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

Yawei Ji, Shengyi Sun, Aimin Xu, Prerna Bhargava, Liu Yang, Karen S. L. Lam, Bin Gao, Chih-Hao Lee, Sander Kersten, and Ling Qi

From the Division of Nutritional Sciences, Graduate Program in Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853, the Department of Medicine and Center for Heart, Brain, Hormone, and Healthy Aging, University of Hong Kong, Hong Kong, China, the Departments of Genetics and Complex Diseases and Nutrition, Harvard University School of Public Health, Boston, Massachusetts 02115, the Laboratory on Alcohol Abuse and Alcoholism, Rockville, Maryland 20852, and the Nutrition, Metabolism, and Genomics Group, Division of Human Nutrition, Wageningen University, 6700EV, Wageningen, The Netherlands

Background: Obesity is associated with a state of chronic low grade inflammation. Natural killer T (NKT) cells are important therapeutic targets in various disease models and are under clinical trials for cancer patients. However, their function in obesity and type 2 diabetes remains unclear. Our data show that adipose tissues of both mice and humans contain a population of type 1 NKT cells, whose abundance decreases with increased adiposity and insulin resistance. Although loss-of-function of NKT cells had no effect on glucose tolerance in animals with prolonged high fat feeding, activation of NKT cells by lipid agonist α-galactosylceramide enhances alternative macrophage polarization in adipose tissue and improves glucose homeostasis in animals at different stages of obesity. Furthermore, the effect of NKT cells is largely mediated by the IL-4/STAT6 signaling axis in obese adipose tissue. Thus, our data identify a novel therapeutic target for the treatment of obesity-associated inflammation and type 2 diabetes.

Results: Activation of natural killer T (NKT) cells attenuates inflammation in adipose tissue and improves systemic glucose homeostasis in mice at different stages of obesity.

Conclusion: Upon activation, NKT cells have significant impact on inflammatory responses and systemic glucose tolerance in obesity.

Significance: NKT-activating glycolipids may be useful in treating obesity-associated complications.

Natural killer T (NKT) cells have been implicated in autoimmune, microbial infection, and cancer and hence represent an important immunotherapeutic target (1). Unlike conventional CD4+ and CD8+ T cells, NKT cells recognize and are activated by lipid antigens presented by the MHC class I homologue molecule CD1d on antigen presenting cells such as macrophages and dendritic cells (2–5). Among different types of NKT cells, type 1 or invariant NKT cells are the most abundant and best characterized (6). The prototypical lipid antigen is the marine sponge-derived α-galactosylceramide (αGalCer) (7), which is not found in mammals (7) and has been used widely to specifically study type 1 NKT cells in vivo (8). Upon αGalCer activation, NKT cells secrete large amounts of TH1 cytokines, IFN-γ and TNF-α, and TH2 cytokines, IL-4 and IL-13 (9). αGalCer challenge has been shown to protect against the development of type 1 diabetes (10) and autoimmune encephalomyelitis (11), although some of these findings remain controversial (8).

The ability of NKT cells to secrete both TH1 and TH2 cytokines upon activation underlies their unique regulatory functions that bridge innate and adaptive immunity (8, 12). It is important to note that secretion of TH1 and/or TH2 cytokines by NKT cells is context-dependent (8, 12) as the nature of the lipid antigens, the subsets of NKT cells, and the microenvironment of the tissues may have significant influences on their cytokine profiles (11, 13). Indeed, studies have shown that NKT cells may promote or suppress immune processes by skewing adaptive immune responses toward either a TH1 or TH2 response (8, 14). However, whether and how NKT cell activation affects obesity-associated inflammation remains to be characterized.
Targeting NKT Cells in Obesity

Obesity is associated with a state of chronic low-grade inflammation that significantly contributes to the pathogenesis of this disorder and its associated complications. At late stages of obesity, a variety of immune cells, most notably macrophages (15, 16), CD8+ T (17, 18), mast cells (19), B cells (20), and myeloid-derived suppressor cells with immunosuppressive functions (21) infiltrate adipose tissue during diet-induced obesity, with concurrent down-regulation of other immune cells such as regulatory T cells (22). Some of these immune cells may affect the polarization of macrophages to classical (M1) or alternative (M2) activation status via directly or indirectly influencing the local T11 or T12 cytokines in the adipose microenvironment (23–25). Unlike M1, M2 macrophages may contribute to improved insulin sensitivity due to their capacity to resolve inflammation (i.e. anti-inflammation) and facilitate wound healing (25–28). Bias toward M2 polarization can be promoted by immunomodulatory T12 cytokines such as IL-4 and IL-13. Studies have identified adipocytes, CD4+ T cells, and eosinophils in adipose tissue as a potential source of T12 cytokines (29, 30).

An early study reported the presence of NKT cells in adipose tissue, whose abundance seems decreased with obesity (31). Two recent studies demonstrated the lack of metabolic effect in NKT-deficient CD1d−/− mice following long term HFD feeding (32, 33). In light of these results, we asked whether gain-of-function of NKT cells affects obesity-associated glucose homeostasis. Here, we show that αGalCer-mediated activation of NKT cells enhances alternative macrophage polarization in adipose tissue and improves glucose homeostasis in animals at different stages of obesity following the feeding of 60% HFD for 4 days and 8 and 24 weeks. The beneficial effect of NKT cell activation is largely mediated by the IL-4 signaling pathway. Pointing to their significance in human obesity, levels of adipose tissue and improves glucose homeostasis in animals at

**EXPERIMENTAL PROCEDURES**

**Mouse Models**—WT C57/B6 (catalog no. 000664), B6.V-Leprob/J (ob/ob, catalog no. 000632), B6.129S6-Cd1d1/J (CD1d1+/+13580) (CD1d−/−, catalog no. 008881), and B6.129P2-IL4tm1Cgn/J (IL-4−/−, catalog no. 002253) were purchased from The Jackson Laboratory and bred at our facility. The latter three have been backcrossed at The Jackson Laboratory to the B6 background over 45, 13, and 12 times, respectively. Myeloid cell-specific PPARγ−/− or PPARδ−/− mice were generated by crossing Pparγfloxed/floxed or Pparδfloxed/floxed mice to the B6.129P2-Ly22m1Cgo/J mice (catalog no. 004781, The Jackson Laboratory) (29) and have been backcrossed to the C57BL/6 background over 10 times. Mice were housed in microisolators in our brand new pathogen-free facility with sterile 13% LFD with 13% fat, 67% carbohydrate, and 20% protein from Harlan Teklad (catalog no. 2914) or 60% HFD with 59% fat, 26% carbohydrate, and 15% protein from Bio-Serv Inc. (F3282). All animal procedures were approved by the Cornell and Harvard IACUC.

**Human Subjects**—39 healthy premenopausal adult Chinese women, including 25 lean individuals with BMI <25 kg/m2 and 14 overweight/obese subjects with BMI ≥25 kg/m2, undergoing elective abdominal surgery for benign, noninfective gynecological conditions at Queen Mary’s hospital, University of Hong Kong, were recruited. Pre-operative assessment was carried out within 1 week of the operation. Anthropometric parameters (body weight, height, waist circumference, and blood pressure) were measured, and body composition was determined by bioelectric impedance analysis (Tanita Body Composition Analyzer TBF-410, Japan). All subjects underwent an oral glucose tolerance test (OGTT) with 75 g of glucose. Fasting plasma glucose and 2-h glucose levels at OGTT were measured by hexokinase method on a Hitachi 747 analyzer (Roche Applied Science). Insulin was measured by microparticle enzyme immunoassay (Abbott). HOMA index was calculated to estimate insulin resistance (IR): HOMA-IR = fasting glucose (mmol/l) × fasting insulin (mIU/liter)/22.5 (35). During the operation, visceral adipose tissues (about 8 cm3 each) were collected aseptically and transported immediately to the laboratory for RNA extraction and Q-PCR analysis (see below). The study was approved by the Ethics Committee of the University of Hong Kong and adhered to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

**Animal Experiments**—6-Week-old male mice were fed either 13% LFD or 60% HFD for an indicated period of time from 4 days to 24 weeks. αGalCer (Toronto Research Chemicals) was dissolved in pyridine at 2.5 mg/ml and then diluted 1:250 in PBS to prepare 10 µg/ml working solution prior to use. In all experiments, mice were injected intraperitoneally with 200 µl of αGalCer (100 ng of αGalCer per g body weight). The vehicle control group was injected with PBS with 0.4% pyridine. The dosage and frequency of αGalCer injection were the same for all the gain-of-function experiments unless indicated. HFD mice were given intraperitoneal αGalCer or vehicle at day 0 and day 2. One day following the GTT (day 4), adipose tissues were harvested, frozen for Q-PCR or Western blot, and fixed for H&E staining. Flow cytometric analyses of NKT levels and other immune cells were performed on day 4. For GTT, mice were fasted for 16–18 h followed by injection of glucose (Sigma) at 1 g/kg body weight. Blood glucose was monitored using One-Touch Ultra Glucometer. Fasting insulin levels were measured following a 6-h fast.

**Antibodies and Reagents for Flow Cytometry**—Fluorochrome- or biotin-conjugated antibodies against CD3 (145-2C11), TCRβ (H57-597), CD4 (GK1.5), CD8 (YTS169), F4/80 (BM8), CD11b (M1/70), CD45 (30-F11), BrdU-FITC (PRB-1), avidin-PerCP, and isotype control antibodies were purchased from BioLegend, University of California San Francisco Flow Core Facility or BD Biosciences. αGalCer-loaded CD1d-tetramer-phycoerythrin was generously provided by the Tetramer Facility, National Institutes of Health. Data were analyzed using CellQuest software (BD Biosciences) and Flowjo.

**Quantitation of Immune Cells in Adipose Tissue Using Flow Cytometric Analysis**—Single cell suspensions from stromal vascular cells of adipose tissue were prepared as described (21). Stromal vascular cells from two fat pads per mouse were diluted

**Cytometric Analysis**—Cells were stained with antibodies against CD3 (145-2C11), TCRβ (H57-597), CD4 (GK1.5), CD8 (YTS169), F4/80 (BM8), CD11b (M1/70), CD45 (30-F11), BrdU-FITC (PRB-1), avidin-PerCP, and isotype control antibodies. The data were analyzed using CellQuest software (BD Biosciences) and Flowjo.

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in 120–200 μl of PBS, from which one-tenth was used for the subsequent staining. Following incubation with anti-CD16/ CD32 antibody to block Fc receptors, 1 × 10⁶ cells were incubated with 20 μl of antibodies diluted at optimal concentrations for 20 min at 4 °C. Cells were washed three times with PBS and then resuspended in 200 μl of PBS for analysis using the FACSCalibur flow cytometer (BD Biosciences). NKT cells were defined as CD45⁺ αGalCer-loaded CD1d-tetramer⁺ CD3/ TCRβ⁺ lymphocytes (supplemental Fig. S1A); CD8⁺ T cells were defined as CD8⁺ CD45⁺ cells (supplemental Fig. S1B), and macrophages were as CD45⁺ F4/80⁺ CD11b⁺ cells. During the run, samples were completely run out, and the total number of CD45⁺ lymphocytes or immune cells was gated and counted. The total cell number for various immune cells per g of adipose tissue was calculated as (% cells in total CD45⁺ lymphocytes or immune cells × total CD45⁺ lymphocytes or immune cells)/g of adipose tissue.

Intracellular Flow Cytometric Analysis—For the BrdU staining, mice were injected (intraperitoneally) with αGalCer (100 ng/g of body weight) at day 0 and then with BrdU (Sigma, 0.6 mg/10 g of body weight) at 36 and 12 h prior to sacrifice at day 3. Stromal vascular cells of adipose tissues were purified, labeled with cell surface antibodies, and then fixed in cold 70% ethanol at −20 °C overnight. The rest of steps were performed as the regular flow cytometric analysis using BrdU-FITC antibody and DNA dye 7-aminoactinomycin D (Anaspec).

RNA Extraction and Q-PCR—RNA extraction from cells and murine tissues and Q-PCR were carried out as described previously (36) using TRIzol (Invitrogen) for liver and TRIzol plus QIAeasy kit (Qiagen) for adipose tissues with DNase digestion (Roche Applied Science). Q-PCR data collected on the Light-Cycler 480 (Roche Applied Science) were normalized to ribosomal l32 gene in the corresponding sample. Primer sequences are listed in supplemental Table S1.

Analysis of NKT Cells in Human Adipose Tissues by Q-PCR—Following extraction using the Qiagen mini-RNA purification kit, 1 μg of total RNA from each sample was reverse-transcribed. The relative abundance of each gene was determined by Q-PCR on a Prism 7000 sequence detection system (Applied Biosystems) and was normalized against 18 S rRNA. Two primers were used in the analysis: one detects the splicing event of TCRαβ chain constant region, chromosome 14) (37), and the other detects the use of TCRγ chain constant 2, chromosome 7) (38) was used as a control for all T cells. Primer sequences are listed in supplemental Table S1.

Table 1

| Overweight/obese subjects (n = 14) | Lean subjects (n = 25) | p value |
|-----------------------------------|-----------------------|---------|
| Age                              | 44.9 ± 5.6 years      | 42.5 ± 7.3 years | 0.089 |
| BMI                               | 28.6 ± 3.3 kg/m²      | 21.7 ± 2.1 kg/m² | <0.001 |
| Fasting glucosea                  | 4.8 ± 0.5 mmol/liter  | 4.7 ± 0.4 mmol/liter | 0.363 |
| 2-h glucose                       | 7.5 ± 1.0 mmol/liter  | 5.8 ± 0.9 mmol/liter | <0.01 |
| Fasting insulinb                  | 0.9 ± 0.4 mIU/liter   | 3.8 ± 0.6 mIU/liter | 0.016 |
| HOMA-IRc                         | 1.8 ± 0.3             | 1.2 ± 0.2         | <0.01 |

* BMI is BMI cutoff from lean and overweight/obese is 25.
* Fasting glucose/insulin is glucose or insulin levels after an overnight 16-h fast.
* 2-h glucose is glucose levels at the end of OGTT, i.e. the 2-h time point.
* HOMA-IR = (fasting glucose (mmol/l) × fasting insulin (mIU/liter))/22.5.
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Microarray Analysis—Microarray analyses of WAT were performed as described previously (39) and in the supplemental material with four groups (n = 3–4 mice each) as follows: WT + vehicle, WT + αGalCer, CD1d−/− + vehicle, and CD1d−/− + αGalCer, all of which were on 4-day HFD feeding with two αGalCer injections at day 0 and day 2. For human studies, statistical analysis was performed with the SPSS 11.5 statistical software package. tigroup comparisons. For human studies, statistical analysis with the Bonferroni post-tests of the PRISM software for mul-
two-tailed Student’s t test of the EXCEL software for two-group exposure.

RESULTS

Adiposity in Two Obese Mouse Models—To study the impact of obesity on NKT cells in adipose tissue, we place 6-week-old B6 mice on an HFD containing 60% calories derived from fat or a 13% LFD as a control cohort (supplemental Fig. S1). Strikingly, Vα24 NKT cells in adipose tissue decreased with BMI (Fig. 2A), whereas total T cells (CD4, CD8, and NKT cells) exhibited an opposite trend (Fig. 2B). Bivariant correlation analysis further revealed that the levels of Vα24-containing mRNA or Va24-associated Va24-Ca splicing event in adipose tissue (supplemental Fig. S2). Strikingly, Va24 NKT cells in adipose tissue decreased with BMI (Fig. 2A), whereas total T cells (CD4, CD8, and NKT cells) exhibited an opposite trend (Fig. 2B).

Levels of Adipose-resident Type 1 NKT Negatively Correlate with Insulin Resistance in Humans—We next analyzed the abundance of NKT cells in visceral adipose tissues collected from 25 lean and 14 overweight/obese Asian female subjects (Table 1). As most CD1d-restricted type 1 human NKT cells express an invariant TCR Vα24-Jβ111 splicing event in adipose tissue, we placed 6-week-old B6 mice on an HFD containing 60% calories derived from fat or a 13% LFD as a control cohort (supplemental Fig. S1). Strikingly, Vα24 NKT cells in adipose tissue decreased with BMI (Fig. 2A), whereas total T cells (CD4, CD8, and NKT cells) exhibited an opposite trend (Fig. 2B). Bivariant correlation analysis further revealed that the levels of Vα24-containing mRNA or Va24-associated Va24-Ca splicing event in adipose tissue (supplemental Fig. S2). Strikingly, Va24 NKT cells in adipose tissue decreased with BMI (Fig. 2A), whereas total T cells (CD4, CD8, and NKT cells) exhibited an opposite trend (Fig. 2B).

AlphaGalCer Injection Activates Adipose-resident NKT Cells and Improves Systemic Glucose Homeostasis in Obesity—In line with two recent studies (32, 33), NKT-deficient CD1d−/− mice exhibited no changes in glucose tolerance following an 8-week exposure.

H&E Histology—Adipose tissues were fixed in 4% formalde-
hyde, embedded in paraffin, and sectioned by the Cornell His
tology Core Facility. Pictures were taken using the Axiowert 200 M microscope (Zeiss).

ELISA—Blood was collected in animals upon 6 h of fasting during the day. Circulating insulin levels were measured using the kit from Millipore per the supplier’s protocols.

Statistical Analysis—Results are expressed as means ± S.E. Comparisons between groups were made using either unpaired two-tailed Student’s t test of the EXCEL software for two-group comparisons or the one- or two-way analysis of variance test with the Bonferroni post-tests of the PRISM software for mul-
tigroup comparisons. For human studies, statistical analysis was performed with the SPSS 11.5 statistical software package. p < 0.05 was considered as statistically significant.

FIGURE 2. Correlation between adipose NKT cells and metabolic parameters in humans. Correlations are shown between Vα24 type 1 NKT cells and BMI (A), total T cells and BMI (B), NKT cells and insulin resistance as measured by HOMA (C), and glucose levels measured at the 2-h point of OGTT (D). NKT cells in visceral adipose tissues of 39 human subjects were analyzed by two oligonucleotide sets: oligos 1–2 measured the Vα24CR mRNA levels of type 1 NKT cells. A control oligo 3 measured the TCR mRNA levels of total T cells. Oligonucleotide positions at the genomic loci of human TCR genes and PCR product sizes are shown in supplemental Fig. S2. a.u., arbitrary units.

Abundance of Adipose-resident Type 1 NKT Decreases with Adiposity in Two Obese Mouse Models—To study the impact of obesity on NKT cells in adipose tissue, we place 6-week-old B6 mice on an HFD containing 60% calories derived from fat or a 13% LFD as a control cohort (supplemental Fig. S1C). HFD feeding progressively increased body and epididymal fat pad weights (Fig. 1A) as well as fasting glucose levels (Fig. 1B). Interestingly, unlike CD8+ T cells, abundance of type 1 NKT cells in adipose tissue, in terms of the percentage in total lymphocytes and total cell number per g of adipose tissue, gradually decreased with HFD (Fig. 1, C and D).

This observation was further confirmed in the adult murine ob/ob genetic obesity model; the percentage of NKT cells in adipose tissues was significantly reduced in ob/ob mice (Fig. 1, E and F). The reduction of NKT in adipose tissue of ob/ob mice was not due to defects in NKT cell development or emigration from the thymus as the level of NKT cells in lymphoid organs such as spleen was not affected by obesity (Fig. 1G). Taken together, these data suggest that HFD progressively decreases the abundance of NKT cells in adipose tissue.

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HFD (supplemental Fig. S3A). However, to address whether these cells can be targeted therapeutically, we took a gain-of-function approach by stimulating NKT cells in vivo with GalCer. Early studies have established that significant expansion of NKT cells was observed from 2 to 3 days up to 7 days after the initial GalCer injection, a process associated with sustained cytokine production up to 7 days (43). Accordingly, we challenged HFD-fed male C57BL/6 mice with two GalCer on days 0 and 2 followed by GTT analysis on day 4 (Fig. 3A). To address the therapeutic effect of NKT cell activation at different stages of obesity, we performed these studies in animals that have been on HFD for 4 days and 8 and 24 weeks, which represent short term, long term, and chronic HFD feeding models, respectively.

Indeed, although GalCer injection had no discernible effect on body and epididymal fat weights (supplemental Fig. S3B), it caused massive proliferation of NKT cells in adipose tissue (Fig. 3B) with over a 4-fold increase in percentage (Fig. 3C) and 70-fold increase in total cell number (supplemental Fig. S4A) in 4-day HFD mice. It also caused milder increases of macrophages and CD8+ T cells (supplemental Fig. S4A). In line with these observations, H&E staining of WAT sections revealed a significant increase in the number of cells surrounding adipocytes following GalCer injection (Fig. 3D) and were abolished by CD1d deficiency (Fig. 3E), suggesting that the expansion of immune cells, including CD8+ T cells and macrophages, is NKT-dependent. Similar observations were made in 8- and 24-week-old HFD mice (Fig. 3, F and G, and supplemental Figs. S3, C and D, and S4, B and C).

Metabolically, GalCer challenge improved systemic glucose tolerance in mice at all stages of the HFD (Fig. 4, A–C) but no effect on fasting insulin levels (Fig. 4D). The GalCer effect on glucose tolerance of HFD mice is NKT cell-dependent as it had no effect in CD1d−/− mice (Fig. 4E). Pointing to the importance of HFD in NKT cell effect, GalCer injection in mice on LFD had no effect on glucose tolerance (Fig. 4F). Thus, NKT cell activation by GalCer improves systemic glucose tolerance in obese animals.

**Activation of NKT Cells Enhances M2 Macrophage Polarization in Adipose Tissue**—To explore possible mechanisms underlying the beneficial effect of NKT cell activation, we assessed the inflammatory status of adipose tissue by examining the status of macrophage polarization. GalCer challenge caused a marked 50–100-fold induction of a subset of M2 genes...
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FIGURE 4. NKT cell activation improves systemic glucose homeostasis in diet-induced obesity mice. A–C, GTT of WT mice on LFD or HFD injected with vehicle (veh) or αGalCer. A, n = 12–15 mice each cohort with three repeats; B, n = 9–11 mice of each cohort with two repeats; C, n = 3–4 mice per HFD cohort and n = 10 mice for LFD. * refers to the p values compared between HFD + vehicle (veh) and HFD + αGalCer groups. D, fasting insulin levels in mice under 8-week HFD. n = 9–10 mice each, two repeats. E, GTT of CD1d−/− mice on LFD or HFD injected with vehicle (veh) or αGalCer. n = 9–10 mice each cohort with two repeats. F, GTT of WT mice on LFD with vehicle or αGalCer injection. n = 10 mice each, two repeats. Values represent mean ± S.E. *, p < 0.05, and ***, p < 0.005.

Indeed, the expression of 1556 genes was significantly increased by more than 2-fold in an absolute NKT-dependent manner (Fig. 6A). GSEA indicated pronounced induction of many pathways related to immune functions, including the “T<sub>H1</sub>/T<sub>H2</sub> differentiation” and the “IL-4 pathway” (supplemental Fig. S6B). In comparison with a pre-existing dataset (40), we found that 40% of genes up-regulated by IL-4 in macrophages were also induced by αGalCer in WAT (Fig. 6C). Furthermore, most components of the canonical IL-4 signaling pathway were induced by αGalCer in WAT (supplemental Fig. S6C). Pointing to a potential role of IL-4, 7 of the top 10 αGalCer-induced genes in WAT were also highly up-regulated in IL-4-treated macrophages (40), and many were classical M2 genes such as Chi3l3 (138-fold, αGalCer versus vehicle in WT WAT), pdccl1lg2 (53-fold), and Arg1 (32-fold) (Fig. 6B).

The array data were further supported by Q-PCR and Western blot analyses of Arg1 levels of WAT (Figs. 5A and 6, D and E). Providing direct support for the elevated IL-4 signaling in WAT, Il4 mRNA levels in WAT were highly induced by αGalCer treatment in an NKT-dependent manner (Fig. 6F). Thus, we conclude that αGalCer-mediated NKT cell activation increases IL-4 signaling in adipose tissue.

NKT Cell Effect Is Mediated by the IL-4/STAT6 Signaling Axis in Adipose Tissue—We next determined the physiological importance of IL-4 in αGalCer-mediated NKT cell activation using IL-4−/− mice. Although both body and adipose weights were comparable among WT and IL-4−/− cohorts following 4-day HFD (Fig. 7A), loss of IL-4 completely abolished the αGalCer effect in glucose tolerance (Fig. 7, B and C) and reduced the total number of infiltrating immune cells in adipose tissue following αGalCer injection (supplemental Fig. S7A). Indeed, total lymphocytes in WAT of IL-4−/− mice were reduced by ~40% relative to WT cohorts (supplemental Fig. S7B).

Loss of IL-4 markedly attenuated the αGalCer-mediated induction of M2 genes Arg1, Chi3l3, and Pdccl1lg2 (Fig. 7D) and Arg1 protein in WAT (Fig. 7, E and F). Moreover, loss of IL-4 completely abolished tyrosine phosphorylation (Tyr(P)) of STAT6 but not Tyr(P) of STAT3, in WAT following αGalCer-mediated NKT activation (Fig. 7, E and F). Providing further support to the significance of adipose tissue in mediating NKT effect in vivo, hepatocyte STAT6 was not activated by αGalCer (Fig. 7G).

Finally, it has been shown that the IL-4/STAT6 effect may be mediated by the activities of nuclear receptors (PPAR<sub>y</sub> or PPAR<sub>β/δ</sub>) in macrophages (44). However, αGalCer-mediated induction of M2 genes was not affected in WAT of myeloid cell-specific PPAR<sub>y</sub> or PPAR<sub>β/δ</sub>-deficient animals when compared with WT control littermates (supplemental Fig. S7C). Thus, NKT cells regulate macrophage polarization and exert metabolic control largely through the IL-4/STAT6 axis in obese adipose tissue, in a PPAR<sub>y</sub>- and PPAR<sub>β/δ</sub>-independent manner.

DISCUSSION

Our understanding of the role of NKT cells in regulating obesity-associated inflammation remains incomplete. Our data using mice fed with a 60% HFD for 8 weeks confirmed the findings of two previous reports where no metabolic effect was seen in CD1d<sup>−/−</sup> mice upon feeding of 45 or 60% HFD for 26 or
8–16 weeks (32, 33). These loss-of-function studies suggest that NKT cells are dispensable for glucose homeostasis at chronic obesity. In contrast, our gain-of-function study demonstrated that αGalCer-mediated NKT cell activation promotes M2 macrophage polarization and exerts a salutary effect on systemic glucose homeostasis through the IL-4/STAT6 signaling axis. These gain-of-function data are in line with the known function of NKT cells in altering TH1/TH2 responses in...
The lack-of-effect of NKT cells at late stages of obesity in the absence of stimulation is not surprising given the massive infiltration and expansion of other cells such as CD8⁺ T cells and macrophages (23, 24) and given the concomitant reduction of NKT cells (Fig. 1). Thus, these studies suggest that in the absence of a strong agonist, NKT cell effect in adipose tissue in the context of obesity is limited and may be dispensable; however, upon activation by a potent stimulus such as αGalCer, NKT cells have significant impact on inflammatory responses in adipose tissue and systemic glucose tolerance in obese animals.

Using the β₂-microglobulin-deficient mice that lack all MHC class I molecules, including CD1d, a recent study concluded that NKT cells infiltrate adipose tissue and that loss of NKT cells reduces inflammation in obesity, whereas activation of NKT cells by one injection of αGalCer had mild effect on glucose tolerance in adipose tissue (45), opposite to our findings. However, interpretation of their results is confounded by the fact that the β₂-microglobulin knock-out mice not only are deficient in NKT but also CD8⁺ T cells (46, 47). Accordingly, the absence of CD8⁺ T cells may account for the reported phenotypes (18).

In line with the role of IL-4 in T₉₂ responses, our data suggest the IL-4 is an important mediator of NKT cell function in adipose tissue in the context of obesity. However, it does not exclude the role of other T₉₂ cytokines such as IL-13, which is known to be secreted by NKT cells (9). This is supported by our observation that, unlike their hepatic counterparts, adipose-resident NKT cells have a T₉₂-biasing effect upon αGalCer activation is very intriguing. The difference may be related to the nature of antigen presenting cells, tissue microen-

**FIGURE 7.** NKT cell activation signals through the IL-4/STAT6 axis in adipose tissue. 6–7-Week-old mice were placed on either LFD or HFD for 4 days with two αGalCer or vehicle (Veh) injections prior to metabolic phenotyping. A, weights of body and epididymal adipose tissues. B and C, GTT, n = 7–8 mice each cohort with two repeats. D, Q-PCR analysis of M1 and M2 genes in WAT. n = 6 mice each cohort with two repeats. E and F, Western blot analysis of Arg1, Tyr(STAT6) and Tyr(STAT3) in WAT of WT and IL-4⁻/⁻ mice (n = 3–5 mice each cohort with two repeats). The ratios of Arg1 to HSP90, Tyr(STAT3/6 to total STAT3/6 were quantitated in F, G. Western blot analyses of Tyr(STAT3/6 proteins in the liver of the WT mice under 4-day HFD without or with αGalCer injection (n = 4–5 mice each). Each lane represents an independent sample. Values represent mean ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.005; n.s., not significant; a.u., arbitrary units.
vironment, and/or the different lineage of NKT cells in adipose tissue (8). Our preliminary data showed that unlike CD4+ CD8− NKT cells in the liver, the majority of NKT cells in adipose tissue are CD4− CD8+ (4), and thus may be associated with specific functional changes. Indeed, earlier studies have shown that CD4− CD8+ versus CD4+ CD8− NKT cells likely represent functionally separate lineages that may promote different T1/2 response with distinct capacities to secrete T1/2 and T1/2 cytokines (8, 49–51). Alternatively, the tissue-specific NKT effect may reflect a unique microenvironment of adipose tissue in terms of antigen presenting cells. Intriguingly, as adipocytes express CD1d transcript (data not shown), they may be able to present lipids to and directly activate NKT cells, whereas Kupffer cells in the liver have been shown to be important for αGalCer-mediated NKT cell activation (52). Finally, as NKT cell activation by bacterial lipid antigens may be toll-like receptor 4-dependent and IL-12-mediated (53, 54), it will be interesting to investigate whether different cytokine environments of the liver versus adipose tissue may explain the tissue-specific NKT effect upon αGalCer activation.

Our data show that αGalCer-mediated NKT activation leads to activation of the IL-4/STAT6 signaling axis in adipose tissue, promoting M2 macrophage polarization. How IL-4 mediates macrophage polarization in adipose tissue remains unclear. It has been shown that STAT6 may modulate the activities of nuclear receptors (PPARγ or PPARβ/δ) (44) or induce the expression of histone H3K27 demethylase Jmjd3 (encoded by Kdm6b gene) (55, 56) to influence M2 macrophage polarization. Our data show that M2 marker genes induced by αGalCer are not affected by lack of either PPARγ or PPARβ/δ in myeloid cells, consistent with two recent studies demonstrating that PPARγ and PPARβ/δ in macrophages or myeloid cells are dispensable for the T1/2 responses (40, 57). The discrepancies may be due to different genetic backgrounds of mice, fatty acid composition of diets, or gut microbiota. It will be interesting to delineate whether the combined actions of PPARγ and PPARβ/δ or more intriguingly epigenetic regulation by Jmjd3 may be required for the NKT cell effect in adipose tissue. Additionally, besides its effect on macrophage polarization, IL-4 may affect differentiation of naïve T cells and B cells. In light of recent studies showing important roles of various T and B cells in the pathogenesis of obesity (18, 20), the interplay among different cell types in obese adipose tissue is an interesting area of future studies.

In line with the animal data, the abundance of type 1 NKT cells in adipose tissue decreases in obese humans. Although this was initially reported by an earlier study (31), our data using a much larger cohort further demonstrate that abundance of type 1 NKT cells in adipose tissue negatively correlates with BMI, insulin resistance, and OGTT glucose levels. The near-perfect correlations between adipose NKT and metabolic parameters suggest that these cells may be involved in the regulation of insulin sensitivity in obese humans. As αGalCer is not toxic and is well tolerated in humans and often stimulates the expansion of residual NKT cell populations in other disease settings (12, 34), our data suggest that NKT-activating glycolipids may have clinical application in treating type 2 diabetes. Therapies using NKT lipid agonists may skew T1/2 bias in adipose tissue and hence delay or ameliorate the development of inflammation and type 2 diabetes in obese patients. Further studies are required to determine the optimal dosage, feeding routes, and frequency of αGalCer or other T1/2-biasing lipid agonists in humans.

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