

# Adipose Tissue Invariant NKT Cells Protect against Diet-Induced Obesity and Metabolic Disorder through Regulatory Cytokine Production

Lydia Lynch,<sup>1,2,5,\*</sup> Michael Nowak,<sup>1</sup> Bindu Varghese,<sup>1</sup> Justice Clark,<sup>1</sup> Andrew E. Hogan,<sup>2</sup> Vasillis Toxavidis,<sup>1</sup> Steven P. Balk,<sup>1</sup> Donal O'Shea,<sup>3,4</sup> Cliona O'Farrelly,<sup>5</sup> and Mark A. Exley<sup>1</sup>

<sup>1</sup>Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA

<sup>2</sup>Education Research Centre

<sup>3</sup>Department of Endocrinology

St. Vincent's University Hospital, Dublin 4, Ireland

<sup>4</sup>Conway Institute, University College Dublin, Dublin 4, Ireland

<sup>5</sup>School of Biochemistry and Immunology and School of Health Sciences, Trinity College, Dublin 2, Ireland

\*Correspondence: llynch1@bidmc.harvard.edu

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## SUMMARY

Invariant natural killer T (iNKT) cells are evolutionarily conserved innate T cells that influence inflammatory responses. We have shown that iNKT cells, previously thought to be rare in humans, were highly enriched in human and murine adipose tissue, and that as adipose tissue expanded in obesity, iNKT cells were depleted, correlating with proinflammatory macrophage infiltration. iNKT cell numbers were restored in mice and humans after weight loss. Mice lacking iNKT cells had enhanced weight gain, larger adipocytes, fatty livers, and insulin resistance on a high-fat diet. Adoptive transfer of iNKT cells into obese mice or *in vivo* activation of iNKT cells via their lipid ligand, alpha-galactocylceramide, decreased body fat, triglyceride levels, leptin, and fatty liver and improved insulin sensitivity through anti-inflammatory cytokine production by adipose-derived iNKT cells. This finding highlights the potential of iNKT cell-targeted therapies, previously proven to be safe in humans, in the management of obesity and its consequences.

## INTRODUCTION

The discovery that tumor necrosis factor alpha (TNF- $\alpha$ ) was elevated in obesity and correlated with insulin resistance was a seminal finding that kick-started the field of inflammation and immunometabolism in 1993 (Hotamisligil et al., 1993). It is now accepted that inflammation, particularly within adipose tissue itself (Hotamisligil, 2006; Hotamisligil et al., 1993; Nishimura et al., 2008; Shoelson et al., 2006), is critically linked to obesity and its accompanying metabolic disorders, including impaired glucose tolerance, insulin resistance, hepatic steatosis, dyslipidemia, and eventually type 2 diabetes (Reaven, 1988). Adipose tissue is immunologically dynamic, containing resident CD4<sup>+</sup> (Winer et al., 2009) and CD8<sup>+</sup> T cells (Nishimura et al., 2009), regulatory

T (Treg) cells (Feuerer et al., 2009), B cells (Winer et al., 2011), and macrophages (Lumeng et al., 2007; Weisberg et al., 2003; Wentworth et al., 2010), each of which have been shown to play positive or negative roles in metabolic dysregulation and the development of obesity (Feuerer et al., 2009; Lumeng et al., 2007; Nishimura et al., 2009; Wentworth et al., 2010; Winer et al., 2011; Winer et al., 2009).

Chronic low-grade inflammation in obese fat may activate resident innate immune cells, leading to inappropriate immune responses and the development of insulin resistance (Xu et al., 2003). Likewise, inflammation associated with adiposity is partly generated by fat-resident immune cells, the most widely studied being macrophages, which infiltrate fat during obesity and undergo phenotypic switching, leading to the development of insulin resistance (Lumeng et al., 2007; Weisberg et al., 2003). Macrophage activation is modified by T cells. Interferon- $\gamma$  (IFN- $\gamma$ ) production by T cells enhances proinflammatory M1 macrophage differentiation, whereas T cells secreting anti-inflammatory cytokines such as interleukin-4 (IL-4), IL-13, and IL-10 promote anti-inflammatory M2 macrophage development (Tiemessen et al., 2007; Kim et al., 2008). Classical CD11c-expressing M1 macrophages (Fujisaka et al., 2009; Lumeng et al., 2007) often aggregate around necrotic adipocytes and produce excess proinflammatory cytokines, such as IL-6 and TNF- $\alpha$ , characteristic of obese adipose tissue in humans (Wentworth et al., 2010) and mice (Lumeng et al., 2007). Alternatively activated M2 macrophages (F4/80<sup>+</sup>CD11c<sup>-</sup>CD206<sup>+</sup>) found in lean adipose tissue generate high amounts of anti-inflammatory cytokines, such as IL-10, but are decreased in obesity (Fujisaka et al., 2009; Lumeng et al., 2007). Although the importance of macrophages and their interaction with adipocytes and other immune cells is now accepted as key in the development of adipocyte and metabolic dysfunction, the mechanisms regulating these interactions are not well understood.

We have explored a role for the innate T lymphocyte population of invariant natural killer T (iNKT) cells in obesity and have previously shown that iNKT cells are enriched in human adipose tissue but are decreased in human obesity (Lynch et al., 2009). iNKT cells constitute a unique innate T cell population, which is highly conserved and expresses invariant T cell receptors (TCRs) V $\alpha$ 24J $\alpha$ 18, paired with V $\beta$ 11 in humans, and V $\alpha$ 14J $\alpha$ 18,

coupled with TCR V $\beta$ 7, V $\beta$ 8.2, or V $\beta$ 2 in mice. These specific TCRs recognize glycolipid ligands presented by the major histocompatibility complex-like molecule CD1d (Brigl and Brenner, 2004; Gumperz, 2006; Matsuda et al., 2000). The most studied lipid antigen is alpha-galactosylceramide ( $\alpha$ GC) (Matsuda et al., 2000), as physiological lipid ligand(s) have yet to be fully defined (Fox et al., 2009; Speak et al., 2007). A striking feature of iNKT cells is their rapid production of both T helper 1 (Th1) and Th2 cell cytokines upon activation with  $\alpha$ GC (Bendelac et al., 2007; Berzins et al., 2011; Matsuda et al., 2008), probably accounting for their immunoregulatory potential. Indeed, iNKT cell cytotoxicity and rapid production of IFN- $\gamma$  have been shown to protect against tumor development, and iNKT cells also play immunoregulatory roles in type 1 diabetes, multiple sclerosis, and rheumatoid arthritis (Berzins et al., 2011; Swann et al., 2007; Wu and Van Kaer, 2009). Our previous study reported that iNKT cells are enriched in human fat at higher numbers than elsewhere in the body (Lynch et al., 2009). Here, we have shown that, like that of humans, murine adipose tissue was enriched with iNKT cells. Under physiological conditions of clonal abundance, the number of iNKT cells probably exceeds a given number of antigen-specific T cells by up to 10,000-fold, and thus it is probable that iNKT cells will play an important immune regulatory role in adipose tissue. Furthermore, we showed that iNKT cells resident in fat represent a unique subset with distinct Th2 cell cytokine production compared to iNKT cells in the liver and spleen. Given the potent influence of other fat-resident immune cells on the development of obesity and metabolic outcomes, the conservation of iNKT cells in fat in mammals, and the unique anti-inflammatory profile of fat-resident iNKT cells, we hypothesized that local fat-resident iNKT cell populations would play a physiological role in adipose tissue regulation. Our data confirm this hypothesis and show that iNKT cells confer protection against the development of metabolic syndrome and inflammation following a high fat diet (HFD) challenge.

## RESULTS

### iNKT Cells Are Depleted in Fat and Liver during the Development of Obesity

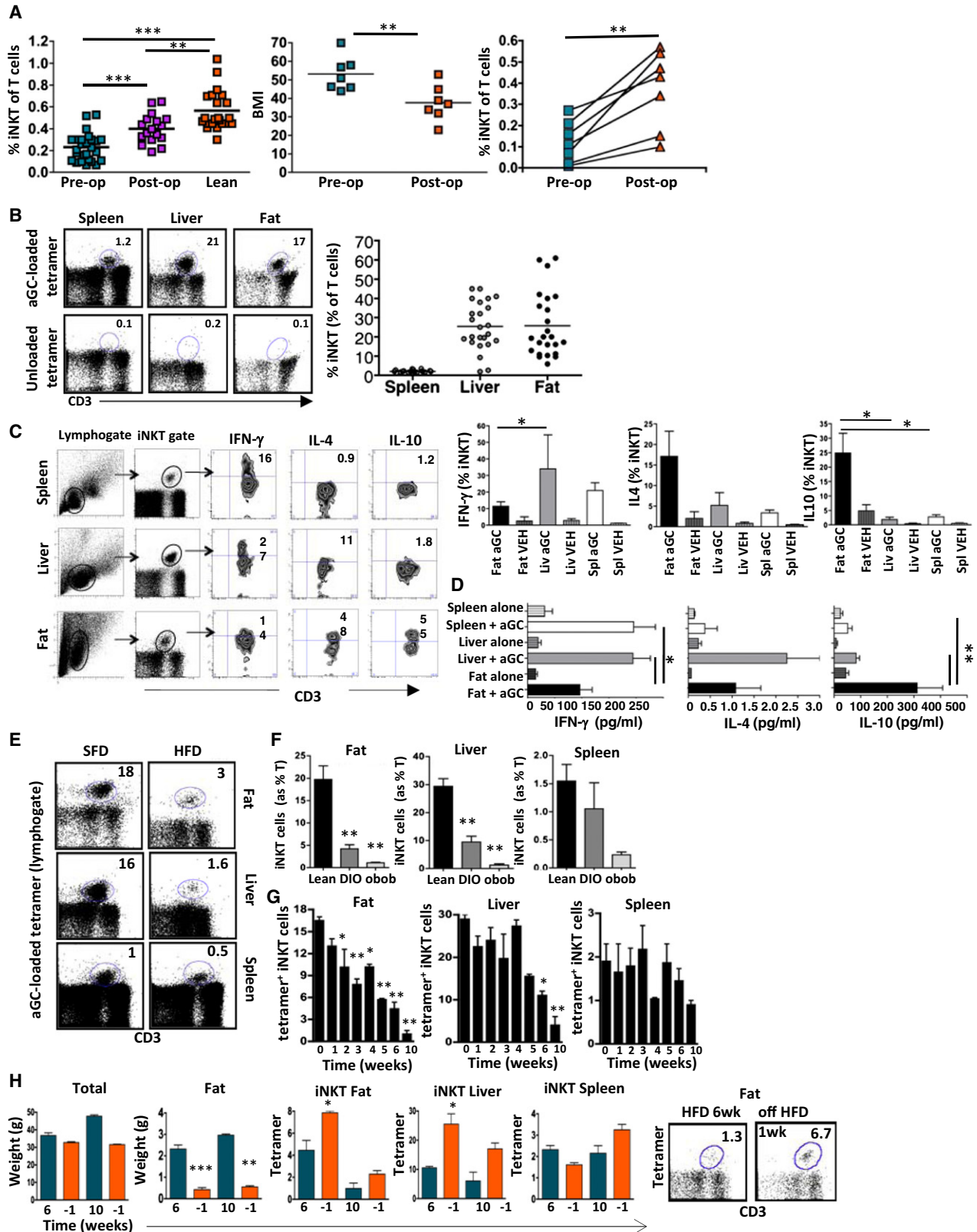
We have previously shown that iNKT cells are enriched in human fat but are depleted in obesity (Lynch et al., 2009). As it was not possible to obtain adipose tissue from patients at multiple time points after bariatric surgery, we looked in human peripheral blood and found that iNKT cells were also reduced in the circulation of obese patients compared to lean, healthy, age-matched controls (Figure 1A). Cross-sectional analysis found that circulating iNKT cell numbers were increased in obese patients who had lost weight after Roux-en-Y gastric bypass (RYGB) surgery compared to obese patients pre-RYGB surgery, although iNKT cells were still reduced compared to lean controls (Figure 1A). We then followed a group of patients ( $n = 7$ ) longitudinally pre- and post-RYGB surgery whose body mass index (BMI) decreased from grade III obesity (mean BMI  $>50$  kg/m $^2$ ) to grade II obesity (mean BMI 35–40 kg/m $^2$ ) 18 months postsurgery (Figure 1A). Peripheral iNKT cell numbers increased in each patient after weight loss (Figure 1A).

To further explore the relationship between obesity and iNKT cells, we turned to murine models. We first determined whether

iNKT cells were also present in murine fat. As in humans, iNKT cells are enriched in murine adipose tissue (Figure 1B) at numbers equivalent to those found in murine liver. Fat-derived iNKT cells produced significantly less IFN- $\gamma$  and more IL-4 and IL-10 compared to iNKT cells from the liver and spleen following  $\alpha$ GC activation in vivo (Figure 1C) and in vitro (Figure 1D). We also compared other lymphoid populations from adipose tissue to those from liver and spleen. Similar to liver, fat had high levels of NK and NKR $^+$  T cells and high numbers of CD4 $^-$ CD8 $^-$  double negative (DN) T cells, reflecting the increased numbers of DN iNKT cells in liver and fat (Figure S1A available online). Furthermore, of the iNKT cells, there were fewer CD4 $^+$  iNKT and more DN iNKT cells in fat compared to those in the liver and spleen (Figure S1B). Adipose tissue contained a small but significant population of Treg (FoxP3 $^+$ CD25 $^+$ CD4 $^+$ ) cells (6%), similar to those in the spleen (7% of T cells) and in agreement with numbers previously reported (Feuerer et al., 2009) (Figures S1A and S1C). iNKT cells were previously shown to express FoxP3 in certain circumstances (Monteiro et al., 2010). Given the high levels of iNKT cells and the substantial population of Treg cells in fat, it was necessary to determine whether there was any overlap between both populations. Figure S1C shows that iNKT cells are distinct from FoxP3 $^+$  Treg cells. Thus, the immune repertoire in fat is unique, but with similarities both to liver in NK, NKR $^+$ T, and iNKT levels and to spleen in Treg cell levels.

We next investigated the effects of obesity on iNKT cells using two murine models of obesity: diet-induced obesity (DIO) and obesity due to leptin deficiency (ob/ob). Mice fed a HFD for 6 weeks had markedly reduced numbers of iNKT cells in adipose tissue and liver (Figures 1E and 1F). iNKT cell depletion was even more pronounced in ob/ob mice (Figure 1F), which were heavier and had higher fasting blood glucose than DIO mice. We next analyzed iNKT cell numbers during the development of obesity. iNKT numbers in fat were reduced at as early as week 2 of the HFD and steadily declined each week during the course of the HFD (Figure 1G). iNKT levels also declined in the liver upon HFD challenge, with significant differences seen from week 6 onward. Splenic iNKT cell levels fluctuated, with a trend toward depletion at week 10 (Figure 1G). We also calculated absolute numbers of iNKT cells per organ and per gram of fat (Table S1). We estimate that lean adipose tissue contains  $7.3 \times 10^4$  iNKT cells, and in obesity the numbers were reduced to  $2.2 \times 10^4$  iNKT cells per two epididymal fat pads. This compares with  $7.3 \times 10^4$  iNKT in liver from lean mice and  $3 \times 10^4$  iNKT in liver from obese mice. We observed no changes in activation markers CD69 and CD25 on iNKT cells in obesity (data not shown). We also looked at fluctuations in other T cells in obese fat. CD8 $^+$  T cells increased in obese fat, there were no significant obesity-induced changes in other T cells in liver, and there was a decrease in CD4 $^+$  T cells and an increase in CD8 $^+$  T cells in the spleens of obese mice (Figure S1D).

HFD was replaced by a standard fat diet (SFD) for a week after 6 weeks or 10 weeks, which caused only a slight drop in total weight but a dramatic reduction in fat-pad weight (Figure 1H). After to the switch to SFD after 6 weeks of HFD, there was a significant increase in iNKT cells in fat and liver; iNKT levels also began to increase after the switch to SFD after 10 weeks of HFD (Figure 1H). These findings indicate that murine and human iNKT cell responses to obesity and weight loss are similar.



**Figure 1. iNKT Cells Are Depleted in Obesity, but Are Restored Following Weight Loss in Mice and Humans**

(A) iNKT cells (expressed as a % of T cells) in peripheral blood of obese patients before bariatric surgery (pre-op) (BMI > 50, n = 26) compared to lean age-matched controls (BMI = 20-25, n = 22) and unmatched patients 18 months after surgery (post-op) (n = 18, p = 0.0002; Mann-Whitney test). Seven patients were analyzed

### Contribution of iNKT Cells to the Development of Obesity

We next explored the hypothesis that iNKT cells may protect against obesity and related metabolic consequences.  $J\alpha 18$ -deficient mice lacking iNKT cells but having an otherwise normal immune system were fed a HFD alongside age-matched wild-type (WT) mice on HFD or SFD.  $J\alpha 18$ -deficient mice were significantly larger before HFD challenge. They also gained significantly more weight than WT mice on HFD, and had significantly larger fat pads, whereas lean mass was unchanged (Figure 2A). Food intake was similar between  $J\alpha 18$ -deficient and WT mice (Figure 2B). Adipocytes were larger in  $J\alpha 18$ -deficient mice compared to WT mice on HFD (Figures 2C and 2D). Furthermore,  $J\alpha 18$ -deficient mice on HFD had higher fat deposition in the liver (Figure 2E), an elevated fasting blood glucose, an impaired glucose tolerance test (GTT), and increased insulin resistance compared to WT mice on HFD (Figure 2F). Serum leptin concentrations were equivalent in WT and  $J\alpha 18$ -deficient mice on HFD compared to SFD (Figure 2G).

We examined DIO in another mouse model lacking iNKT cells,  $Cd1d1^{-/-}$  mice, which lack the restriction element for iNKT cells and therefore also selectively lack iNKT cells.  $Cd1d1^{-/-}$  mice gained significantly more weight than WT mice on a HFD (Figure S2A).  $Cd1d1^{-/-}$  mice on HFD had higher fasting blood glucose and worse glucose handling than WT on HFD. Fasting insulin and insulin resistance were not significantly higher, possibly due to higher variability inherent in these insulin assays (Figures S2B and S2C).  $Cd1d1^{-/-}$  mice also had larger adipocytes, as measured by immunohistochemistry (Figure S2D).

The above experiments were all performed on males. As gender differences have been reported in some of the metabolic complications of obesity (Fox et al., 2007; Medrikova et al., 2012), we also investigated metabolic outcome in female  $J\alpha 18$ -deficient versus female WT mice on HFD (Figure S3). Like males, female  $J\alpha 18$ -deficient mice gained significantly more weight in the first 4 weeks of HFD challenge; thereafter, weight gain was increased but not significantly different compared to WT mice (Figure S3A). This correlated with eating behavior; at 4 weeks, female  $J\alpha 18$ -deficient mice had a reduced food intake compared to WT females, unlike males, which had almost identical food intake patterns in WT and  $J\alpha 18$ -deficient mice (Figure S3B). Lean mass was similar, but both overall fat mass and fat-pad weight were significantly higher in  $J\alpha 18$ -deficient females (Fig-

ure S3A). Adipocytes were significantly larger and fewer in number (Figure S3C), and the degree of fatty liver was greater in  $J\alpha 18$ -deficient females compared to WT on HFD (Figure S3D). However, in contrast to males, fasting glucose was unchanged and GTT was not impaired in  $J\alpha 18$ -deficient females compared to WT females on HFD (Figure S3E). Our findings agree with other obesity studies (Fox et al., 2007; Medrikova et al., 2012) that illustrate that female mice have larger fat pads and adipocytes but are less susceptible to HFD-induced glucose impairment and insulin resistance than male mice.

### iNKT Cell Numbers Correlate Inversely with Macrophage Infiltration in Adipose Tissue

iNKT cells can recruit and regulate other immune cells (Cerundolo et al., 2009). We investigated the influence of adipose-derived iNKT cells on macrophage infiltration and activation. As expected, proinflammatory macrophages ( $F4/80^+CD11c^+$ ) increased in adipose tissue during the development of obesity, with significant increases detectable as early as 1 week into HFD. After cessation of HFD for 1 week, proinflammatory macrophages were significantly decreased in fat (Figure 3A). We found a strong inverse correlation between iNKT cell levels in fat and proinflammatory macrophages (Figure 3A).

To determine whether iNKT cells play a causal role in the infiltration and phenotype of macrophages, we investigated macrophage numbers in  $J\alpha 18$ -deficient mice in obesity. In the absence of iNKT cells, total macrophage numbers were higher in adipose tissue (Figures 3B–3D). Adipose tissue macrophages displayed a trend toward increased proinflammatory (CD11c) and decreased anti-inflammatory (CD206) phenotypes in  $J\alpha 18$ -deficient mice compared to WT mice on HFD (Figure 3B).  $Cd1d1^{-/-}$  mice had similarly high numbers of F4/80 macrophages in adipose tissue compared to WT mice on HFD (Figure S2E) but had significantly more proinflammatory macrophages than WT mice on HFD (Figure S2F).

### Mice Lacking iNKT Cells Show Metabolic Disorder on SFD

Both  $J\alpha 18$ -deficient and  $Cd1d1^{-/-}$  mice have overtly normal immune systems with no pathological susceptibilities, unless challenged with certain pathogens or tumors. We investigated whether there was any evidence of metabolic syndrome in  $J\alpha 18$ -deficient and  $Cd1d1^{-/-}$  mice fed ad libitum for 4–5 months

both pre- and postbariatric surgery. Middle panel, BMI of patients before and 18 months after operation. Right panel, Peripheral iNKT cell levels in each patient ( $n = 7$ , paired t test).

(B) Left panel, iNKT ( $\alpha$ GC-loaded tetramer<sup>+</sup>) as % of T cells (top row) and unloaded CD1d tetramer negative control (bottom row) in a representative sample of matched spleen, liver, and fat. Right panel, iNKT cells (expressed as a % of T cells) in each individual WT spleen, liver, and fat from 6–10-week-old mice ( $n = 24$ ).

(C) Left panel, representative flow cytometric plots showing the percentage of iNKT cells producing each cytokine intracellularly following  $\alpha$ GC injection (representative of  $n = 4$ ). Right panel, concentrations of intracellular cytokines production by iNKT cells; mean  $\pm$  SD ( $n = 4$ ).

(D) iNKT cytokine production after in vitro stimulation with  $\alpha$ GC-loaded CD1d-transfected C1R cells (three independent experiments were performed in duplicate).

(E) Representative dot plots of iNKT cell ( $\alpha$ GC-loaded tetramer<sup>+</sup>) numbers in matched fat, liver, and spleen from 10–14-week-old mice on an SFD or HFD (representative of 11 experiments).

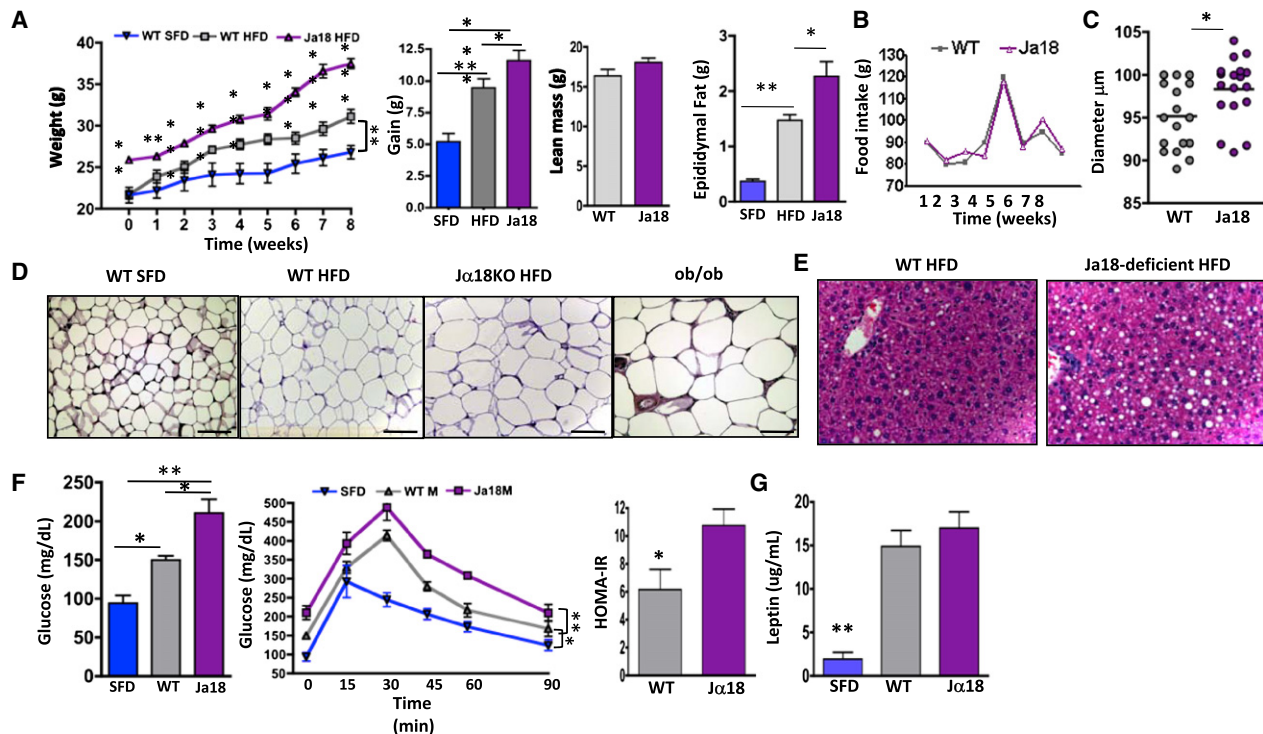
(F) iNKT cells in fat, liver, and spleen from mice on HFD for 8 weeks ( $n = 11$ ) or ob/ob mice ( $n = 3$ ) compared to age-matched mice on SFD ( $n = 11$ ).

(G) iNKT cell numbers measured each week on HFD in matched fat, liver, and spleen ( $n = 4$  per week). See also Figure S2.

(H) Mice were removed from HFD after 6 weeks ( $n = 4$ ) and after 10 weeks ( $n = 4$ ). Graphs show overall weight (total) and fat-pad weight during HFD (green bars) and after removal from HFD (orange bars) after 6 weeks ( $p = 0.0007$ ) or 10 weeks ( $p = 0.001$ ). iNKT numbers in fat, liver, and spleen after removal from HFD. Graphs show mean  $\pm$  SD ( $n = 4$ ).

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ .





**Figure 2. Impact of iNKT Cell Deficiency on Weight Gain, Glucose Tolerance, Adipocyte Size and Number, and Fat Accumulation in Liver**

(A) Weight of  $J\alpha 18^{-/-}$  and WT mice on commencement of and during 8 weeks of HFD compared to WT on SFD ( $n = 4$  per group per week). Overall weight gain after 8 weeks of HFD. Lean mass and epididymal fat-pad weight of WT and  $J\alpha 18^{-/-}$  mice on HFD; WT mice on SFD are shown for comparison.

(B) HFD food intake of WT and  $J\alpha 18$ -deficient mice.

(C) Adipocyte diameter was measured on osmium-fixed adipocytes with a particle counter. Adipocyte size from  $J\alpha 18^{-/-}$  and WT mice on HFD (four samples per mouse, four mice per group).

(D) Histology of adipocytes from epididymal fat. Adipocyte size from WT mice on SFD, WT mice on HFD, and  $J\alpha 18^{-/-}$  mice on HFD. ob/ob mice are also shown for comparison. Scale bars represent 100  $\mu\text{m}$ .

(E) Histology of fat infiltration in liver of WT and  $J\alpha 18^{-/-}$  mice on HFD (Representative of four individual experiments).

(F) Fasting glucose (left), glucose tolerance (middle), and insulin resistance (right) in WT mice on SFD, WT mice on HFD, and  $J\alpha 18$ -deficient mice on HFD for 6 weeks ( $n = 4$  per group, t tests, and two-way ANOVA with Tukey for GTTs). Insulin resistance, as measured by HOMA-IR (t test).

(G) Serum leptin levels in WT and  $J\alpha 18$ -deficient mice on HFD compared to WT mice on SFD ( $n = 4$  per group, ANOVA). Graphs show mean  $\pm$  SD.

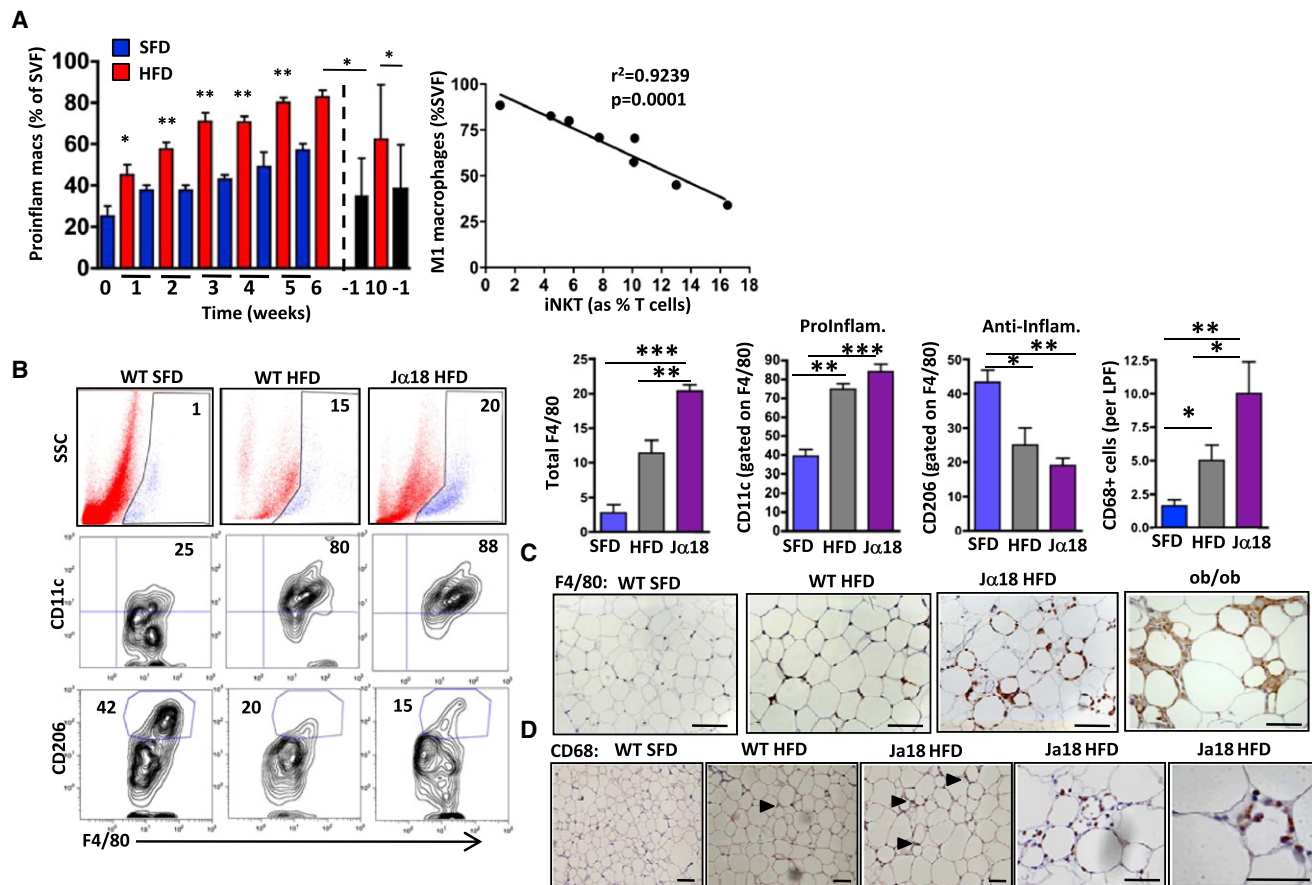
\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ .

on SFD. Both  $J\alpha 18$ -deficient mice and  $Cd1d1^{-/-}$  mice consistently weighed more (Figure 4A) and had larger adipocytes on SFD compared to aged-matched WT mice (Figure 4B). Adipose tissue macrophages were markedly increased in both types of iNKT cell-deficient mice, but had similar proinflammatory (data not shown) and anti-inflammatory phenotypes in each group on SFD (Figure 4C). Consistent with these findings, both  $J\alpha 18$ -deficient and  $Cd1d1^{-/-}$  mice had greatly increased serum triglyceride numbers and TNF- $\alpha$  concentrations as well as somewhat elevated IL-6 concentrations (Figure 4D).  $J\alpha 18$ -deficient mice and  $Cd1d1^{-/-}$  mice on SFD had elevated fasting glucose, and GTT was slightly impaired in  $Cd1d1^{-/-}$  mice, although these differences were not significant (Figure 4E).

#### Transfer of iNKT Cells in Obese $J\alpha 18$ -Deficient Mice Improves Glucose Handling

To directly test the hypothesis that iNKT cells play a protective role against the development of obesity-induced metabolic syndrome, we adoptively transferred  $5 \times 10^5$  WT hepatic iNKT

cells into obese mice (>8 weeks on HFD), who were continued on HFD for 4 days. Adoptive transfer of  $5 \times 10^5$  NKT cell-depleted T cells or PBS were used as controls. Adoptive transfer of iNKT cells resulted in significant weight loss and a dramatic reduction in adipocyte size, compared to transfer of T cells or PBS (Figures 5A and 5B). Mice that received NKT cells had similar numbers of macrophages as mice that received T cells but had less proinflammatory marker CD11c expression (Figure S4). Food intake was similar between mice receiving iNKT cell transfer or PBS alone (data not shown). Mice that received iNKT cells had significantly smaller fat pads than PBS controls, whereas fat-pad size in the T cell recipients was intermediate between PBS and iNKT cells (Figure 5C). Importantly, lean control mice that received the same number of iNKT cells did not lose weight (Figure 5D) or have hypoglycemia (Figure 5E) compared to PBS alone. Obese mice that received iNKT cells had significantly lower fasting glucose (Figure 5F) and improved GTT (Figure 5G) compared to mice receiving PBS, whereas T cell recipients were again intermediate between PBS and iNKT cell



**Figure 3. The Relationship between Adipose iNKT Cells and Macrophages**

(A) Left panel, proinflammatory macrophage (Proinflamm. macs; F4/80<sup>+</sup>CD11c<sup>+</sup>MMR<sup>+</sup>) levels in fat (as % SVF) of WT mice fed HFD (red) or SFD (blue) for 10 weeks. Dashed line shows when HFD was replaced with SFD (black). Right panel, correlation between iNKT cell levels and macrophage numbers in fat; Pearson  $r = -0.9612$ ,  $p = 0.0001$ .

(B) Left panel, representative dot plots of % F4/80<sup>+</sup> total macrophages per fat pad (top), the % macrophages that are CD11c<sup>+</sup> (proinflammatory) (middle), and the % macrophages that are CD206<sup>+</sup> (anti-inflammatory) (bottom) in WT mice on SFD, WT mice on HFD, and J $\alpha$ 18-deficient mice on HFD. Right panel, levels and phenotypes of macrophages from individual mice groups (n = 4 mice per group), including number of CD68<sup>+</sup> macrophages as measured by immunohistochemistry (n = 20 low power fields per group).

(C) Immunohistochemical staining of F4/80<sup>+</sup> macrophages in fat from WT mice on SFD, WT mice on HFD, J $\alpha$ 18<sup>-/-</sup> mice on HFD, and ob/ob mice on SFD (representative of four mice per group).

(D) Immunohistochemical staining of CD68<sup>+</sup> M1 macrophages in fat from WT on SFD and WT and J $\alpha$ 18-deficient mice on HFD (representative of four mice per group). Scale bars represent 100  $\mu$ m.

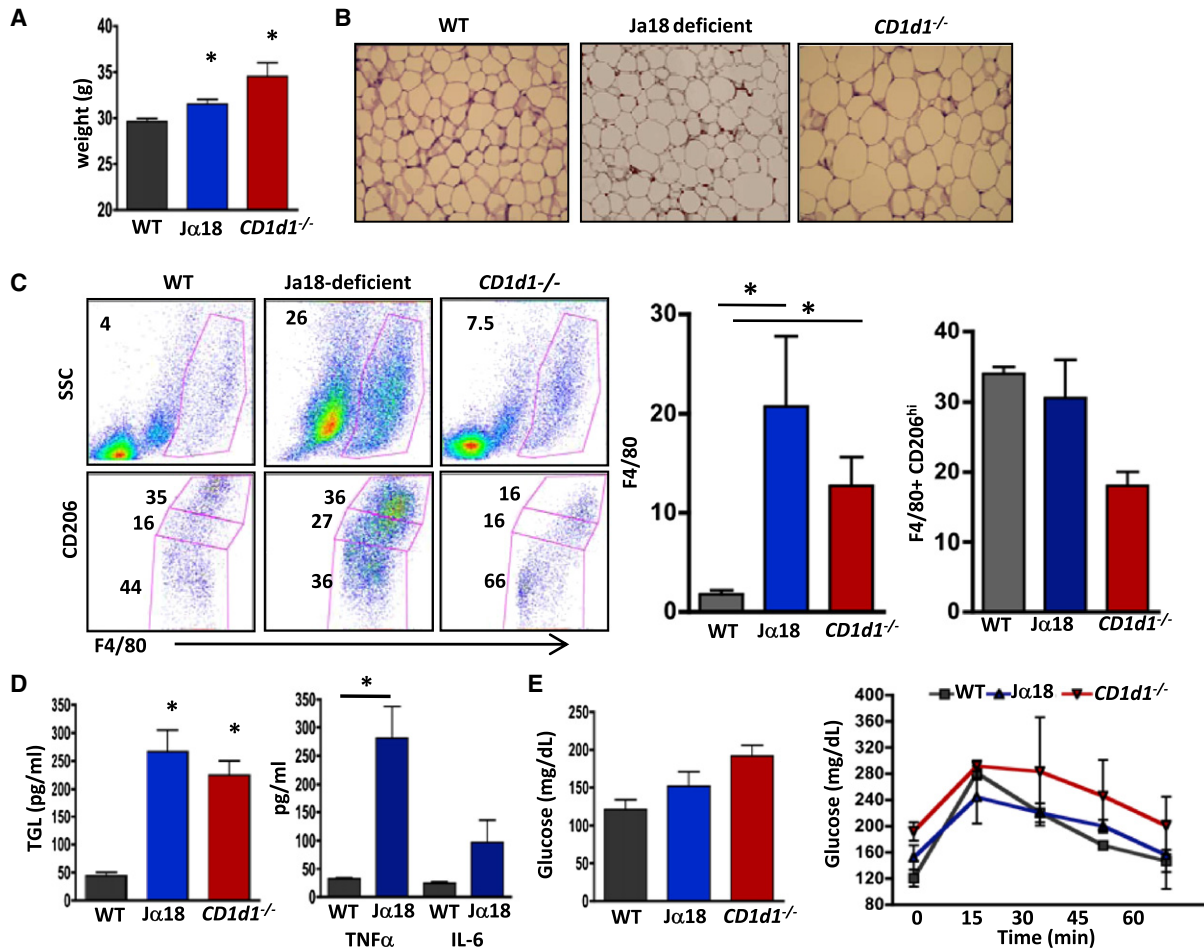
\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$

recipients. Insulin sensitivity improved after iNKT cell transfer, but not T cell transfer (Figure 5H). Next, we cultured fat pads overnight from mice that received NKT cells or PBS in vivo and performed an array analysis on the supernatant from the cultured fat pads. Mice that received NKT cells had similar amounts of the adipokine resistin but had significantly less leptin and significantly more adiponectin. Furthermore, angiopoietin-like 3 (ANGPTL3) production was greatly reduced, and IL-10 production was increased, probably produced by iNKT cells (Figure 5I).

#### **$\alpha$ GC Treatment Causes Weight Loss and Adipocyte Hypertrophy and Improves Fatty Metabolic Disorder**

We investigated whether  $\alpha$ GC, the prototypical ligand for iNKT cells, could activate residual iNKT cells in obesity and subsequently improve metabolic outcome. Following one injection of

$\alpha$ GC into WT obese mice (>8 weeks on HFD) and continued HFD for 4 days, mice lost a significant amount of weight; their percentage body fat also decreased significantly, although lean mass did not change (Figures 6A and 6B).  $\alpha$ GC also caused a rapid reduction in adipocyte size (Figure 6C). Food intake was similar between treatment and control.  $\alpha$ GC caused a marked reduction in fasting blood glucose compared to vehicle control (Figure 6D). Obese J $\alpha$ 18-deficient mice lacking iNKT cells did not lose weight after  $\alpha$ GC treatment (Figure 6A) or have improved glucose concentrations, which were extremely high (Figure 6D).  $\alpha$ GC returned GTT to almost normal in WT mice on HFD, unlike in obese J $\alpha$ 18-deficient mice.  $\alpha$ GC treatment did not significantly affect fasting glucose or GTT in normal-weight mice on SFD (Figure 6D).  $\alpha$ GC significantly improved insulin resistance as measured by homeostatic model of assessment insulin



**Figure 4. iNKT Null Mice Have More Proinflammatory Cytokines and Macrophages on an SFD**

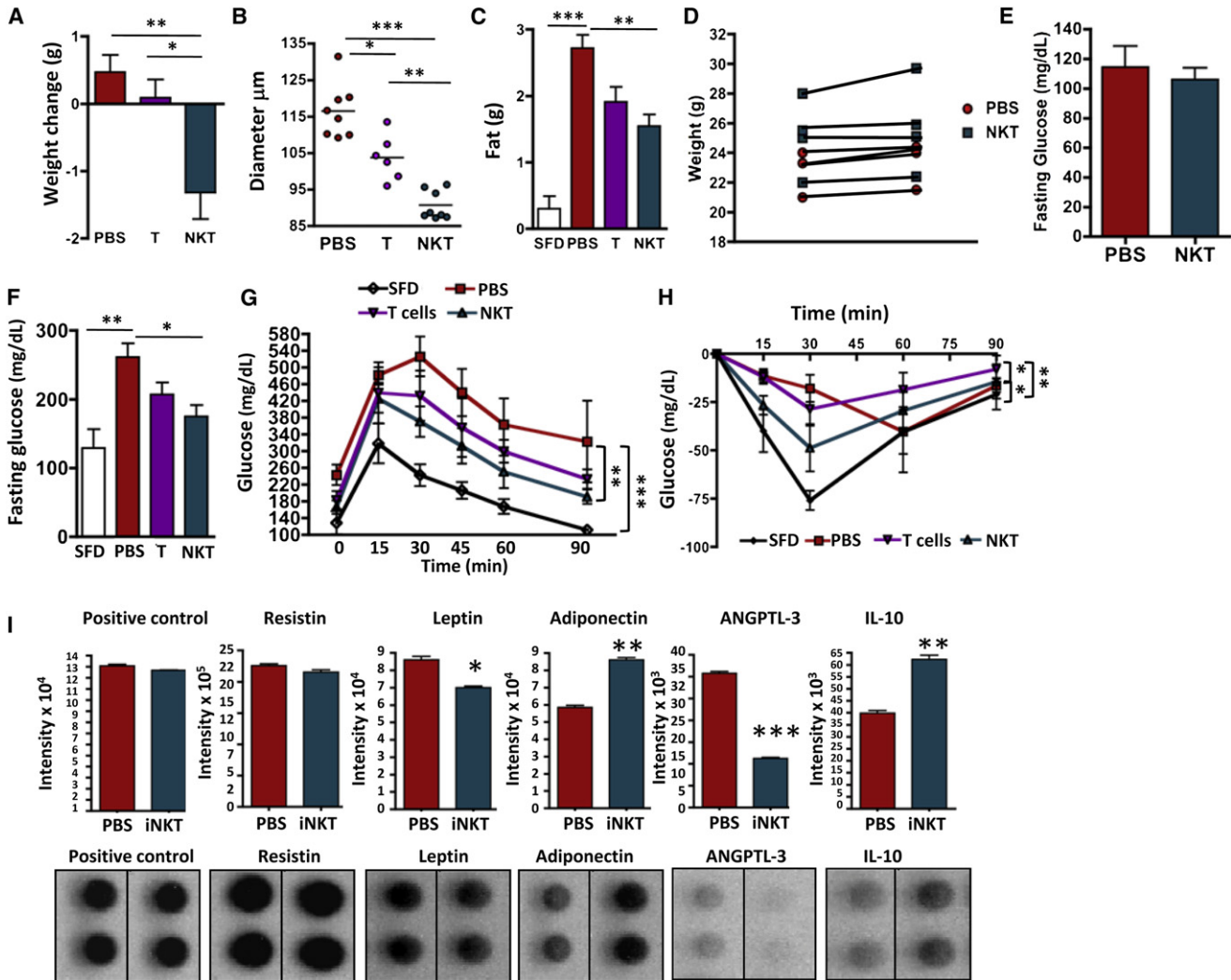
(A) Weight of WT,  $J\alpha 18^{-/-}$ , and  $CD1d1^{-/-}$  mice fed SFD ad libitum until 20 weeks of age ( $n = 3$  per group, one-way ANOVA with post hoc Tukey). (B) Adipocyte size in WT,  $J\alpha 18$ -deficient, and  $CD1d1^{-/-}$  mice on SFD (representative of three mice per group). (C) Macrophage level and phenotype in the three mice groups on SFD. Left top, total macrophages; left bottom, M2 (CD206<sup>hi</sup>) macrophages; CD206<sup>hi</sup> (top gate), CD206<sup>lo</sup> (middle gate), and CD206<sup>hi</sup> (bottom gate). SSC, side scatter. (D) Fasting serum triglyceride (TGL) concentration in the three groups ( $n = 3$ , all one-way ANOVA with post hoc Tukey). Serum TNF- $\alpha$  and IL-6 concentration in WT and  $J\alpha 18^{-/-}$  mice on SFD ( $n = 3$ , t test);  $CD1d1^{-/-}$  mice not tested. (E) Fasting glucose and glucose tolerance of 20 week old WT,  $J\alpha 18^{-/-}$ , and  $CD1d1^{-/-}$  mice on SFD. Graphs show mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ .

resistance (HOMA-IR), although the insulin tolerance test (ITT) was not significantly different due to variability in baseline fasting insulin (Figure 6E).  $\alpha$ GC lowered serum triglyceride numbers, circulating leptin, and IL-6 in obese WT mice (Figure 6F). Surprisingly, TNF- $\alpha$  concentrations were increased (Figure 6F). IL-4 was not significantly increased after  $\alpha$ GC, and serum IL-10 was below the detection range (data not shown).  $\alpha$ GC treatment improved fatty liver; fat droplets were smaller and less frequent, compared to mice that received vehicle (Figure 6G).

We examined iNKT cells 4 days post-treatment with  $\alpha$ GC, at the time of metabolic analysis.  $\alpha$ GC caused dramatic expansion in numbers of iNKT cells in fat, as well as a smaller but significant expansion in spleen and liver (Figure 7A).  $\alpha$ GC activated iNKT cells to produce anti-inflammatory cytokines (Figure 7A), probably leading to the improvement in metabolism despite HFD

feeding (Figures 6A–6E), although iNKT cells also produced some IFN- $\gamma$  following  $\alpha$ GC. Furthermore, iNKT cells in adipose tissue were still producing cytokines 4 days postinjection, unlike those in spleen and liver (Figure 7A). We next investigated whether  $\alpha$ GC could mediate metabolic protection if IL-4 and IL-10 were neutralized before  $\alpha$ GC injection. When both IL-4 and IL-10 were neutralized immediately prior to  $\alpha$ GC treatment, mice still lost weight (Figure 7B); however, fasting glucose concentrations were elevated compared to  $\alpha$ GC treatment alone (Figure 7C). Furthermore, neutralizing these cytokines resulted in increased glucose concentrations during the GTT at 60 and 90 min, like vehicle control, and unlike  $\alpha$ GC alone, which significantly improved GTT at each time point. Blocking these cytokines resulted in increased insulin resistance at 15 and 30 min time points compared to  $\alpha$ GC treatment alone (Figure 6E). We





**Figure 5. Adoptive Transfer of iNKT Cells Protects from Weight Gain and Adipocyte Hypertrophy and Reverses Obesity-Associated Metabolic Disorders**

WT iNKT cells (>95% pure), iNKT cell-depleted T cells (T), or no-treatment (PBS) control were injected i.p. into 18–20-week-old DIO mice on HFD for 12 weeks; they continued on HFD for 4 days.

(A) Difference in weight pre- and 4 days postadoptive transfer of mice that received iNKT cells (n = 10), PBS (n = 6), or T cells (n = 6).

(B) Adipocyte diameter of obese mice that received iNKT cells versus T cells or PBS control (two samples per mouse; n = 4 for PBS and NKT, n = 3 for T cells; ANOVA with Tukey).

(C) Epididymal fat weight after iNKT transfer (n = 10) compared to PBS (n = 6) or T cell transfer (n = 6), with WT mice on SFD for comparison (n = 4).

(D and E) Weight change (D) and fasting glucose (E) in lean WT mice that received NKT cell transfer.

(F and G) Fasting glucose (ANOVA) (F) and glucose tolerance (G) following iNKT transfer (n = 8) compared to PBS (n = 4) or T cell transfer (n = 4), with WT mice on SFD for comparison (n = 4, two-way ANOVA).

(H) ITT in obese mice was measured 4 days post transfer of iNKT cells (n = 4), PBS (n = 4), T cells, or WT on SFD (n = 3) (two-way ANOVA with Tukey post hoc).

(I) Following adoptive transfer of iNKT cells for 3 days, adipose tissue was harvested and cultured. Production of resistin, leptin, adiponectin, ANGPTL3, and IL-10 were measured by protein array (n = 2 of two pooled mice each).

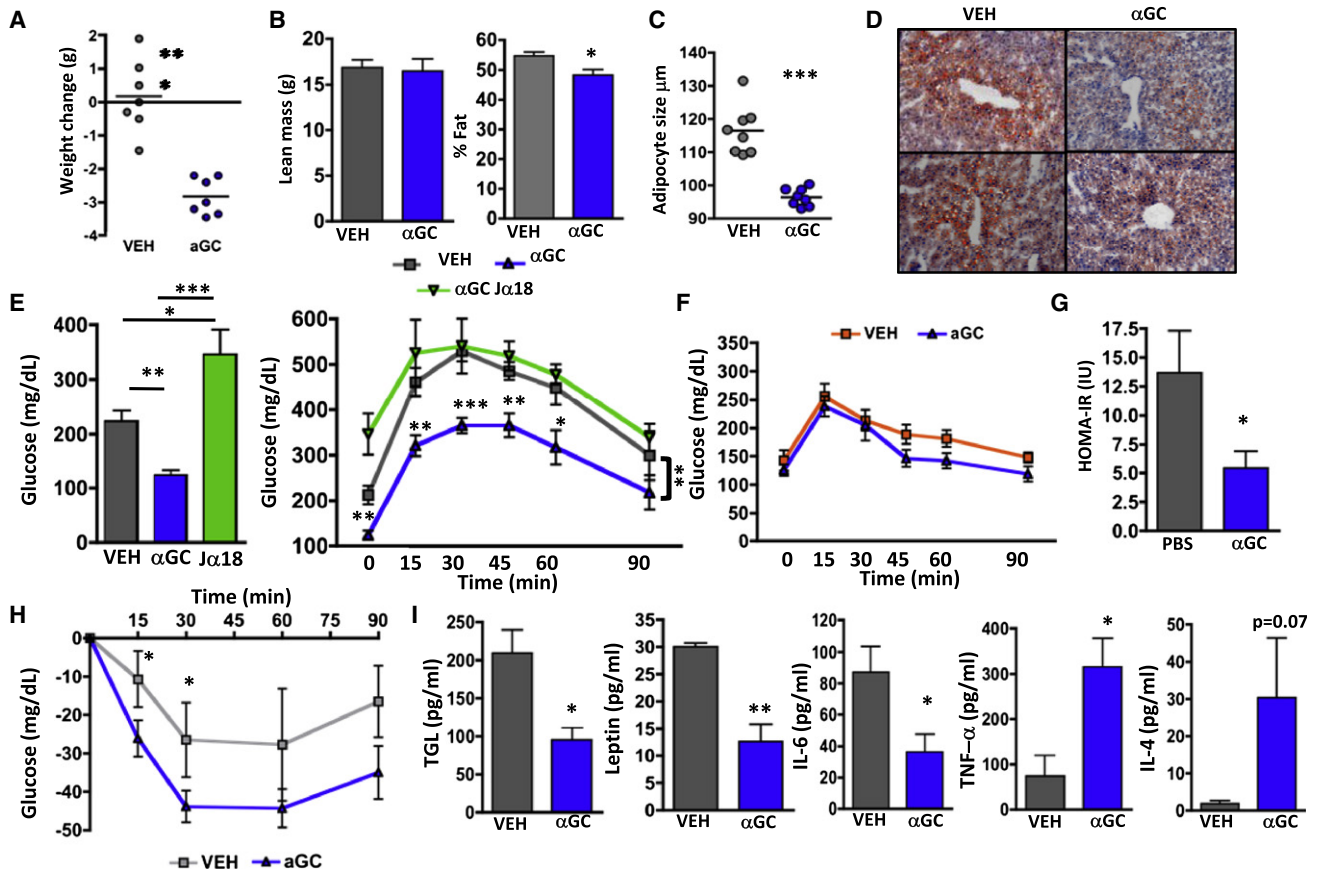
Graphs show mean ± SD. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.0001.

next blocked these cytokines individually. Neutralization of IL-4 alone prior to αGC partly prevented the improvement in fasting glucose seen with αGC (Figures S6A and S6C). It is possible that blocking IL-4 or IL-10 without αGC could also be beneficial through another pathway, such as macrophages; however, neutralizing antibody treatment alone without αGC did not improve or worsen GTT compared with PBS (Figure S5D).

## DISCUSSION

We have identified a role for iNKT cells in the regulation of body weight and metabolic state. Our results indicate that iNKT cells may act through regulation of inflammation in adipose tissue, although whether this effect is direct or indirect is not fully clear. Previous data from human and murine studies suggest that



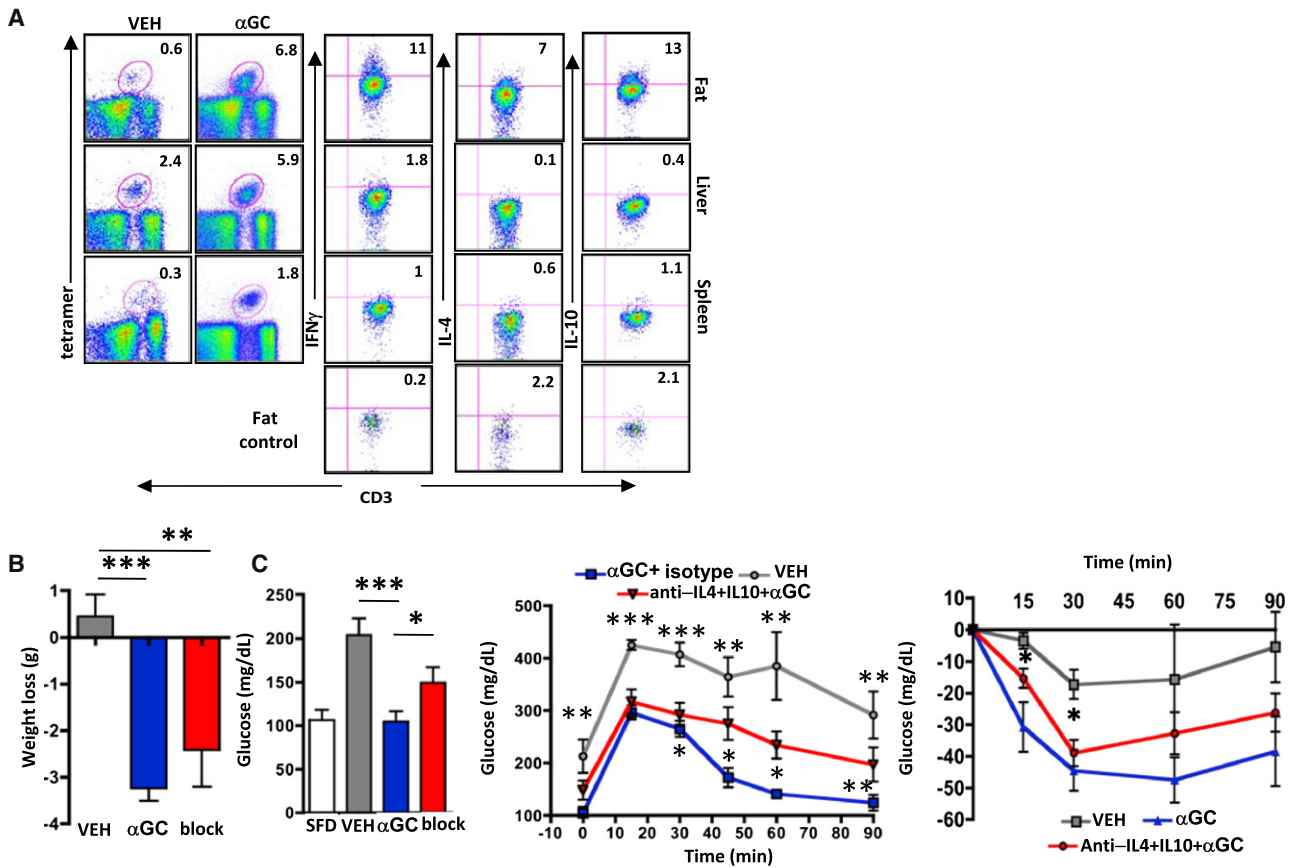


**Figure 6. αGC Treatment Reverses Obesity-Associated Metabolic Disorders**

(A and B) Effect of αGC treatment on weight gain and adipocytes. (A) Difference in weights before and 4 days after αGC or vehicle (VEH) treatment (n = 7 per group, t test). (B) Lean mass and % body fat measured by DEXA following αGC treatment (n = 5, t test). (C) Adipocyte size following αGC treatment (two samples per mouse, n = 4 mice, t test). (D) Oil red O staining in liver samples from WT obese mice 4 days post αGC injection. Two representative images per treatment (representing five mice per group). (E) Fasting glucose and GTT of WT obese mice 4 days post αGC injection (n = 5, fasting glucose: t test; GTT: two-way ANOVA with post hoc shown; area under curve \*\*\*p = 0.0007). (F) GTT following αGC treatment into lean WT mice on SFD (n = 4 per group). (G and H) Insulin resistance as measured by HOMA-IR (G) and ITT (H) following αGC treatment (n = 4). (I) Circulating triglyceride (TGL), leptin, IL-6, TNF-α, and IL-4 following αGC treatment (n = 5 each, all t tests). Graphs show mean ± SD. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.0001.

adiposity due to diet (and negative energy expenditure) is the trigger for increased adipose tissue inflammation, which subsequently leads to insulin resistance and metabolic disorder. In obesity, excess lipid leads to larger, stressed adipocytes that produce proinflammatory adipokines and cytokines, leading to downstream negative effects on insulin sensitivity. Influencing the function of adipocytes has potentially significant health benefits. We and others have shown that increasing adipocyte size correlates with insulin resistance, triglyceride levels, and the degree of hepatic steatosis (Lönn et al., 2010; O’Connell et al., 2010). One hypothesis is that adipocytes are dysfunctional when filled to capacity (~3 μg lipid per cell) (Danforth, 2000), causing “overflow” of lipids to muscle and liver (Gregor and Hotamisligil, 2007) and leading to overproduction of glucose and high fatty-acid levels, which can activate the kinases JNK and IKK, leading to insulin resistance at these

sites (reviewed by Guilherme et al., 2008; Hotamisligil and Erbay, 2008). iNKT cell-deficient mice had larger adipocytes, even on SFD, and when iNKT cells were reintroduced or activated, adipocytes rapidly reduced in size. Importantly, iNKT cell transfer did not cause weight loss or hypoglycemia in lean control mice. Furthermore, mice that received iNKT cells had significantly higher levels of adipose tissue adiponectin, an insulin-sensitizing adipokine produced largely by adipocytes. Moreover, fat from iNKT cell transfers had significantly less leptin and ANGPTL2 production, which are key mediators of chronic adipose tissue inflammation and insulin resistance. The production of anti-inflammatory cytokines by iNKT cells may have direct effects on adipocytes. IL-10, which was increased in adipose tissue following iNKT cell transfer, can protect adipocytes from the physiological insulin-desensitizing effects of TNF-α seen in obesity (Hong et al., 2009; Lumeng



**Figure 7.  $\alpha$ GC Treatment Expands Adipose iNKT Cells and Activates Their IL-4 and IL-10 Production, Resulting in Improved Metabolic Health**

(A) Left panel, iNKT cell levels in adipose tissue, liver, and spleen at 4 days post  $\alpha$ GC injection. Right panel, IFN- $\gamma$ , IL-4, and IL-10 production by iNKT cells from each organ postactivation. Quadrant percentages represent cytokine production by iNKT cells after subtraction of control (control for fat shown on bottom row). (B) Obese mice received neutralizing IL-4 and IL-10 (n = 7) antibodies before  $\alpha$ GC treatment. Weight loss post treatment with or without neutralizing antibodies is shown. (C) GTT and insulin resistance test in obese mice where IL-10 and IL-4 were neutralized prior to  $\alpha$ GC (n = 7, two-way ANOVA and t test).

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.0001.

et al., 2007) and probably leads to “healthier” adipocytes, reflected in lower levels of circulating proinflammatory cytokines and leptin.

iNKT cells may also indirectly affect adipocytes through their effects on macrophage function. The production of IL-10 was unique to iNKT cells in adipose tissue, and IL-10 and IL-4 are potent inhibitors of proinflammatory cytokines (Williams et al., 2004). IL-10 promotes a phenotypic switch in macrophage activation toward an anti-inflammatory phenotype (Williams et al., 2004). Adipocyte hypertrophy can lead to cell death, triggering an inflammatory response from macrophages, which form crown-like structures surrounding dead adipocytes. This causes a phenotypic switch in macrophages from M2 to M1, which also contributes to adipose tissue inflammation (Dalmás et al., 2011). iNKT numbers were strongly inversely correlated with proinflammatory macrophage numbers in fat, and reintroduction or activation of iNKT cells caused a marked decrease in adipose tissue-associated macrophages. These findings and another recent study (Winer et al., 2009) show that adoptive transfer of conventional T cells offered some relief in the metabolic

syndrome, compared to PBS, although the effect was not as potent as in iNKT cell transfer. The partially protective effect of T cell transfer into obese mice is not surprising given that CD4<sup>+</sup> T cell transfer into obese mice reversed weight gain and insulin resistance (Winer et al., 2009), an improvement that was found to be due to both FoxP3 positive and negative CD4<sup>+</sup> T cells. Another recent study found that Treg cells alone did not significantly restore all metabolic function in obesity (Feuerer et al., 2009), although this could be due to difficulties in complete gain or loss experiments involving Treg cells. Our conventional T cell transfer also included Treg cells, which likely contributed to the benefits in metabolism. Conversely, it is possible that iNKT cells were likely a contaminant in previous studies that used adoptive transfer of CD4<sup>+</sup> T cells in obesity, given that iNKT cells are a subpopulation of CD4<sup>+</sup> T cells in fat, liver, and spleen. From these combined data, we could draw the conclusion that iNKT cells are sufficient and possibly more effective at improving glucose handling, but are not the only immune cell type in adipose tissue that is involved in metabolic regulation.

Our results from two iNKT cell-deficient models show that mice lacking iNKT cells are more affected by HFD and, even on an SFD, gained more weight with age than controls did. In this study, we did not use littermate controls in our iNKT-deficient models, due to lack of availability. Therefore, there is a possibility that weight gain or metabolic changes seen could be wholly or partly due to different weaning or early changes, which have been shown to be very important in metabolism during the life of animals. In this case, we believe this is unlikely, because iNKT cells are thought to arise postnatally at day 5 in the thymus and at 1–2 weeks in the periphery (Pellicci et al., 2002). We found that WT and mutant mice had similarly sized litters with similar birth weights; additionally, it was only with age (>4 months) that differences were seen on an SFD. Our results also suggest that the severe obesity in leptin deficient ob/ob mice causes iNKT cell depletion. The presence of leptin itself does not appear to be essential for iNKT cell survival, as leptin levels are elevated in obesity, whereas iNKT cells are depleted. Previous studies show that ob/ob mice have aberrant hematopoiesis and immune defects (Fantuzzi and Faggioni, 2000; Macia et al., 2006), including decreased iNKT cells in livers from ob/ob mice (Yang et al., 2007). Given that iNKT cells are thought to be absent at birth (Kronenberg and Gapin, 2002; Pellicci et al., 2002), and ob/ob mice are already significantly larger than their WT littermates very soon after birth, effects of excess adiposity cannot be ruled out.

The role of iNKT cells in regulation of metabolism is just emerging, although it has previously been described that iNKT cells are reduced in livers of obese mice (Ma et al., 2008; Yang et al., 2007), leading to hepatic insulin resistance, and that increasing iNKT cells in obese liver improved hepatic steatosis and glucose tolerance (Ma et al., 2008; Elinav et al., 2006). During the review of our manuscript, Ji et al. (2012) also reported that iNKT cells are decreased in human and murine obesity, confirming our previous (Lynch et al., 2009) and current findings. Furthermore, both our groups found that activation of iNKT cells with  $\alpha$ GC led to macrophage polarization to an M2 phenotype and improved glucose sensitivity through anti-inflammatory cytokine signaling (Ji et al., 2012). Similarly, Kotas et al. (2011) found that iNKT cells were selectively depleted in obese livers and that *Cd1d1*<sup>-/-</sup> mice had slight but significantly increased steatosis and impaired hepatic glucose tolerance. Their findings, however, were not as dramatic as our results in obese iNKT-deficient mice. Furthermore, the protective role of iNKT cells was further questioned when Ohmura et al. (2010) reported that mice lacking iNKT cells were protected against obesity. However, this study used  $\beta$ 2-microglobulin-deficient mice, which lack both CD8<sup>+</sup> T cells and iNKT cells, and CD8 T cells are reported to be pathogenic in obesity (Nishimura et al., 2009). To address this, Mantell et al. (2011) used *CD1d*<sup>-/-</sup> mice, and in contrast to our and others' studies, found no difference between *CD1d*<sup>-/-</sup> and WT mice on a HFD, although the conclusion was drawn that the deletion of NKT cells was insufficient to protect against the development of the metabolic abnormalities of obesity. Another study (Sato et al., 2012) described that *CD1d*<sup>-/-</sup> mice gained less weight than WT mice with less steatohepatitis, implying that NKT cells are pathogenic in obesity, in contrast to previously published studies (Elinav et al., 2006; Ji et al., 2012; Kotas et al., 2011; Ma et al., 2008;

Yang et al., 2007). These discrepancies between iNKT-deficient studies are difficult to resolve, although there are some key differences in experimental approach between studies. Most importantly, Mantell et al. (2011) used NK1.1<sup>+</sup> T cells as a marker of iNKT cells, although not all invariant NKT cells express NK1.1, and in fat, iNKT cells express less NK1.1 than elsewhere; lastly, not all NK1.1<sup>+</sup> T cells are CD1d-restricted. However, this study found that iNKT cells were depleted in obesity, similar to ours and others that describe a loss of iNKT cells in obesity that leads to a worsening metabolic outcome. The discrepancies in results with iNKT-deficient mice between different laboratories may not yet be explained, but the adoptive transfer of iNKT cells into obese mice and the specific activation of iNKT cells with  $\alpha$ GC provide a more conclusive demonstration of the positive benefits of iNKT cells against obesity-related disorders. Furthermore, our murine data parallel our human results on the effects of weight gain and loss on iNKT cells.

In humans, iNKT cell levels correlate negatively with weight gain (Lynch et al., 2009). Following the return to SFD in mice or weight loss in humans, iNKT levels are increased, demonstrating the reversibility of iNKT cell defects. Likewise, despite severe numerical defects, the reduced iNKT cell pool in murine obesity is still capable of marked expansion and production of anti-inflammatory cytokines, leading to dramatically positive effects on adipocytes and metabolism. Whether treatments that increase or activate iNKT cells will have beneficial effects in humans with metabolic syndrome remains to be determined. The use of  $\alpha$ GC to activate iNKT cells in humans has been employed in multiple cancer settings. It has proven to be safe, with no reports of hepatotoxicity, unlike in mice, wherein repeated higher doses in older animals can cause hepatotoxicity (Exley et al., 2011). The observation that targeting the iNKT cell system with  $\alpha$ GC in mice did not cause hypoglycemia in the diabetic or euglycemic model suggests a restoration of physiological balance. This is potentially very attractive from a therapeutic perspective.

Immuno-metabolic interaction in obesity is now established as a key factor in adipose tissue inflammation and subsequent development of type 2 diabetes. It also holds exciting possibilities for the development of a new treatment paradigm for this disorder, which is currently at epidemic proportions. Our study supports the emerging view that T cells and macrophages play important roles in adipose tissue function and identifies iNKT cells as a major regulatory T cell population in fat. Further studies exploring the therapeutic potential of iNKT cells in obesity and metabolic syndrome are warranted.

## EXPERIMENTAL PROCEDURES

### Mice

Male (and where indicated, female) WT C57BL/6 and ob/ob mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). *J $\alpha$ 18*-deficient mice and C57BL/6J *CD1d1*<sup>-/-</sup> mice have been described (Exley et al., 2003). *J $\alpha$ 18*-deficient (F9) and *CD1d1*<sup>-/-</sup> (F12) mice were backcrossed on the C57BL/6 background. Mice were housed adjacently from birth and fed the same chow (SFD or HFD). In general, experiments began with 6-week-old male mice. For metabolic studies, the mice received either SFD or HFD (Research Diets, 60 kcal% fat for the HFD) from 6 weeks of age, for 6 weeks, or 12 weeks where indicated. Mice were housed under specific pathogen-free conditions. Animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

### Subjects

Ten milliliters of peripheral blood were obtained from 26 consecutive obese subjects who were referred to our hospital-based weight-management clinic (mean age 47, range 24–60 years; mean BMI 48), 18 patients attending the weight management clinic 18 months after bariatric surgery (mean age 46, range 36–54 years; mean BMI 38), and 22 lean healthy controls (mean age 39, range 23–54 years; mean BMI 24). All blood samples were obtained with written informed consent. The ethics committee at St. Vincent's University Hospital, Dublin granted approval for this study.

### Reagents

$\alpha$ GC analog PBS-57-loaded or empty CD1d tetramers were provided by the National Institutes of Health (NIH) tetramer facility (Emory Vaccine Center, Atlanta).  $\alpha$ GC (KRN 7000) was kindly provided by Kirin, Japan. Immune cells were cultured in RPMI-1640 and adipose tissue-derived cells in Dulbecco's modified Eagle's media (DMEM) supplemented with penicillin, streptomycin (Mediatech, Manassas, VA, USA), and 5% fetal bovine serum (HyClone, Logan, UT, USA).

### Diet and Metabolic Studies

WT,  $\alpha$ 18-deficient, and *CD1d1*<sup>-/-</sup> mice were weighed weekly, and food intake was monitored on HFD. Body fat content was measured by a dual-energy X-ray absorptiometry (DEXA) scan performed after mice were sacrificed. Whole abdominal adipose fat pads were weighed after dissecting out the testes and lymph nodes. After 6 weeks on HFD, fasting blood glucose (OneTouch Ultra) and insulin concentrations (Crystal Chem ELISA) were measured. For glucose tolerance tests, fasted (10 hr) mice received 1 g glucose per kg body weight intraperitoneally (i.p.), and glucose levels were measured every 15 min for 90 min. For insulin resistance, HOMA-IR calculation (Matthews et al., 1985) was used: fasting blood glucose  $\times$  fasting insulin  $\div$  22.5. Two samples of 5 mm liver were collected and fixed in formalin overnight, prior to paraffin mounting and preparation of hematoxylin and eosin (H&E)- or oil red O-stained slides for measurement of fatty liver. For H&E and oil red O staining, biopsies were viewed using the 20 $\times$  objective. The degree of fatty liver was measured by oil red O staining intensity around five portal-tract areas per slide.

### Adipocyte Size

Adipocyte size and number were measured by osmium and immunohistochemistry. Two samples of 20–30 mg of adipose tissue per mouse were immediately fixed in osmium tetroxide (3% solution in 0.05 M collidine), minced into 1 mm pieces and incubated in the dark at room temperature for 48 hr. Adipose cell size and number were determined by a Beckman Coulter Multisizer 3 with a 400  $\mu$ m aperture. Adipose tissue was also fixed in formalin overnight, prior to paraffin mounting and preparation of H&E slides. The adipocyte number was counted per field of view, in ten fields per sample, and related back to the original weight of each fat pad.

### Spleen, Liver, and Adipose Tissue and Human Blood Preparations

Isoflurane-anesthetized mice were systemically perfused with PBS. Single-cell suspensions from spleens were prepared by standard techniques. Liver mononuclear cells (LMNCs) were isolated as previously described without collagenase digestion (Nowak et al., 2010). In brief, livers were perfused with PBS and minced, and iNKT cells were enriched by centrifugation in a two-step Percoll gradient. Enriched populations typically contained 20%–30% iNKT cells. Epididymal adipose tissue was dissected, carefully avoiding lymph nodes, minced with opposing scalpels, and digested with collagenase (Sigma-Aldrich, 0.2 mg/ml<sup>1</sup> in DMEM for 45 min at 37°C on a rotary shaker). The digests were filtered through 40  $\mu$ m cell strainers and pelleted to enrich fat-associated lymphocytes in the stromovascular fraction (SVF). Cell yields and viability were measured with trypan blue staining. Ten milliliters of venous blood was collected in heparinized tubes for measurement of iNKT cell levels. Peripheral blood mononuclear cells were prepared by standard density gradient centrifugation over Lymphoprep (Nycomed) at 400 g for 25 min. Cells were then washed twice with Hank's balanced salt solution supplemented with HEPES buffer solution (Invitrogen Life Technologies) and antibiotics. Cell pellets were resuspended in 1 ml of RPMI-1640 medium, and cell yields and viability were assessed by ethidium bromide and acridine orange staining.

The cell suspension was adjusted to  $1 \times 10^6$  cells/ml in RPMI for staining (100  $\mu$ l/tube).

### Flow Cytometry

Single-cell suspensions of splenocytes, LMNCs, and adipose SVF were blocked with CD16/32 monoclonal antibody (mAb) and stained for 30 min at 4°C in the dark with PBS-57-loaded or empty CD1d tetramer-PE (NIH tetramer facility) and CD3 (1:150 dilution, eBioscience). Macrophages were labeled with phycoerythrin (PE)-conjugated antibody to F4/80 (1 in 100), CD11c (1 in 200), and CD206 (1 in 200) to differentiate M1 from M2 macrophages in the SVF, as previously described (Fujisaka et al., 2009). For human peripheral blood, mouse anti-human CD3 combined with the iNKT TCR (6B11) and isotype-matched controls were used (BD Biosciences). iNKT cells were also stained with V $\alpha$ 24 and V $\beta$ 11 TCR chains from Coulter Immunotech (Marseilles, France). Cells were washed and fixed in 1% paraformaldehyde and acquired on an LSR II flow cytometer (BD Biosciences) and with FlowJo and Kaluza software.

### iNKT Cell Isolation and Adoptive Transfer

Hepatic mononuclear cells were stained with CD1d tetramer-PE and sorted to >95% purity using a FACSAria II (Becton Dickinson, San Jose, CA, USA). Purified iNKT cells ( $5 \times 10^5$ ) were injected i.p. into  $\alpha$ 18-deficient mice that had been on HFD for 8 weeks. Metabolic parameters were analyzed after 4 days, mice were sacrificed, adipose tissue was weighed, and adipocytes were measured by osmium and immunohistochemistry.

### In Vivo Stimulation of iNKT Cells and Intracellular Cytokine Staining

Mice were injected i.p. with 2  $\mu$ g of  $\alpha$ GC or vehicle alone and sacrificed after 5 hr or 4 days, at the time of metabolic analysis. Single-cell suspension of splenocytes, LMNCs, and adipose tissue SVFs were obtained as before, but with the inclusion of Brefeldin A in all media. First, single-cell suspensions of splenocytes or LMNCs were stained with cell-surface-labeling CD3 mAb and  $\alpha$ GC-loaded CD1d tetramer. Cells were then fixed, permeabilized, and stained intracellularly for IL-4, IL-10, and IFN- $\gamma$  using the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions. To neutralize cytokines prior to  $\alpha$ GC treatment, anti-IL-4 (11B11) or anti-IL-10 (JES5-2A5) were injected i.p. prior to injection of  $\alpha$ GC.

### Statistical Analyses

Error bars represent SEM. The statistical significance of differences between two groups was determined using the Mann-Whitney *U* test or Student's *t* tests where appropriate following determination of Gaussian distribution of the data. Differences among mice groups (>2) were evaluated using one-way or two-way ANOVA followed by post hoc Tukey tests. Values of *p* < 0.05 were considered significant.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2012.06.016>.

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