Increased levels of invariant natural killer T lymphocytes worsen metabolic abnormalities and atherosclerosis in obese mice

Savitha Subramanian, Michael S. Turner, Yilei Ding, Leela Goodspeed, Shari Wang, Jane H. Buckner, Kevin O’Brien, Godfrey S. Getz, Catherine A. Reardon, and Alan Chait

Division of Metabolism, Endocrinology and Nutrition and Division of Cardiology, University of Washington, Seattle, WA; Translational Research Program, Benaroya Research Institute, Seattle, WA; and Department of Pathology, University of Chicago, Chicago, IL.

Abstract  Obesity is a chronic inflammatory state characterized by infiltration of adipose tissue by immune cell populations, including T lymphocytes. Natural killer T (NKT) cells, a specialized lymphocyte subset recognizing lipid antigens, can be pro- or anti-inflammatory. Their role in adipose inflammation continues to be inconclusive and contradictory. In obesity, the infiltration of tissues by invariant NKT (iNKT) cells is decreased. We therefore hypothesized that an excess iNKT cell complement might improve metabolic abnormalities in obesity. Vα14 transgenic (Vα14tg) mice, with increased iNKT cell numbers, on a LDL receptor-deficient (Ldlr−/−) background and control Ldlr−/− mice were placed on an obesogenic diet for 16 weeks. Vα14tg.Ldlr−/− mice gained 25% more weight and had increased adiposity than littermate controls. Transgenic mice also developed greater dyslipidemia, hyperinsulinemia, insulin resistance, and hepatic triglyceride accumulation. Increased macrophage Mac2 immunostaining and proinflammatory macrophage gene expression suggested worsened adipose inflammation. Concurrently, these mice had increased atherosclerotic lesion area and aortic inflammation. Thus, increasing the complement of iNKT cells surprisingly exacerbated the metabolic, inflammatory, and atherosclerotic features of obesity. These findings suggest that the reduction of iNKT cells normally observed in obesity may represent a physiological attempt to compensate for this inflammatory condition.—Subramanian, S., M. S. Turner, Y. Ding, L. Goodspeed, S. Wang, J. H. Buckner, K. O’Brien, G. S. Getz, C. A. Reardon, and A. Chait. 

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Obesity, a chronic inflammatory state, is characterized by immune cell infiltration into expanded adipose tissue in mice and humans (1, 2). It is widely accepted that the inflammation occurring within obese adipose tissue is linked to accompanying metabolic disorders, such as insulin resistance, hepatic steatosis, type 2 diabetes, and cardiovascular disease (3). Adipose tissue is immunologically dynamic, containing a variety of resident immune cells, such as macrophages (1, 2), T cells (4, 5), and B cells (6), alterations of which can modulate immune responses and metabolic function. While adipose tissue macrophages have garnered much of the focus over the past decade as a contributor to the sterile inflammatory response in obesity, recently there has been increased interest in the role of other immune cells, including T lymphocytes.

T-cell activation involves peptide antigen presentation via major histocompatibility complex (MHC) class II (CD4+) or MHC class I (CD8+). A unique highly conserved subset of T cells called natural killer T (NKT) cells, respond to lipid or glycolipid antigens (7–9). These cells express both natural killer receptor 1.1 (NK1.1) and T-cell receptors (TCR). Based on these characteristics, they are classified broadly as type 1 or invariant iNKT cells and type 2 or variant NKT (vNKT) cells. vNKT cells express more diverse TCRs, whereas the iNKT-cell TCR is characterized by the semi-invariant Vα14/Jα18 chain paired with the VB8, VB7, or VB2 β chains in the mouse (Vα24/α18/Vβ11 in humans). iNKTs react with various lipid antigens whereas the antigen specificity of vNKTs is less clear. iNKT cells recognize and undergo activation when lipid antigen is presented via the MHC class I-like molecule CD1d on mouse

Abbreviations: ALT, alanine aminotransferase; HFHSC, high-fat, high-sucrose diet with 0.15% cholesterol; iNKT, invariant NKT; MHC, major histocompatibility complex; NKT, natural killer T; TCR, T-cell receptor; TUNEL, terminal deoxynucleotidyl transferase nick-end labeling; vNKT, variant NKT.

1To whom correspondence should be addressed.

e-mail: ssubrama@u.washington.edu

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antigen-presenting cells and recognized by Vα14Jα18/Vβ8 on responding T cells (10, 11). Upon activation, NKT cells can rapidly and robustly secrete either proinflammatory (Th1) or anti-inflammatory (Th2) cytokines, which can activate antigen-presenting cells as well as lymphocytes. iNKT cells are therefore uniquely poised as a bridge between the innate and the adaptive immune systems (7) and can have opposing roles in promoting or resolving inflammation.

The reported role for iNKT cells in obesity is controversial. However, a reduction in iNKT numbers has been observed in adipose tissue and livers of obese mice and humans (12–15). Since iNKT-cell numbers are reduced in obesity and since iNKT cells could potentially recognize lipid antigens in expanded adipose tissue of obesity and beneficially modulate inflammation, we hypothesized that increasing iNKT-cell numbers would improve metabolic abnormalities and atherosclerosis. We utilized Vα14Jα18 TCR transgenic mice which have an increased complement of iNKT cells (16). These mice were studied on a LDL receptor-deficient (Ldlr−/−) background, since the Ldlr−/− mouse is a model of the metabolic syndrome when fed diets rich in fat and refined carbohydrates and also allows for evaluation of atherosclerosis (17). In contrast to our expectation, we show here that increasing iNKT-cell numbers worsens the metabolic complications that accompany obesity in this mouse model.

METHODS

Animals and diet

Vα14 transgenic (Vα14tg) mice from Dr. Albert Bendelac (University of Chicago) (16) were crossed with Ldlr−/− mice as described previously (18). All animals were in the C57BL/6J background. Littermate Ldlr−/− mice were used as controls. Age-matched 10-week-old male mice were fed either standard chow or a high-fat, high-sucrose diet with 0.15% cholesterol (HHFSC) (BioServ F4997, Frenchtown, NJ) for 16 weeks (n = 10 per group). Mice were maintained in a temperature- and light-controlled facility in cages with microisolator filter tops. Body weights were measured weekly. Food intake was recorded after 10 weeks of diet and calculated as an average of three sequential days from a known amount of food given. The food was weighed daily, and the amount of food consumed was calculated. When the animals were euthanized, harvested tissues were snap-frozen in liquid nitrogen and stored at −70°C or were fixed with 10% neutral-buffered formalin and embedded in paraffin wax. All experimental procedures were undertaken with approval from the Institutional Animal Care and Use Committee of the University of Washington.

Isolation of leukocytes and flow cytometry

Mouse adipose tissue, livers, and spleens were collected and weighed after gentle perfusion with PBS. Tissues were minced in flow buffer (2% FBS in PBS), and adipose and livers were digested with Collagenase type IV (Sigma, St. Louis, MO) for 30 min at 37°C with shaking. Spleens were processed without collagenase treatment. Adipose stromal vascular cells (SVC), hepatic nonparenchymal cells, or splenocytes thus obtained were passed through a 70 μm strainer and centrifuged at 300 g for 5 min. Pellets were incubated with erythrocyte lysis buffer for 5 min, centrifuged, and suspended in flow buffer.

Cell suspensions (1 × 10⁶ cells/sample) were preincubated with CD16/32 (FcR Block, BD Biosciences, San Jose, CA) for 15 min at 4°C, then stained with fluorescent-labeled antibodies or IgG isotype controls for 30 min at 4°C. Antibodies used for lymphocyte phenotyping were as follows: FITC-conjugated anti-CD3ε, APC-conjugated anti-CD69 (eBioscience, San Diego, CA); PerCP-conjugated anti-CD4 (Biolegend, San Diego, CA); and PE-conjugated CD1d tetramer (NIH Tetramer Core Facility). Cells were washed in flow buffer twice, resuspended in 0.5% paraformaldehyde, and analyzed using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA). Unstained and singly stained control cells were used to set up compensation and gates. Data were analyzed using FACSDiva software. A minimum of 50,000 events were analyzed for each sample.

Analytical procedures

Metabolic variables were measured in blood samples obtained from the retro-orbital sinus after a 5–6 h fast. Cholesterol and triglycerides in plasma and fast-phase liquid chromatography (FPLC) fractions were measured using colorimetric assay kits. Lipoproteins were separated from pooled plasma samples by FPLC. Plasma insulin levels were measured using an ELISA kit (Millipore, Billerica, MA). Alanine aminotransferase (ALT) was measured using an autoanalyzer through the Nutrition and Obesity Research Center at the University of Washington. Tissue lipids were extracted using the Folch technique (19). Intraperitoneal glucose and insulin tolerance tests were performed after a 5 h fast at weeks 13 and 14 of diet feeding, respectively, as previously described (17). Insulin resistance index was calculated using the formula fasting glucose (mmol/l) × fasting insulin (mU/l) / 22.5. Body composition was performed on conscious, immobilized mice using quantitative magnetic resonance imaging (EchoMRI whole-body composition analyzer, Echo Medical Systems, Houston, TX).

Real-time quantitative PCR

Total RNA was extracted from ~100 mg of whole adipose, liver, or aortae using a commercially available RNA extraction kit according to the manufacturer’s protocol (Agilent Technologies, Santa Clara, CA). After spectroscopic quantification, 2 μg of RNA was reverse-transcribed, and cDNA thus obtained was analyzed by real-time quantitative PCR. Primers specific for individual genes were purchased from Applied Biosystems (Assay-on-Demand, Life Technologies, Carlsbad, CA). GAPDH was used as the control housekeeping gene, levels of which did not change with diet. Relative amounts of the target gene were calculated using the ΔΔCt formula.

Histology, immunohistochemistry, and atherosclerosis quantification

The extent of atherosclerosis was measured in pinned aortas using the en face technique as previously described (17). Formalin-fixed, paraffin-embedded adipose tissues were sectioned and stained with Masson’s Trichrome stain using standard protocols. Macrophages in adipose tissue were detected using a rat monoclonal antibody (Mac2; titer 1:2500, Cedarlane Laboratories, Burlington, NC). Adipocyte size was measured using a modification of techniques described previously (16). Liver sections were stained with Masson’s Trichrome stain using standard protocols. Liver cell apoptosis was assessed using the terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay according to the manufacturer’s instructions (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Millipore, Billerica, MA) as previously described (20). Quantification of aortic root lesion area was performed as described previously (21). Quantification
of lesion area was performed on digital images of Movat-stained sections using image analysis software (Image Pro Plus software, Media Cybernetics, Bethesda, MD).

**Statistical analyses**

Data were analyzed using the GraphPad Prism 5 program (GraphPad Software Inc., La Jolla, CA) and are represented as means and standard errors. Student t-test was used to detect differences within groups when applicable. One-way ANOVA (ANOVA) was used to compare differences among all groups, and Bonferroni post-hoc testing was used to detect differences among mean values of the groups. P<0.05 was considered statistically significant.

**RESULTS**

**Obesity alters iNKT-cell levels in tissues**

We investigated the effects of diet-induced obesity on tissue iNKT-cell numbers in Ldlr<sup>−/−</sup> mice. In line with other studies, Ldlr<sup>−/−</sup> mice fed HFHSC diet for 16 weeks demonstrated a reduced number of iNKT cells in the liver, where iNKT cells represent up to 40% of total resident lymphocytes in mice (Fig. 1A–E) (7). Similarly, as has been demonstrated by others, we observed a decrease in iNKT cells in visceral (perigonadal) adipose tissue of obese Ldlr<sup>−/−</sup> mice. We calculated absolute iNKT-cell numbers per tissue (Fig. 1C) and per gram of tissue (Fig. 1D). Livers from lean Ldlr<sup>−/−</sup> mice contained ~2.8 × 10<sup>5</sup> iNKT cells per gram compared with 0.9 × 10<sup>5</sup> cells per gram in obese Ldlr<sup>−/−</sup> mice (Fig. 1D). In comparison, perigonadal adipose tissue from lean Ldlr<sup>−/−</sup> mice contained approximately 4.3 × 10<sup>4</sup> iNKT cells per gram, whereas obese Ldlr<sup>−/−</sup> mice contained ~0.9 × 10<sup>4</sup> cells per gram (Fig. 1D). Interestingly, iNKT populations were not altered in the spleen in Ldlr<sup>−/−</sup> mice with 16 weeks of high-fat feeding (Fig. 1A, B).

**V<sub>a14tg.Ldlr<sup>−/−</sup></vsub> mice have an increased complement of iNKT cells and develop excess weight gain with high-fat, high-sucrose diet feeding**

To evaluate the role of iNKT cells in obesity-associated metabolic derangements, we utilized a gain-of-function approach. As anticipated, V<sub>a14tg</sub> mice have a selective increase in iNKT-cell numbers (16) in the spleen, liver, and adipose tissue (Fig. 1F–J). The HFHSC diet did not alter the levels of iNKT cells in spleen, liver, or adipose tissues in the V<sub>a14tg</sub> mice (not shown).

We next evaluated the hypothesis that excess iNKT cells may beneficially impact obesity and related metabolic consequences. We previously showed that Ldlr<sup>−/−</sup> mice fed a HFHSC diet for 24 weeks developed obesity, hyperinsulinemia, and hyperlipidemia as well as significant atherosclerosis (17). V<sub>a14tg.Ldlr<sup>−/−</sup></vsub> and littermate Ldlr<sup>−/−</sup> mice were placed on standard chow or HFHSC for 16 weeks. These mice were fertile and appeared healthy. No differences in body weight or adiposity were observed between chow-fed V<sub>a14tg.Ldlr<sup>−/−</sup></vsub> and control Ldlr<sup>−/−</sup> mice; however, when challenged with the HFHSC diet, weight gain was significantly higher in V<sub>a14tg.Ldlr<sup>−/−</sup></vsub> mice compared with control Ldlr<sup>−/−</sup> mice (P < 0.001, Fig. 2A). Caloric intake was equivalent between the HFHSC diet groups (not shown). There were no differences in the perigonadal (intra-abdominal) fat-pad weights between the transgenic mice and their controls either in lean (0.4 ± 0.05 g versus 0.5 ± 0.03 g) or obese (2.4 ± 0.1 g versus 2.3 ± 0.1 g) mice. However, body composition analysis revealed increased generalized body fat distribution in the transgenic mice fed the HFHSC diet (Fig. 2B, C) compared with obese Ldlr<sup>−/−</sup> mice, suggesting increases in other fat depots. No differences in lean body mass were observed between the obese groups.

**Increased complement of iNKT cells causes marked metabolic dysregulation in obesity**

Hypercholesterolemia and hypertriglyceridemia observed in Ldlr<sup>−/−</sup> mice on the HFHSC were amplified in the transgenic mice. Plasma cholesterol levels were 30% higher in V<sub>a14tg.Ldlr<sup>−/−</sup></vsub> mice compared with littermate high-fat-fed controls; similarly, plasma triglyceride levels were 50% higher in V<sub>a14tg.Ldlr<sup>−/−</sup></vsub> mice (Fig. 3A, B). Differences in plasma cholesterol and triglycerides were observed as early as four weeks of diet feeding (not shown). Lipoprotein profiles showed increased VLDL/IDL and LDL particles in the HFHSC-fed transgenic mice (Fig. 3C, D). Fasting hyperglycemia was observed in the transgenic mice on HFHSC feeding; these mice also demonstrated significant fasting hyperinsulinemia (Fig. 3E, F). Correspondingly, the insulin resistance index was increased in the transgenic mice, suggesting a greater degree of insulin resistance in states of lipid excess (Fig. 3G). Glucose tolerance testing revealed a modest worsening of glucose excursions in the V<sub>a14tg.Ldlr<sup>−/−</sup></vsub> compared with Ldlr<sup>−/−</sup> HFHSC-fed control mice with significant hyperinsulinemia (Fig. 3H, I). No differences in lipids or insulin sensitivity were observed in the chow-fed animals between the two genotypes.

**Adipose tissue inflammation is worsened in obese mice with excess iNKT cells**

Since activated iNKT cells can interact with tissue macrophages (12), we examined whether the presence of excess iNKT cells influenced the macrophage content of adipose tissue. Obese V<sub>a14tg.Ldlr<sup>−/−</sup></vsub> mice accumulated more macrophages in perigonadal (intra-abdominal) adipose tissue compared with littermate controls, as demonstrated by increased immunostaining for the macrophage-specific protein Mac2 (Fig. 4A, B). Adipocyte size was decreased in the obese V<sub>a14tg.Ldlr<sup>−/−</sup></vsub> mice (Fig. 4C). Analysis of genes involved in inflammation in whole adipose tissue revealed increased expression of a variety of genes in obese mice, although the increase was significantly more pronounced for the chemotactic factor (Mcp1) gene as well as macrophage-specific genes, such as Emr1 (F4/80) and Mac2 (Fig. 4D). Overall, the accumulated macrophages showed a phenotype of proinflammatory M1 activation, as evidenced by increased Tnfα and Ccl5 expression with decreased expression of Retnla, an anti-inflammatory activation M2 marker (Fig. 4D). Taken together, these findings indicate an increase in adipose inflammatory changes in obese mice with excess iNKT cells.
mice (Fig. 5C), suggesting increased hepatocyte damage, although we did not observe an increase in TUNEL-positive cells (Fig. 5D). Plasma circulating SAA levels, an inflammatory marker derived from the liver, were increased in both groups of obese mice (Fig. 5E). Additionally, gene expression analysis did not reveal evidence of increased hepatic inflammation in transgenic mice (Fig. 5E).

Aortic atherosclerosis and inflammation is worsened in obese Vα14tg.Ldlr−/− mice

Mice on the Ldb−/− background are particularly susceptible to atherosclerosis when placed on high-fat diets. iNKT cells have been shown to enhance atherosclerotic lesion development (22). To assess atherosclerosis in our mouse model of excess iNKT cells, we first measured aortic atherosclerotic lesion area using the en face technique.
Chow-fed mice in both groups did not show any evidence of atherosclerosis. As anticipated, significant atherosclerosis was observed in the Ldlr−/− mice on the HFHSC diet, and it was substantially amplified in the obese Vα14tg.Ldlr−/− mice (P < 0.002, Fig. 6A). A similar trend was also observed in the aortic root lesion area, although it did not reach statistical significance (58,645 ± 19,999 µm² versus 87,712 ± 13,274 µm²). In line with these observations, aortic expression of Il6 and Vcam1 genes was significantly increased in the obese transgenic mice compared with the obese Ldlr−/− mice, which in turn were much higher than in the aortae of the lean animals (Fig. 6B). Aortic lesion area correlated with plasma cholesterol (r = 0.58, P < 0.05) and triglyceride levels (r = 0.70, P < 0.01) in the obese animals fed high-fat diets.

**DISCUSSION**

We previously showed that Ldlr−/− mice develop many features of the metabolic syndrome when fed diets rich in saturated fat and refined carbohydrates, effects that can be exacerbated by the addition of moderate amounts of dietary cholesterol (17). We utilized this model to evaluate the effects of iNKT-cell excess on weight gain and glucose homeostasis, as well as to evaluate atherosclerosis. Here we show that obesity and features of the metabolic syndrome are aggravated by the presence of increased numbers of invariant NKT cells, as achieved by crossing the Vα14 TCR transgenic mouse with the Ldlr−/− mouse. To our knowledge, this is the first reported study in which this transgenic mouse has been employed for the study of obesity and its associated pathophysiology. Contrasted with control Ldlr−/− mice, the transgenic Ldlr−/− mice exhibited increased weight gain, hyperlipidemia, adiposity and inflammation, glucose intolerance, and insulin resistance, as well as increased aortic atherosclerosis.

In obesity, adipocyte expansion in adipose tissue is accompanied by infiltration of macrophages, as well as an altered balance of T cells, including alterations in iNKT cells, CD8+ T cells, and CD4+ regulatory T cells. Changes in adipose tissue mass and inflammation are associated with the release of cytokines and adipokines and with metabolic dysfunction, in which glucose intolerance, insulin resistance, and increased hepatic lipid content are most extensively studied. The molecular mediators of these changes are not always clear.

There has been much recent investigation of the potential role of NKT cells in obesity using a variety of approaches. These include the examination of adipose tissue for the presence of NKT cells in lean and obese mice; stimulation of iNKT cells with its characteristic exogenous agonist α-galactosylceramide; and the response to diet-induced obesity of mice lacking iNKT cells only (Ldlr−/− mice) or lacking both iNKT and vNKT cells (CD1d−/− mice). The outcome of these studies has not been consistent in all cases. Some of these studies suggest that iNKT cells reduce the development of obesity and its metabolic accompaniments (12, 13, 15). On the other hand, one study showed that, in the absence of iNKT cells, there was a reduction in
Fig. 3. iNKT-cell excess worsens metabolic abnormalities in obesity. Plasma cholesterol (A) and triglycerides (B) in mice fed chow or HFHSC diets. Plasma lipoprotein cholesterol (C) and triglyceride (D) distribution in lean Ldlr<sup>−/−</sup> and HFHSC-fed V<sub>a14tg</sub>Ldlr<sup>−/−</sup> and control mice. (E) Plasma fasting glucose, (F) fasting insulin, and (G) calculated insulin resistance index in lean and obese mice. (H) Intraperitoneal glucose tolerance and area under the curve. (I) Plasma insulin levels at 30 min after glucose injection. n = 5–10 per group. *P < 0.05, **P < 0.001, ***P < 0.0001 versus lean mice; #P < 0.05, ##P < 0.001, ###P < 0.0001 versus HFHSC-fed Ldlr<sup>−/−</sup> mice.
obesity on a high-fat diet (23). A recent study evaluating the effect of iNKT-cell deficiency in lean mice suggests that adipose tissue NKT cells may protect against the development of insulin resistance (15). Others have shown that activation of iNKT cells with α-galactosylceramide exacerbates adipose tissue inflammation, insulin resistance, and hepatic steatosis in obese mice (24, 25). Ours is the first example of the use of a transgenic model overrepresenting iNKT cells. Each of the above-cited studies were performed with Ldlr+/− mice, whereas ours employed Ldlr−/− mice. Reasons for discordant results between laboratories include variations in experimental protocol, such as diet composition, level of enrichment in dietary fat, and duration of dietary therapy, and experimental location, which could influence the gut microbiota, which are known to be influenced by obesity (26). Some investigators have employed the commonly used Western-type diet containing 21% fat (40% kcals from fat), others have used a slightly higher dietary fat (45% kcals) (15), while most have employed diets in which 60% kcals are from fat (12, 13, 25, 27, 28). None of these studies used diets supplemented with cholesterol. We used a high-fat, high-sucrose diet (HFHSC), providing 60% calories as fat derived mostly from lard, supplemented with dietary cholesterol (0.15% in the HFHSC diet). In a separate study (data not shown), we found that with feeding the Western-type diet (40% calories from milk fat and 0.2% cholesterol), the transgenic animals exhibited no differences in body weight, hypercholesterolemia, or atherosclerosis over the Ldlr−/− controls. Overall, our results indicate that the overabundance of iNKT cells interacts critically with the diet and the presence of obesity in relation to the observed pathophysiological consequences.

The evolution of obesity with time involves a very dynamic network of cellular changes. Like the liver, white adipose tissue is highly enriched in NKT cells compared with the spleen and other lymphoid tissues. With the feeding of high-fat diets, there is a decline in the NKT-cell complement in two tissues: adipose and liver (13, 25). This was more pronounced in one study (13) than another (25), in which the reduction was only seen after 20 weeks of high-fat feeding. The restoration of chow to high-fat-fed animals rapidly increased adipose tissue iNKT cells. In our hands, the HFHSC diet also caused a reduction in iNKT-cell numbers in liver as well as adipose tissue. However, our data suggests that increasing the numbers of iNKT cells via enforced expression of a transgenic TCR, which drives positive thymic selection, may overwhelm the regulatory effects of obesity on iNKT. Nonetheless, in our experiments, it is clear that the exacerbation of obesity and metabolic syndrome was a result of the HFHSC diet acting through the NKT cells rather than an intrinsic effect of increased iNKT numbers, because there was no difference
between chow-fed Va14tg.Ldlr⁻/⁻ mice and their control Ldlr⁻/⁻ counterparts.

The relative contributions of the increased adipocyte number/size and of adipose inflammation to metabolic dysfunction are not clear. Both increase with the duration of the high-fat feeding. In one study, four days of a high-fat diet was sufficient to induce an increment in the size of adipocytes and enrichment of iNKT cells in the adipose tissue, resulting in impaired glucose tolerance and insulin sensitivity, though an increase in plasma insulin was only seen at eight weeks of high-fat feeding (27). In another study, three days of high-fat feeding resulted in increased adipocyte size and glucose intolerance (29). High-fat feeding in Rag⁻/⁻ mice lacking all T cells, pro- and anti-inflammatory, led to elevated fat mass and a small degree of insulin resistance (5). These authors noted an increase in SAA3 in adipose tissue with the three-day feeding but did not report on this with prolonged exposure to the high fat. Our Ldlr⁻/⁻ mice also exhibited increased adipose SAA3 expression when fed the HFHSC diet for 16 weeks, but the effect of HFHSC on SAA3 was attenuated in Vα14tg.Ldlr⁻/⁻ mice.

The macrophages that accumulated in mice with NKT-cell enrichment in our experiments have features of proinflammatory, M1 macrophages that may contribute to the metabolic dysfunction observed. In experiments with short-term, high-fat feeding (four days), the macrophages of the adipose tissue were predominantly of the M2 subtype.

**Fig. 5.** Hepatic steatosis is worsened in obese mice with excess iNKT cells. (A) Liver sections stained with Trichrome, 10× magnification. (B) Hepatic triglyceride content. (C) Plasma alanine aminotransferase levels. (D) TUNEL staining of hepatocytes. (E) Plasma SAA levels. (F) Gene expression of F4/80 (Emr1), Mcp1 (Ccl2), Tnf, and Cd3e in liver. n = 10 per group. *P < 0.05, **P < 0.01, ***P < 0.001 versus lean Ldlr⁻/⁻; ##P < 0.01 versus HFHSC Ldlr⁻/⁻.
mediated by IL-4/Stat6 activation (12). A study of the time-dependent evolution of macrophage subtypes in the adipose tissue has not been undertaken and would be of interest, since these cells may modify their expression patterns with the duration of fat feeding. Also included in the variety of cellular players in obesity are regulatory T cells, levels of which are decreased in obese adipose tissue, thus contributing to insulin resistance (30). Recent reports suggest a reciprocal cross-talk between iNKT cells and regulatory T cells, which can suppress cytokine production and cytotoxic activity of iNKT cells (31, 32). Thus, it is conceivable that the increase in iNKT-cell numbers accompanied by a likely decrease in regulatory T cells could account for the accelerated proinflammatory response that we observed in our obese transgenic mice.

One approach to the activation of iNKT cells involves the administration of the Cd1d agonist α-galactosylceramide. One might expect that this would yield results similar to those observed with NKT-cell enrichment. However, as with other studies of the role of NKT cells in obesity, the results of agonist administration are not fully consistent. The response to the agonist is not seen in fat 18−/− mice deficient in iNKT cells. Wu and colleagues noted that while weekly injections of agonist for eight weeks into obese mice did not increase body weight, worsening of insulin sensitivity and increased liver fat was observed (25). The same agonist given to obese mice in another study (29) reduced body weight and fat, with a decrease in adipocyte size, an improvement in glucose tolerance, and a reduction in plasma and liver triglycerides. Similar results were reported by Ji and colleagues (12). Ohmura and colleagues found that α-galactosylceramide administration resulted in a major accumulation of iNKT cells in adipose tissue, moderate glucose intolerance, and increased mRNA for Ifng, Tnfa, and Mep-1 (24). Their results are similar to ours reported here with iNKT-cell enrichment. The activation of iNKT cells by endogenous agonists presumably accounts for the activity of these cells in diet-induced obesity. The nature of these agonists is not known, although a dietary origin must be considered, given the variability of results with diets containing various lipids and fatty acids.

We used mice on the Ldlr−/− background for our studies to evaluate atherosclerosis. A role for the LDL receptor in lipid antigen presentation has been suggested. In a series of elegant studies, apoE was shown to be essential for exogenous lipid antigen uptake and presentation by antigen-presenting cells (33). The same authors also demonstrated a diminished IFNγ response in Ldlr−/− mice treated with the lipid antigen galactosyl galactosylceramide, but this response was nonetheless ~8-fold higher than in control mice. This finding suggests that the LDL receptor is not required for exogenous lipid antigen uptake. In corroboration, recently it was shown that the absence of the LDL receptor does not affect lipid antigen uptake; Ldlr−/− mice also demonstrated appropriate IFNγ responses to α-galactosylceramide in vivo (34). For some natural antigens, these authors implicated scavenger receptors as an important recognition receptor. Taken together, these data point to the presence of other less studied pathways by which lipids can be presented to iNKT cells and that the LDL receptor plays a role in but is not necessary for lipid antigen uptake and presentation.

Evidence that iNKT cells mediate an increase in atherosclerosis has been demonstrated previously. Absence of Cd1d and thus NKT cells resulted in decreased atherosclerosis in Ldlr−/− and ApoE−/− mice fed a high-fat diet (35–37). Adoptive transfer of splenocytes from Vα14H4g to Rag−/− mice led to an increase in atherosclerotic lesions (18). With the HFFSC diet used in our study, we noted a marked enhancement of aortic atherosclerosis with NKT-cell enrichment, in contrast to what we observed in the same strains fed the Western-type diet (data not shown). This again indicates a strong interaction between the former diet and NKT-cell enrichment in promoting atherosclerosis likely mediated in part by the enhanced hyperlipidemia, including increase in VLDL/IDL particles in the transgenic mice. We previously noted that atherosclerosis is well correlated with VLDL cholesterol levels (38). While hyperlipidemia developed in Ldlr−/− mice fed HFFSC diets, hypercholesterolemia and hypertriglyceridemia were worsened in the obese transgenic mice. Another contributor to the increased atherosclerosis could be related to the presence of increased LDL particles,
which have been demonstrated to stimulate the Vα14/4β18 TCR (18).

In conclusion, the role of iNKT cells in diet-induced obesity and its sequelae remains inconclusive. Results vary across a large spectrum, presumably depending upon the level and nature of the fat in the diet, the duration of high-fat feeding, the possible differences in gut microbiota, and other unrecognized factors. The resolution of the differences noted in this discussion will require careful further experimentation, in which the composition of the diet and its duration of feeding are carefully standardized, preferably in the same laboratory microenvironment. The activity of iNKT cells at various stages in the evolution of the obesity also needs to be considered. However, our findings are that the enrichment of tissues, including the adipose with iNKT cells as a result of transgenesis results in increased weight gain, adiposity, adipose tissue inflammation, liver steatosis, hyperlipidemia, hyperglycemia, glucose intolerance, insulin resistance, as well as a notable increment in aortic atherosclerosis, compared with control Ldlr−/− mice fed the same diet. Rather than knocking out a population that may have opposing physiological effects, our approach was to use a system that employs the expansion of iNKT numbers, while allowing them to respond naturally to endogenous physiological cues. Therefore, our data represent an augmentation of the natural role of iNKT cells and suggest that they act to promote diet-induced obesity and its related comorbidities when fed a diet rich in saturated fat and refined carbohydrates.

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