Natural Killer T Cells Are Involved in Adipose Tissues Inflammation and Glucose Intolerance in Diet-Induced Obese Mice

Kazue Ohmura; Naoki Ishimori; Yoshinori Ohmura; Satoshi Tokuhara; Atsushi Nozawa; Shunpei Horii; Yasuhiro Andoh; Satoshi Fujii; Kazuya Iwabuchi; Kazunori Onoé; Hiroyuki Tsutsui

Background—Macrophage and lymphocyte infiltration in adipose tissue may contribute to the pathogenesis of obesity-mediated metabolic disorders. Natural killer T (NKT) cells, which integrate proinflammatory cytokines, have been demonstrated in the atherosclerotic lesions and in visceral adipose tissue.

Objective—To determine whether NKT cells are involved in glucose intolerance and adipose tissue inflammation in diet-induced obese mice.

Methods and Results—To determine whether NKT cells are involved in the development of glucose intolerance, male β2-microglobulin knockout (KO) mice lacking NKT cells and C57BL/6J (wild-type) mice were fed with a high-fat diet (HFD) for 13 weeks. Body weight and visceral obesity were comparable between wild-type and KO mice. However, macrophage infiltration was reduced in adipose tissue and glucose intolerance was significantly ameliorated in KO mice. To further confirm that NKT cells are involved in these abnormalities, α-galactosylceramide, 0.1 µg/g body weight, which specifically activates NKT cells, was administered after 13 weeks of HFD feeding. α-Galactosylceramide significantly exacerbated glucose intolerance and macrophage infiltration as well as cytokine gene expression in adipose tissue.

Conclusion—NKT cells play a crucial role in the development of adipose tissue inflammation and glucose intolerance in diet-induced obese mice.

Key Words: obesity ■ natural killer T cells ■ macrophages ■ visceral adipose tissues ■ glucose intolerance
producing a mixture of T-helper type 1 (Th1) and Th2 cytokines, such as interferon (IFN) \( \gamma \) and interleukin (IL) 4 in shaping subsequent adaptive immune responses. Thus, NKT cells can function as a bridge between the innate and adaptive immune systems. Caspar-Bauguil et al\(^7\) have reported the presence of significant levels of NKT cells in the stromal-vascular fraction of white adipose tissue by cytofluorometric analysis. However, they have not determined whether NKT cells are involved in adipose tissue inflammation and the development of metabolic disorders, including glucose intolerance in HFD-induced obesity.

Some of the processes involved in adipose tissue inflammation resemble inflammatory processes in atherogenesis.\(^8\) Inflammation during the development of an atherosclerotic lesion is also characterized by monocyte/macrophage and lymphocyte infiltration.\(^8\) These lymphocytes are mainly CD4-positive lymphocytes that express proinflammatory Th1 cytokines, such as IFN-\( \gamma \), and orchestrate the inflammatory response in the vascular wall by activating other cells. Previous studies,\(^9,10\) including our own studies, demonstrated that NKT cells were present in atherosclerotic lesions and are critically important in atherogenesis. These findings suggest that NKT cells can also be involved in inflammation within adipose tissue. However, to date, it remains unclear whether NKT cells play a similar role in adipose tissue inflammation.

In the present study, we determined whether NKT cells are involved in HFD-induced glucose intolerance and adipose tissue inflammation by using \( \beta_2 \) microglobulin knockout (KO) mice lacking NKT cells. Moreover, we further examined the effects of NKT cell activation by \( \alpha \)-galactosylceramide (\( \alpha \)GC), a specific activator for NKT cells,\(^11\) on glucose intolerance and adipose tissue inflammation in HFD-induced obese mice.

**Methods**

Expanded materials and methods are available in the supplemental files (available online at http://atvb.ahajournals.org).

**Experiment 1: The Effects of NKT Cell Depletion on Metabolic Disorders**

Male wild-type (WT) (Charles River Japan, Inc, Yokohama, Japan) and KO mice, which lack NKT and T cells on the C57BL/6 background (The Jackson Laboratory, Bar Harbor, Maine), aged 8 weeks, and KO mice, which lack NKT and T cells on the C57BL/6 background (The Jackson Laboratory, Bar Harbor, Maine), aged 8 weeks, were injected using PBS (n = 5) or an HFD containing 21% fat and 0.15% cholesterol/HFD for 13 weeks. Animals were euthanized and organs, including visceral adipose tissue, were dissected. Other WT mice, aged 8 weeks, were fed with an SD (n = 15) or an HFD (n = 15) for 2, 4, or 6 weeks. Afterward, animals underwent euthanasia and organs, including visceral adipose tissue, were dissected.

**Experiment 2: The Effects of NKT Cell Activation on Metabolic Disorders**

After feeding male WT and KO mice, aged 8 weeks, with an HFD for 13 weeks, phosphate-buffered saline (PBS) (WT-PBS, n = 5; and KO-PBS, n = 5) or \( \alpha \)GC, 0.1 \( \mu \)g/g body weight (Kirin Brewery Company, Ltd, Tokyo, Japan) (WT-\( \alpha \)GC, n = 5; and KO-\( \alpha \)GC, n = 5), was injected intraperitoneally. After 8 to 9 days, ipGTT was performed and visceral adipose tissues were dissected. Other WT mice, aged 8 weeks, were injected using PBS (n = 9) or \( \alpha \)GC, 0.1 \( \mu \)g/g body weight (n = 11) intraperitoneally and organs, including visceral adipose tissues, were dissected 1, 4, and 7 days after the injection.

The animal care and procedures for the experiments were approved by the Committee of Hokkaido University Graduate School of Medicine on Animal Experimentation.

**Results**

**NKT Cell Depletion Ameliorates Metabolic Disorders in HFD-Fed Mice**

To characterize the role of NKT cells in the pathogenesis of HFD-induced glucose intolerance and visceral adipose tissue inflammation, WT and KO mice were fed with either SD or HFD for 13 weeks.

The quantification of NKT cells by V\( \alpha \)14/\( \alpha \)18 gene expression confirmed that NKT cell infiltration was significantly enhanced in adipose tissue from HFD mice and, more important, was completely abolished in KO groups (Figure 1A).

An HFD did not affect fasting plasma levels of glucose, insulin, and HOMA-IR in WT-HFD and KO-HFD compared with WT-SD (Table 1). However, plasma glucose levels during ipGTT were significantly increased in WT-HFD than in WT-SD, and these values were significantly lower in KO-HFD (Figure 1B). The area under the curve values of plasma glucose...
WT-HFD mice and an M2 phenotype in KO-HFD mice

Infiltrating macrophages possess a predominantly M1 phenotype in macrophage, respectively, demonstrated that infiltrating macrophage activation, M1 macrophage, and M2 gene expression were measured in the hepatic tissues. Hepatic gluconeogenesis and macrophage infiltration was enhanced in adipose tissue from WT-HFD and that this increase was significantly ameliorated in KO-HFD accompanied by a phenotypic change into M2 macrophage.

To examine the temporal relationship between infiltrating NKT cells and macrophages within obese adipose tissues, WT mice were fed with SD or HFD for 2, 4, or 6 weeks. Quantification of NKT cells by Vα14/18 gene expression demonstrated that NKT cell infiltration was significantly increased after 6 weeks of HFD feeding, whereas macrophages quantified by F4/80 gene expression were not increased during the same period in visceral adipose tissues (supplemental Figure 1A and B). Similarly, in subcutaneous fat tissues, NKT cell infiltration was significantly increased after 4 weeks of HFD feeding, whereas macrophages were not increased during the same period (supplemental Figure 1C and D). More important, macrophages were significantly increased at 13 weeks of HFD feeding. Combining the data from weeks 2 to 6 (supplemental Figure 1) with those from week 13 (Figure 2B), the infiltration of NKT cells preceded the occurrence of glucose intolerance and macrophage infiltration into adipose tissue from HFD-induced obese mice is mediated by NKT cells.

To examine the role of NKT cells in gluconeogenesis, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase gene expression were measured in the hepatic tissues. Hepatic gluconeogenesis tended to be suppressed in KO-SD compared with WT-SD, which did not reach statistical significance (supplemental Figure 2).

### Table. Animal Characteristics in Experiment 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Animal Group</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT-SD (n=10)</td>
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<tr>
<td>Body weight, g</td>
<td>29.4 (0.5)</td>
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<tr>
<td>Fasting plasma glucose level, mg/dL</td>
<td>78 (8)</td>
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<tr>
<td>Insulin level, ng/mL</td>
<td>0.49 (0.09)</td>
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<tr>
<td>HOMA-IR level</td>
<td>2.10 (0.22)</td>
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<td>Total cholesterol level, mg/dL</td>
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<tr>
<td>Leptin level, ng/mL</td>
<td>2.2 (0.5)</td>
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<tr>
<td>TNF-α level, pg/mL</td>
<td>37 (5)</td>
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<tr>
<td>Adiponectin level, μg/mL</td>
<td>22 (2)</td>
</tr>
<tr>
<td>Glucagon level, pg/mL</td>
<td>478 (25)</td>
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<tr>
<td>Visceral adipose tissue</td>
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<td>Visceral adipose tissue weight, mg</td>
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<td>Visceral adipose tissue weight/body weight, mg/g</td>
<td>19.7 (1.7)</td>
</tr>
<tr>
<td>Adipocyte size, μm²</td>
<td>1697 (156)</td>
</tr>
</tbody>
</table>

Abbreviations: HFD, high-fat diet; KO, β2-microalbumin knockout mice; SD, standard diet; TNF, tumor necrosis factor; WT, wild type (C57BL/6J) mice.

*P<0.05 vs WT-SD.
†P<0.01 vs WT-SD.
‡P<0.05 vs WT-HFD.
§P<0.01 vs WT-HFD.

levels during the ipGTT were significantly increased in WT-HFD, and this increase was attenuated in KO-HFD to the WT-SD levels (Figure 1C). These results demonstrated that glucose intolerance seen in HFD-fed mice was significantly ameliorated by the depletion of NKT cells. Plasma total cholesterol and leptin levels were also significantly increased by HFD but were not altered in KO-HFD. The plasma adiponectin level did not change in WT-HFD compared with WT-SD, which did not reach statistical significance (Table). The plasma glucagon level tended to be lower in KO-SD compared with WT-SD, but significantly increased in KO-HFD. The plasma glucose intolerance seen in HFD-fed mice was significantly ameliorated by the depletion of NKT cells. Plasma total cholesterol and leptin levels were also significantly increased by HFD but were not altered in KO-HFD. The plasma adiponectin level did not change in WT-HFD compared with WT-SD, which did not reach statistical significance (Table).
NKT Cell Activation Exacerbated Metabolic Disorders in HFD-Fed Mice

To further characterize the role of NKT cells in the pathogenesis of HFD-induced glucose intolerance and visceral adipose tissue inflammation, GC was injected intraperitoneally in WT mice fed HFD for 13 weeks.

GC did not affect body weight, visceral adipose tissue weight, and adipocyte size in HFD mice 9 days after injection (supplemental Table). The quantification of NKT cells by V14/J18 gene expression confirmed GC significantly enhanced NKT cell infiltration into adipose tissue (Figure 3A). Plasma glucose levels during ipGTT were significantly increased by GC (15 minutes: 330 [11] vs 296 [11] mg/dL [P<0.05]; and 30 minutes: 326 [9] vs 295 [8] mg/dL [P<0.05]) (Figure 3B).

F4/80-positive macrophage infiltration was significantly increased in the adipose tissues for WT mice by GC (Figure 4A and B). These changes of adipose tissue macrophages by the immunohistochemical analysis were also confirmed by MHC class II and CD11c gene expression (Figure 4C and D). In parallel to macrophage infiltration into the visceral adipose tissue, the injection of GC significantly increased the expression of MCP-1, TNF-α, IFN-γ, and RANTES genes in HFD mice (Figure 5A–D).

To examine the temporal relationship between infiltrating NKT cells and macrophages, WT mice, aged 8 weeks, were injected using PBS or GC intraperitoneally and adipose tissues were dissected 1, 4, and 7 days after the injection. The NKT cells and macrophages tended to increase at 4 and 7 days after GC administration in WT mice (supplemental Figure 3).

To examine the effects of GC treatment on the metabolic phenotypes of genetically induced obese mice, GC was injected intraperitoneally in ob/ob mice. Natural killer T cell and macrophage infiltration were significantly increased in GC-treated ob/ob mice compared with PBS-treated ob/ob mice (supplemental Figure 4A and B). Major histocompatibility complex class II, CD11c, and arginase gene expression were also significantly increased in GC-treated ob/ob mice (supplemental Figure 4C–E). Similar to diet-induced obese mice, the injection of GC significantly enhanced the expression of TNF-α, IFN-γ, and RANTES genes, also in ob/ob mice (supplemental Figure 4G–I). Plasma glucose levels during ipGTT in GC-treated ob/ob mice were comparable to those in PBS-treated ob/ob mice (supplemental Figure 5).

To confirm the specificity of GC treatment for activating NKT cells, GC was injected in KO mice fed HFD for 13 weeks. It did not affect NKT cell and macrophage infiltration in the adipose tissues and plasma glucose levels during ipGTT in KO mice (supplemental Figure 6).

To assess the direct relationship between NKT cell activation and adipose tissue inflammation, splenic CD11b+Gr1+CD4–CD8–B220– cells (macrophage-enriched cells) and liver MHC-classII–CD8–B220– lymphocytes (NKT-enriched cells) were cocultured with or without GC for 48 hours. Macrophages conditioned with activated NKT cells by GC secreted a significantly larger amount of MCP-1 into the coculture.
media compared with unconditioned macrophages (supplemental Figure 7).

Discussion

The present study demonstrated that NKT cells were infiltrated into the visceral adipose tissue in association with macrophages during the development of glucose intolerance in a mouse model of HFD-induced obesity. The depletion of NKT cells in \( /\beta 2\) microglobulin KO mice ameliorated glucose intolerance and visceral adipose tissue inflammation induced by HFD feeding without affecting obesity itself. On the contrary, the activation of NKT cells by \( \alpha\)-galactosylceramide (\( \alpha\)GC) exacerbated glucose intolerance and adipose tissue inflammation, including macrophage infiltration and inflammatory cytokine/chemokine gene expression. Therefore, NKT cells may play a pivotal role in the development of glucose intolerance and adipose tissue inflammation associated with HFD-induced obesity.

Visceral obesity has been demonstrated to be associated with macrophage infiltration and inflammation in adipose tissue.\(^1\)\(^2\)\(^12\) As such, MCP-1 is produced by adipocytes in parallel with increasing adiposity, and mice lacking CCR2, a receptor for MCP-1, exhibit less macrophage infiltration in adipose tissues and a reduction in inflammatory gene expression.\(^3\) However, the development of HFD-induced glucose intolerance was not completely abolished in these mice, suggesting that the other chemokine systems might also contribute to obesity-related adipose tissue inflammation and glucose intolerance.

Early work by cytofluorometric analysis revealed the presence of significant levels of NKT cells in the stromal-vascular fraction of white adipose tissues.\(^7\) However, the changes of these cells by HFD feeding and even their roles in HFD-induced metabolic disorders have not been examined. In the present study, depleting NKT cells significantly ameliorated glucose intolerance after HFD feeding (Figure 1). Therefore, our study has extended the previous information on the significance of NKT cells by demonstrating that the cell infiltration of these cells into the adipose tissue is involved in the recruitment of macrophages and inflammatory cytokine gene expression during the development of HFD-induced glucose intolerance. However, the present results were not consistent with those of the previous study by Elinav et al,\(^13\) which noted that NKT cells ameliorated glucose intolerance in leptin-deficient ob/ob mice. In their study, the oral administration of liver extracts in ob/ob mice increased hepatic NKT cells and serum levels of IL-10, indicating that the extracts activated NKT cells toward the TH2 bias, whereas \( \alpha\)GC injection stimulated NKT cells toward the TH1 slant in the present study. Therefore, the discrepancy between these studies might be the result of the differences in the methods of modulating NKT cells and the resultant effects on the adipose tissue environment.

Figure 4. Macrophage infiltration in adipose tissue in experiment 2. A, Demonstrable figures of F4/80 immunohistochemistry. Scale bar, 20 \( \mu m \). B, The number of F4/80-positive nuclei from mice given phosphate-buffered saline (PBS) and \( \alpha\)-galactosylceramide (\( \alpha\)GC). C and D, Expression of major histocompatibility complex (MHC) class II and CD11c genes, respectively, in visceral adipose tissues. *\( P<0.05\) vs PBS.

Figure 5. A–D, Expression of monocyte chemoattractant protein (MCP) 1, tumor necrosis factor (TNF) \( \alpha\), interferon (IFN) \( \gamma\), and regulated upon activation normal T cell expressed secretion (RANTES) genes, respectively, in visceral adipose tissues from mice given phosphate-buffered saline (PBS) and \( \alpha\)-galactosylceramide (\( \alpha\)GC) in experiment 2. Quantitative reverse transcription (RT)–polymerase chain reaction (PCR) was performed 9 days after PBS or \( \alpha\)GC injection. *\( P<0.05\) vs PBS.
changes of cytokines subsequent to NKT cell activation. The differences in the animal models (HFD-induced obese mice vs leptin-deficient ob/ob mice) might also be involved in this discrepancy because the injection of αGC significantly enhanced the expression of arginase in ob/ob mice but not in HFD-induced obese mice.

Previous studies demonstrated that proinflammatory T lymphocytes are also present in visceral adipose tissue and contribute to adipose tissue inflammation and the development of glucose intolerance before the recruitment of macrophages. A recent elegant study by Nishimura et al elucidated the role of T lymphocytes in adipose tissue inflammation in obesity. In their study, many CD8⁺ effector T cells infiltrated into obese epididymal adipose tissue, preceding macrophage infiltration, in HFD-induced obese mice and initiated the inflammatory cascade that leads to insulin resistance in adipocytes. We could not completely exclude the possibility that T lymphocytes are involved in our model because β₂ microglobulin KO mice used in the present study lack not only NKT cells but also CD8⁺ T lymphocytes. However, the development of both glucose intolerance and adipose tissue inflammation induced by HFD was significantly exacerbated by the specific activation of NKT cells by using αGC, an activator of NKT cells but not T cells (Figures 3–5). Based on these results, we consider that NKT cells are critically involved in glucose intolerance and adipose tissue inflammation in obese mice.

The NKT cells are a specialized lineage of T cells that recognize glycolipid antigens presented by the MHC class I-like molecule CD1d. The NKT cells mediate various functions rapidly by producing a mixture of Th1 and Th2 cytokines, such as IFN-γ and IL-4, in shaping subsequent adaptive immune responses. The present study demonstrated that accumulated macrophages in adipose tissues in αGC-treated mice were classically activated (M1) macrophages, one of the distinct subsets of macrophages categorized as M1 by CD11c (Figure 4). In agreement with these findings, the activation of NKT cells was associated with increased gene expression of T_h1 cytokine IFN-γ and MCP-1 in HFD-fed mice (Figure 5). Interferon-γ can also promote the recruitment of monocytes by inducing MCP-1 secretion from periodipocytes, and it could activate other cells, such as macrophages. Therefore, cytokines and chemokines, including IFN-γ and MCP-1, were mechanistically involved in the infiltration of macrophages as a result of NKT cell activation. The NKT cells may orchestrate the inflammatory process in adipose tissue in association with the development of glucose intolerance. The beneficial effects of depleting NKT cells are mostly mediated by the reduction of macrophages. It may be informative to examine whether immunosuppressive agents, such as cyclosporine and tacrolimus, which have been shown to suppress αGC-induced cytokine production in murine NKT cells, can ameliorate adipose tissue inflammation and glucose intolerance in our model. However, they also induce glucose intolerance via its toxic effects on the pancreatic islet cells. Therefore, these reagents may not be suitable for investigating the role of NKT cells in glucose intolerance in HFD-induced obesity in vivo.

The underlying mechanisms responsible for the activation of NKT cells by the HFD feeding remain established. Based on our results using αGC, a glycosphingolipid derived from marine sponges, sphingolipid ceramide may be a crucial intermediate linking between excess nutrients by HFD and inflammatory cytokines to induce glucose intolerance. In fact, ceramide has been shown to be synthesized by long-chain fatty acids and to induce both inflammation and insulin resistance. In agreement with our results, Rocha et al reported that the HFD feeding increases a number of T cells and IFN-γ gene expression in adipose tissue, suggesting T-cell priming toward the Th11 slant. However, the HFD feeding has been shown to suppress Th11 responses in B6 mice by inhibiting toll-like receptor–mediated maturation and proinflammatory cytokine production in dendritic cells. The discrepancy between these studies might be the result of the differences in the tissues studied (visceral adipose tissue lymphocytes vs splenic lymphocytes). More important, the contribution of NKT cells is not mediated by the modulation of adipose tissue weight or adipocyte size because these variables did not differ between HFD-fed groups (Table 1 and Supplementary Table); however, adipocyte cell size has been shown to be an independent predictor of glucose intolerance. Activated macrophages secrete TNF-α, which can inhibit insulin signal transduction. Obesity itself can trigger adipose tissue inflammation, which leads to the desensitization of insulin action. We have demonstrated that NKT cells may be important in the evolution of atherosclerotic lesions by communicating macrophages through cell-cell interactions and/or secreting inflammatory cytokines. Some of the inflammatory processes involved in atherogenesis (as shown in our previous study) resemble adipose tissue inflammation in the present study. Therefore, NKT cells are considered to mediate chronic inflammation in vascular and adipose tissues and can represent a direct and common soil for the development of atherosclerotic cardiovascular disease and diabetes. An in vivo transfer experiment with isolated NKT cells may provide more direct evidence of the cause-and-effect relationship between NKT cells and glucose intolerance associated with HFD-induced obesity. Nevertheless, αGC has been established to be a specific activator for NKT cells and, in fact, it has been used in a variety of disease models to elude the pathogenetic role of NKT cells. Therefore, we used αGC administration to activate NKT cells in the present study. There are several limitations to be acknowledged in the present study. First, we only examined the adipose tissue in the present study and did not assess the contribution of liver or skeletal muscle, which can also determine insulin sensitivity. Fasting plasma glucose level and HOMA-IR were significantly lower in KO-SD than in WT-SD (Table). Knockout-SD mice tended to have lower plasma glucose levels and area under the curve values during ipGTT compared with WT-SD (Figure 1B and C); this finding did not reach statistical significance. These data suggested that the absence of NKT cells could improve glucose metabolism in healthy mice, independently of adipose tissue inflammation. It may be possible that NKT cells affect glucose metabolism via the alterations of gluconeogenesis in the liver and skeletal muscle. However, based on the results that the improvement of glucose metabolism is relatively small in KO-SD mice (Figure 1), we consider that NKT cells may impair glucose...
tolerance predominately via promoting adipose tissue inflammation exclusively in HFD-fed mice. Second, there was massive macrophage infiltration in the adipose tissue in our HFD-fed mice even though the weight gain was relatively small. The NKT cell infiltration preceded macrophage infiltration in obese visceral adipose tissues and may play an important role in the early phase of adipose tissue inflammation. Therefore, even though we have not examined how much NKT cells and macrophages infiltrate within adipose tissues during the development of more severe obesity, we consider that the deletion of NKT cells can effectively attenuate the infiltration of macrophages in this setting. In contrast, the activation of NKT cells has been reported to be protective against type 1 diabetes, systemic lupus erythematosus, and infections. Therefore, the inhibition of NKT cells as a therapeutic strategy to prevent and treat metabolic syndrome and cardiovascular disease for obese individuals needs to be used cautiously in the setting of these disease conditions.

In conclusion, the depletion of NKT cells ameliorated chronic inflammation in visceral adipose tissues and suppressed the development of glucose intolerance in HFD-induced obese mice. On the other hand, the activation of NKT cells exacerbated macrophage infiltration in adipose tissue and glucose intolerance with obesity. Therefore, NKT cells enhance chronic inflammation in visceral adipose tissue and contribute to the development of metabolic disorders in obesity. The NKT cells may be the novel therapeutic targets in atherosclerosis, metabolic syndrome, and type 2 diabetes.

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References

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Supplemental Material

Natural Killer T Cells are Involved in Adipose Tissues Inflammation and Glucose Intolerance in Diet-Induced Obese Mice

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Expanded Materials and Methods

Experimental animals

Experiment 1: The effects of NKT cell depletion on metabolic disorders

Male wild type (WT) (Charles River Japan, Inc., Yokohama, Japan) and β2 microglobulin knockout (KO) mice, which lack NKT cells and T cells on the C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME), 8 weeks of age, were fed with a standard diet (SD; WT-SD, n=10 and KO-SD, n=5) or a high fat diet (HFD) containing 21% fat and 0.15% cholesterol (WT-HFD, n=10 and KO-HFD, n=14) for 13 weeks. Animals were metabolically phenotyped including an intraperitoneal glucose tolerance test (ipGTT) using a dose of 1mg/g body weight glucose. Other WT mice, 8 weeks of age, were fed with SD (n=15) or HFD (n=15) for 2, 4 or 6 weeks. Tail vein blood was used for glucose quantification with an automatic blood glucose meter (Glutest Ace, Sanwa chemical, Nagoya, Japan) during ipGTT. Afterwards, animals were euthanized and organs including visceral adipose tissues and subcutaneous fat tissues were dissected.
Experiment 2: The effects of NKT cell activation on metabolic disorders

After feeding male WT and KO mice, 8 weeks of age, with a HFD for 13 weeks, α-galactosylceramide (αGC; n=5) (0.1µg/g body weight; Kirin Brewery Company, Ltd., Tokyo, Japan) or phosphate buffered saline (PBS; n=5) were injected intraperitoneally. After 8-9 days, ipGTT was performed and visceral adipose tissues were dissected. Other WT mice, 8 weeks of age, were injected PBS (n=9) or αGC (0.1µg/g body weight, n=11) intraperitoneally and organs including visceral adipose tissues were dissected 1 day, 4 days and 7 days after the injection. Ob/ob mice, 7 weeks of age, were injected PBS (n=5) or αGC (0.1µg/g body weight, n=5) intraperitoneally. After 7 days, ipGTT was performed and organs including visceral adipose tissues were dissected.

The animal care and procedures for the experiments were approved by the Committee of Hokkaido University Graduate School of Medicine on Animal Experimentation.

Blood chemistry

After fasting for 16 hrs, plasma total cholesterol levels were assayed by enzymatic methods (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma leptin, adiponectin, TNF-α, and glucagon levels were measured by enzyme-linked immunosorbent assay kit (R&D Systems, Inc. Minneapolis, MN and Wako Pure Chemical Industries, Ltd.). Insulin sensitivity was assessed by calculating the Homeostasis Model Assessment (HOMA) score; fasting plasma insulin (ng/mL) × plasma glucose (mg/dL)/22.5.

ipGTT

To assess the glucose tolerance, glucose (1mg/g body weight intraperitoneal injection) was loaded to the mice and blood samples were drawn from the tail vein at baseline and 15, 30, 60, and 120 min after injection. Plasma
glucose levels were measured by using an automatic blood glucose meter (Glutest Ace, Sanwa chemical, Nagoya, Japan) during ipGTT.

**Histological and histochemical analysis of adipose tissues**

The visceral adipose tissues were obtained from the bilateral perigonadal and perirenal adipose tissues and tissue sections were prepared. To measure adipocyte cross-sectional area, three different sections from each tissue block were stained with hematoxylin and eosin. Five different fields from each section were analyzed using image analysis software (Image J version 1.40, National Institutes of Health, Bethesda, MD) and average adipocyte cross-sectional area was calculated for each animal.

To quantify the number of macrophages with adipose tissue, three sections were stained with monoclonal antibody against mouse F4/80, a specific marker for mature macrophage, followed by counter-staining with hematoxylin. The total number of F4/80-expressing macrophage nuclei was counted.

**Quantitative reverse transcriptase PCR**

Total RNA was extracted from adipose and liver tissues with QuickGene-810 (FujiFilm, Tokyo, Japan) according to the manufacture’s instructions. cDNA was synthesized with the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). TaqMan quantitative PCR was performed with the 7300 real-time PCR system (Applied Biosystems) to amplify samples for F4/80, MHC class II (a marker for macrophage activation), cluster of differentiation 11c (CD11c; a marker for M1 macrophages), arginase (a marker for M2 macrophages), Vα14/Jα18 (a specific marker of NKT cells), MCP-1, regulated on activation, normal T cell expressed and secreted (RANTES), TNF-α, and IFN-γ cDNA in visceral adipose tissues and subcutaneous fat tissues and phosphoenolpyruvate
carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (markers for gluconeogenesis) in fasted liver tissues. These transcripts were normalized to GAPDH. The primers were purchased from Applied Biosystems.

**In vitro co-culture of NKT cells and macrophages**

Hepatic mononuclear cells (HMNCs) were isolated from young male WT mice using 33% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) and incubated with a combination of following mAb conjugates: anti-MHC class II (1E4), anti-CD8 (53-6.7), and anti-CD45R/B220 (RA3-6B2). Splenocytes (SPC) were obtained from young male WT mice and incubated with a combination of following mAb conjugates: anti-Ly6G (Gr-1), anti-CD4 (Gk1.5), anti-CD8 (53-6.7), and anti-CD45R/B220 (RA3-6B2). Ab-treated HMNC and SPC were incubated with goat-anti-rat IgG microbeads at 6°C for 15 min and cells that had bound the Ab were depleted with VarioMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Liver MHC-classII−CD8−B220− lymphocytes were used as NKT-enriched cells. Splenic Gr-1−CD4−CD8−B220− cells were further enriched with anti-CD11b (M1/70) microbeads with VarioMACS. Splenic CD11b+Gr-1−CD4−CD8−B220− cells were used as macrophage-enriched cells. NKT-enriched cells (2×10^6) and macrophage-enriched cells (2×10^5) were co-cultured in RPMI 1640 supplemented with 100U/mL penicillin and 100µg/mL streptomycin in a flat-bottomed 6-well plate in the presence of α-GC (0, 10, or 100 ng/mL) for 48 hours at 37°C. After incubation, culture supernatants were harvested from each well and quantified for MCP-1 protein levels with ELISA kits (R&D Systems, Inc.).

**Statistical analysis**

Data were expressed as the means±S.E.. Statistical analysis was performed
using the Student t test or ANOVA with the Bonferroni post-hoc test (GraphPad Prism 4, GraphPad Software, San Diego, CA). A p value <0.05 was considered statistically significant.
Supplemental Figure Legends

Supplemental Figure 1  (A, B) Gene expression of Vα14Jα18 and F4/80 in visceral adipose tissues from WT-SD (n=15) and WT-HFD (n=15) mice 2, 4 or 6 weeks after SD or HFD feeding. (C, D) Gene expression of Vα14Jα18 and F4/80 in subcutaneous fat tissues from WT-SD (n=15) and WT-HFD (n=15) mice 2, 4 or 6 weeks after SD or HFD feeding.* \( p < 0.05 \), † \( p < 0.01 \) vs. WT-SD.

Supplemental Figure 2  (A, B) Gene expression of PEPCK and G6Pase in hepatic tissues from WT-SD (n=10), KO-SD (n=5), WT-HFD (n=10), and KO-HFD (n=14) in Experiment 1. * \( p < 0.01 \) vs. WT-SD.

Supplemental Figure 3  (A, B) Gene expression of Vα14Jα18 and F4/80 in adipose tissues from WT-PBS (n=9) and WT-αGC (n=11) mice, 8 weeks of age, in Experiment 2. Quantitative RT-PCR was performed 1 day, 4 days and 7 days after PBS or αGC injection.

Supplemental Figure 4  (A-I) Gene expression of Vα14Jα18, F4/80, MHC class II, CD11c, arginase, MCP-1, TNF-α, IFN-γ, and RANTES in adipose tissues from PBS-treated \( ob/ob \) mice (n=5) and αGC-treated \( ob/ob \) mice (n=5) in Experiment 2. Quantitative RT-PCR was performed 7 days after PBS or αGC injection. * \( p < 0.05 \) vs. PBS, † \( p < 0.01 \) vs. PBS.

Supplemental Figure 5  Plasma glucose concentrations during ipGTT 7 days after PBS or αGC injection of \( ob/ob \) mice in Experiment 2.

Supplemental Figure 6  (A, B) Gene expression of Vα14Jα18 and F4/80
in adipose tissues from KO-PBS (n=5) and KO-αGC (n=5) mice after feeding a HFD for 13 weeks in Experiment 2. Quantitative RT-PCR was performed 9 days after PBS or αGC injection. (C) Plasma glucose concentrations during ipGTT 8 days after PBS or αGC injection.

**Supplemental Figure 7**  MCP-1 protein levels in the conditioned media of splenic CD11b⁺Gr1⁻CD4⁺CD8⁻B220⁻ cells co-cultured with or without liver MHC-classII⁺CD8⁻B220⁻ lymphocytes activated by 0, 10, or 100 ng/mL of αGC for 48 hours at 37°C (n=4). *p<0.01 vs. non-conditioned medium.
Supplemental Fig. 1

A. Va14Ja18

B. F4/80

C. Va14Ja18

D. F4/80

Arbitrary units

SD HFD

2 weeks 4 weeks 6 weeks

SD HFD

2 weeks 4 weeks 6 weeks

SD HFD

2 weeks 4 weeks 6 weeks

SD HFD

2 weeks 4 weeks 6 weeks
Supplemental Fig. 5

**Graphical Data**

- **Title**: Plasma glucose levels over time in response to different treatments.
- **X-axis**: Time (min) from 0 to 120.
- **Y-axis**: Plasma glucose (mg/dL) from 0 to 300.
- **Legend**:
  - PBS (represented by squares)
  - αGC (represented by squares)
- **Data Points**:
  - Both treatments show an initial peak at 30 minutes, followed by a decrease.
  - αGC treatment maintains a higher glucose level compared to PBS throughout the observation period.
Supplemental Fig. 6

A  

B  

C  

Arbitrary units  

PBS  

αGC  

PBS  

αGC  

Plasma glucose (mg/dL)  

Time (min)
Supplemental Fig. 7

**MCP-1 (pg/mL)**

- **MΦ**: + + + +
- **NKT**: − + + +
- **αGC (ng/mL)**: 0 0 10 100

* denotes significance.
**Online Supplemental Table** Animal characteristics in experiment 2

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<thead>
<tr>
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<th>PBS (n=5)</th>
<th>αGC (n=5)</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>34.9 ± 1.1</td>
<td>32.8 ± 1.2</td>
</tr>
<tr>
<td>Visceral adipose tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visceral adipose tissue weight, mg</td>
<td>1720 ± 98</td>
<td>1782 ± 330</td>
</tr>
<tr>
<td>Visceral adipose tissue weight/Body weight, mg/g</td>
<td>49.2 ± 1.8</td>
<td>54.2 ± 10.0</td>
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<tr>
<td>Adipocyte size, μm²</td>
<td>3398 ± 338</td>
<td>3863 ± 681</td>
</tr>
</tbody>
</table>

PBS: phosphate buffered saline, αGC: α-galactosylceramide.