Deciphering the biosynthetic origin of these α-linked glycolipids seems key to unravel the metabolic effects of endogenous lipid antigens on iNKT cells. Finally, delineating endogenous lipid antigen processing, including environmental cues that potentially inhibit or stimulate this pathway, or influence the specific identity of antigens that are being presented, may reveal novel therapeutic options not only for obesity-induced type 2 diabetes, but also other disorders in which iNKT cells have been implicated [59].

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Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

M.R. R.J.v.E, T.L.G., C.d.H., S.M.W.G., N.H., M.J.F., J.M.F.G.A., H.S.S., M.v.E., M.B. and E.K. designed the experiments; M.R. T.L.G., C.d.H., S.M.W.G., N.H., performed experiments and analyzed the data; M.R. and H.S.S. drafted the manuscript; M.R. and E.K. edited and revised the manuscript; all authors approved the final version of the manuscript.

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Declaration of interest

None.

References

[1] M.A. Exley, L. Hand, D. O'Shea, L. Lynch, Interplay between the immune system and adipose tissue in obesity, J. Endocrinol. 223 (2014) R41–R48.
[2] R.J. van Eijkeren, O. Krabbe, M. Boes, H.S. Schipper, E. Kalkhoven, Endogenous lipid antigens for invariant natural killer T cells hold the reins in adipose tissue homeostasis, Immunology 153 (2018) 179–189.
[3] G. Cildir, S.C. Akincilar, V. Tergaonkar, Chronic adipose tissue inflammation: all immune cells on the stage, Trends Mol. Med. 19 (2013) 487–500.
[4] H.S. Schipper, B. Prakken, E. Kalkhoven, M. Boes, Adipose tissue-resident immune cells: key players in immunometabolism, Trends Endocrinol. Metab. 23 (2012) 407–415.
[5] J.A. Chavez, S.A. Summers, A ceramide-centric view of insulin resistance, Cell Metab. 15 (2012) 585–594.
[6] J.M. Aerts, R.G. Boot, M. van Eijk, J. Groener, N. Bijl, E. Lombardo, F.M. Bietrix, N. Dekker, A.K. Groen, R. Ottenhoff, C. van Roomen, J. Aten, M. Serlie, M. Langeveld, T. Wennekes, H.S. Overkleeft, Glycosphingolipids and insulin resistance, Adv. Exp. Med. Biol. 721 (2011) 99–119.
[7] M. Nagafuku, T. Sato, S. Sato, K. Shimizu, T. Taira, J. Inokuchi, Control of homeostatic and pathogenic balance in adipose tissue by ganglioside GM3, Glycobiology 25 (2015) 303–318.
[8] R.M. McEwen-Smith, M. Salio, V. Cerundolo, CD1d-dependent endogenous and exogenous lipid antigen presentation, Curr. Opin. Immunol. 34 (2015) 116–125.
[9] D.I. Godfrey, D.G. Pellicci, O. Patel, L. Kjer-Nielsen, J. McCluskey, J. Rossjohn, Antigen recognition by CD1d-restricted NKT T cell receptors, Semin. Immunol. 22 (2010) 61–67.
[10] A. Bendelac, P.B. Savage, L. Teyton, The biology of NKT cells, Annu. Rev. Immunol. 25 (2007) 297–336.
[11] D.I. Godfrey, H.R. MacDonald, M. Kronenberg, M.J. Smyth, L. Van Kaer, NKT cells: what's in a name? Nat. Rev. Immunol. 4 (2004) 231–237.
[12] S. Sharif, G.A. Arreaza, P. Zucker, Q.S. Mi, J. Sondhi, O.V. Naidenko, M. Kronenberg, Y. Koezuka, T.L. Delovitch, J.M. Gombert, M. Leite-De-Moraes, C. Gouarin, R. Zhu, A. Hameg, T. Nakayama, M. Taniguchi, F. Lepault, A. Lehuen, J.F. Bach, A. Herbelin, Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes, Nat. Med. 7 (2001) 1057–1062.
[13] D. Sprengers, F.C. Sille, K. Derkow, G.S. Besra, H.L. Janssen, E. Schott, M. Boes, Critical role for CD1d-restricted invariant NKT cells in stimulating intrahepatic CD8 T-cell responses to liver antigen, Gastroenterology 134 (2008) 2132–2143.
[14] J.B. Swann, J.M. Coquet, M.J. Smyth, D.I. Godfrey, CD1-restricted T cells and tumor immunity, Curr. Top. Microbiol. Immunol. 314 (2007) 293–323.
[15] L. Wu, L. Van Kaer, Natural killer T cells and autoimmune disease, Curr. Mol. Med. 9 (2009) 4–14.
[16] F.A. Ververs, E. Kalkhoven, B. Van't Land, M. Boes, H.S. Schipper, Immunometabolic activation of invariant natural killer T cells, Front. Immunol. 9 (2018) 1192.
[17] M. Morita, K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi, H. Fukushima, Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice, J. Med. Chem. 38 (1995) 2176–2187.
[18] Y. Kinjo, D. Wu, G. Kim, G.W. Xing, M.A. Poles, D.D. Ho, M. Tsuji, K. Kawahara, C.H. Wong, M. Kronenberg, Recognition of bacterial glycosphingolipids by natural killer T cells, Nature 434 (2005) 520–525.
[19] J. Mattner, K.L. Debord, N. Ismail, R.D. Goff, C. Cantu 3rd, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, D. Walker, B. Beutler, L. Teyton, P.B. Savage, A. Bendelac, Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections, Nature 434 (2005) 525–529.
[20] L. Kain, B. Webb, B.L. Anderson, S. Deng, M. Holt, A. Constanzo, M. Zhao, K. Self, A. Teyton, C. Everett, M. Kronenberg, D.M. Zajonc, A. Bendelac, P. Savage, L. Teyton, The Identification of the endogenous ligands of natural killer T cells reveals the presence of mammalian α-linked glycosylceramides, Immunity 41 (2014) 543–554.
[21] P.J. Brennan, R.V.V. Tatituri, C. Heiss, G.F.M. Watts, F.-F. Hsu, N. Veerapen, L.R. Cox, P. Azadi, G.S. Besra, M.B. Brenner, Activation of iNKT cells by a distinct constituent of the endogenous glucosylceramide fraction, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 13433–13438.
[22] P.J. Brennan, R.V. Tatituri, M. Brigl, E.Y. Kim, A. Tuli, J.P. Sanderson, S.D. Gadola, F.F. Hsu, G.S. Besra, M.B. Brenner, Invariant natural killer T cells recognize lipid

self antigen induced by microbial danger signals, Nat. Immunol. 12 (2011) 1202–1211.
[23] P.J. Brennan, T.Y. Cheng, D.G. Pellicci, G.F.M. Watts, N. Veerapen, D.C. Young, J. Rossjohn, G.S. Besra, D.I. Godfrey, M.B. Brenner, D.B. Moody, Structural determination of lipid antigens captured at the CD1d-T-cell receptor interface, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 8348–8353.
[24] Y. Ji, S. Sun, A. Xu, P. Bhargava, L. Yang, K.S. Lam, B. Gao, C.H. Lee, S. Kersten, L. Qi, Activation of natural killer T cells promotes M2 Macrophage polarization in adipose tissue and improves systemic glucose tolerance via interleukin-4 (IL-4)/STAT6 protein signaling axis in obesity, J. Biol. Chem. 287 (2012) 13561–13571.
[25] Y. Ji, S. Sun, S. Xia, L. Yang, X. Li, L. Qi, Short term high fat diet challenge promotes alternative macrophage polarization in adipose tissue via natural killer T cells and interleukin-4, J. Biol. Chem. 287 (2012) 24378–24386.
[26] J.Y. Huh, J.I. Kim, Y.J. Park, I.J. Hwang, Y.S. Lee, J.H. Sohn, S.K. Lee, A.A. Alfadda, S.S. Kim, S.H. Choi, D.S. Lee, S.H. Park, R.H. Seong, C.S. Choi, J.B. Kim, A novel function of adipocytes in lipid antigen presentation to iNKT cells, Mol. Cell. Biol. 33 (2013) 328–339.
[27] M.E. Kotas, H.Y. Lee, M.P. Gillum, C. Annicelli, B.A. Guigni, G.I. Shulman, R. Medzhitov, Impact of CD1d deficiency on metabolism, PLoS One 6 (2011) e25478.
[28] K. Ohmura, N. Ishimori, Y. Ohmura, S. Tokuhara, A. Nozawa, S. Horii, Y. Andoh, S. Fujii, K. Iwabuchi, K. Onoe, H. Tsutsui, Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 193–199.
[29] H.S. Schipper, M. Rakhshandehroo, S.F. van de Graaf, K. Venken, A. Koppen, R. Stienstra, S. Prop, J. Meerding, N. Hamers, G. Besra, L. Boon, E.E. Nieuwenhuis, D. Elewaut, B. Prakken, S. Kersten, M. Boes, E. Kalkhoven, Natural killer T cells in adipose tissue prevent insulin resistance, J. Clin. Invest. (2012).
[30] L. Wu, V.V. Parekh, C.L. Gabriel, D.P. Bracy, P.A. Marks-Shulman, R.A. Tamboli, S. Kim, Y.V. Mendez-Fernandez, G.S. Besra, J.P. Lomenick, B. Williams, D.H. Wasserman, L. Van Kaer, Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) E1143–E1152.
[31] L. Lynch, M. Nowak, B. Varghese, J. Clark, A.E. Hogan, V. Toxavidis, S.P. Balk, D. O'Shea, C. O'Farrelly, M.A. Exley, Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production, Immunity 37 (2012) 574–587.
[32] B.V. Martin-Murphy, Q. You, H. Wang, B.A. De La Houssaye, T.P. Reilly, J.E. Friedman, C. Ju, Mice lacking natural killer T cells are more susceptible to metabolic alterations following high fat diet feeding, PLoS One 9 (2014).
[33] M. Rakhshandehroo, E. Kalkhoven, M. Boes, Invariant natural killer T cells in adipose tissue: novel regulators of immune-mediated metabolic disease, Cell. Mol. Life Sci. 70 (2013) 4711–4727.
[34] J.Y. Huh, J. Park, J.I. Kim, Y.J. Park, Y.K. Lee, J.B. Kim, Deletion of CD1d in adipocytes aggravates adipose tissue inflammation and insulin resistance in obesity, Diabetes 66 (2017) 835–847.
[35] M. Satoh, M. Hoshino, K. Fujita, M. Iizuka, S. Fujii, C.S. Clingan, L. Van Kaer, K. Iwabuchi, Adipocyte-specific CD1d-deficiency mitigates diet-induced obesity and insulin resistance in mice, Sci. Rep. 6 (2016) 28473.
[36] M. Rakhshandehroo, S.M. Gijzel, R. Siersbaek, M.F. Broekema, C. de Haar, H.S. Schipper, M. Boes, S. Mandrup, E. Kalkhoven, CD1d-mediated presentation of endogenous lipid antigens by adipocytes requires microsomal triglyceride transfer protein, J. Biol. Chem. 289 (2014) 22128–22139.
[37] S. Ichikawa, H. Sakiyama, G. Suzuki, K.I. Hidari, Y. Hirabayashi, Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 12654.
[38] T. Yamashita, R. Wada, T. Sasaki, C. Deng, U. Bierfreund, K. Sandhoff, R.L. Proia, A vital role for glycosphingolipid synthesis during development and differentiation, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 9142–9147.
[39] M. Amado, R. Almeida, T. Schwientek, H. Clausen, Identification and characterization of large galactosyltransferase gene families: galactosyltransferases for all functions, Biochim. Biophys. Acta 1473 (1999) 35–53.
[40] N. Tokuda, S. Numata, X. Li, T. Nomura, M. Takizawa, Y. Kondo, Y. Yamashita, N. Hashimoto, T. Kiyono, T. Urano, K. Furukawa, K. Furukawa, beta4GalT6 is involved in the synthesis of lactosylceramide with less intensity than beta4GalT5, Glycobiology 23 (2013) 1175–1183.
[41] T. Kumagai, M. Tanaka, M. Yokoyama, T. Sato, T. Shinkai, K. Furukawa, Early lethality of beta-1,4-galactosyltransferase V-mutant mice by growth retardation, Biochem. Biophys. Res. Commun. 379 (2009) 456–459.
[42] T. Kumagai, T. Sato, S. Natsuka, Y. Kobayashi, D. Zhou, T. Shinkai, S. Hayakawa, K. Furukawa, Involvement of murine beta-1,4-galactosyltransferase V in lactosylceramide biosynthesis, Glycoconj. J. 27 (2010) 685–695.
[43] O. Lantz, A. Bendelac, An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans, J. Exp. Med. 180 (1994) 1097–1106.
[44] H.S. Overkleeft, G.H. Renkema, J. Neele, P. Vianello, I.O. Hung, A. Strijland, A.M. van der Burg, G.J. Koomen, U.K. Pandit, J.M. Aerts, Generation of specific deoxynojirimycin-type inhibitors of the non-lysosomal glucosylceramidase, J. Biol. Chem. 273 (1998) 26522–26527.
[45] J.M. Aerts, R. Ottenhoff, A.S. Powlson, A. Grefhorst, M. van Eijk, P.F. Dubbelhuis, J. Aten, F. Kuipers, M.J. Serlie, T. Wennekes, J.K. Sethi, S. O'Rahilly, H.S. Overkleeft, Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity, Diabetes 56 (2007) 1341–1349.
[46] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[47] M. van Eijk, J. Aten, N. Bijl, R. Ottenhoff, C.P. van Roomen, P.F. Dubbelhuis, I. Seeman, K. Ghauharali-van der Vlugt, H.S. Overkleeft, C. Arbeeny, A.K. Groen, J.M. Aerts, Reducing glycosphingolipid content in adipose tissue of obese mice restores insulin sensitivity, adipogenesis and reduces inflammation, PLoS One 4 (2009) e4723.
[48] K. Venken, S. Seeuws, L. Zabeau, P. Jacques, T. Decruy, J. Coudenys, E. Verheugen, F. Windels, D. Catteeuw, M. Drennan, S. Van Calenbergh, B.N. Lambrecht, A. Yoshimura, J. Tavernier, D. Elewaut, A bidirectional crosstalk between iNKT cells and adipocytes mediated by leptin modulates susceptibility for T cell mediated hepatitis, J. Hepatol. 60 (2014) 175–182.
[49] B.T. Bikman, S.A. Summers, Ceramides as modulators of cellular and whole-body metabolism, J. Clin. Invest. 121 (2011) 4222–4230.
[50] J.A. Chavez, M.M. Siddique, S.T. Wang, J. Ching, J.A. Shayman, S.A. Summers, Ceramides and glucosylceramides are independent antagonists of insulin signaling, J. Biol. Chem. 289 (2014) 723–734.
[51] B.L. Anderson, L. Teyton, A. Bendelac, P.B. Savage, Stimulation of natural killer T cells by glycolipids, Molecules 18 (2013) 15662–15688.
[52] Y. Ishibashi, A. Kohyama-Koganeya, Y. Hirabayashi, New insights on glucosylated lipids: metabolism and functions, Biochim. Biophys. Acta 1831 (2013) 1475–1485.
[53] M.M. Venkataswamy, S.A. Porcelli, Lipid and glycolipid antigens of CD1d-restricted natural killer T cells, Semin. Immunol. 22 (2010) 68–78.
[54] Y.K. Park, J.W. Lee, Y.G. Ko, S. Hong, S.H. Park, Lipid rafts are required for efficient signal transduction by CD1d, Biochem. Biophys. Res. Commun. 327 (2005) 1143–1154.
[55] J.C. Lu, Y.T. Chiang, Y.C. Lin, Y.T. Chang, C.Y. Lu, T.Y. Chen, C.S. Yeh, Disruption of Lipid Raft Function Increases Expression and Secretion of Monocyte Chemoattractant Protein-1 in 3T3-L1 Adipocytes, PLoS One 11 (2016) e0169005.
[56] M. Maceyka, S. Spiegel, Sphingolipid metabolites in inflammatory disease, Nature 510 (2014) 58–67.
[57] C.S. Pereira, O. Azevedo, M.L. Maia, A.F. Dias, C. Sa-Miranda, M.F. Macedo, Invariant natural killer T cells are phenotypically and functionally altered in Fabry disease, Mol. Genet. Metab. 108 (2013) 241–248.
[58] W. Mauhin, O. Lidove, E. Masat, F. Mingozzi, K. Mariampillai, J.M. Ziza, O. Benveniste, Innate and Adaptive Immune Response in Fabry Disease, JIMD Rep 22 (2015) 1–10.
[59] A.K. Singh, P. Tripathi, S.L. Cardell, Type II NKT cells: an elusive population with immunoregulatory properties, Front. Immunol. 9 (2018) 1969.