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

Short title: Ohmura et al. Natural Killer T Cells and Glucose Intolerance

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## Abstract

***Objective*** Macrophage as well as 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 also in visceral adipose tissue. We thus determined 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 $\beta_2$ microglobulin knockout mice lacking NKT cells (KO) and C57BL/6J (WT) mice were fed with a high fat diet (HFD) for 13 weeks. Body weight and visceral obesity were comparable between WT 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 (αGC, 0.1μg/g body weight), which specifically activates NKT cells, were administered after 13 weeks of HFD feeding. αGC significantly exacerbated glucose intolerance and also macrophage infiltration as well as cytokine gene expression in adipose tissue.

***Conclusions*** NKT cells play a crucial role in the development of adipose tissue inflammation and glucose intolerance in diet-induced obesity.

**Key Words**: obesity; natural killer T cells; macrophages; visceral adipose tissues; glucose intolerance

## Condensed Abstract

Macrophage as well as lymphocyte infiltration in adipose tissue may contribute to the pathogenesis of glucose intolerance in obesity. Natural killer T cells, which integrate proinflammatory cytokines, are present in adipose tissue. They are involved in the development of adipose tissue inflammation and glucose intolerance in diet-induced obese mice.

## Introduction

Obesity, specifically visceral obesity, increases the risk for metabolic disorders such as type 2 diabetes, dyslipidemia and hypertension as well as atherosclerotic cardiovascular diseases. Previous studies have demonstrated that the accumulation of macrophages within adipose tissue is well-documented in obese individuals and adipose tissue inflammation plays an important role in the pathogenesis of these metabolic disorders.[1, 2] Macrophages are attracted by chemokines like monocyte chemoattractant protein-1 (MCP-1) and contribute to local inflammation through the release of other inflammatory cytokines such as tumor necrosis factor (TNF)-α. In high-fat diet (HFD)-fed obese mice, it has been shown that infiltration of macrophages into adipose tissue coincides with the occurrence of obesity-mediated metabolic disorders.[2] The important role of adipose tissue macrophages in the pathogenesis of metabolic disorders has further been supported by recent data in C-C motif chemokine receptor 2 (CCR2)-deficient mice.[3] $CCR2^{-/-}$ mice exhibited a reduction in adipose tissue macrophages in association with an improvement of glucose homeostasis and insulin sensitivity. However, the abolished monocyte and macrophage recruitment into peripheral tissue via interaction with MCP-1 could not completely inhibit HFD-mediated metabolic disorders, suggesting that other inflammatory cells may play a role in this context. Wu *et al* demonstrated that CD3-positive T-lymphocytes are present in human adipose tissue and RANTES, a T-cell specific chemokine, and its respective receptor CCR5 are expressed in adipose tissue from obese patients.[4, 5] However, the role of other types of lymphocytes in adipose tissue inflammation is largely unexplored.

Natural killer T (NKT) cells are innate-like T lymphocytes that recognize glycolipid antigens and capable to rapidly produce a mixture of T helper type 1 ($T_H1$) and $T_H2$ cytokines such as interferon (IFN)-γ and interleukin (IL)-4 in

shaping subsequent adaptive immune responses.[6] Thus, NKT cells can function as a bridge between the innate and adaptive immune systems. Caspar-Bauguil *et al* have reported the presence of significant levels of NKT cells in the stroma-vascular fraction of white adipose tissue by cytofluorometric analysis.[7] 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 atherosclerotic lesion is also characterized by monocyte/macrophage as well as lymphocyte infiltration.[8] These lymphocytes are mainly CD4-positive lymphocytes which express proinflammatory $T_H1$-cytokines like IFN-γ and orchestrate the inflammatory response in the vascular wall by activating other cells. Previous studies including those of our own demonstrated that NKT cells were present in atherosclerotic lesions and of critical importance in atherogenesis.[9, 10] These findings suggest that NKT cells can be also 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 α-galactosylceramide (αGC), a specific activator for NKT cells,[11] on glucose intolerance and adipose tissue inflammation in HFD-induced obese mice.

## Materials and Methods

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

### Experimental animals

#### 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 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). Other WT mice, 8 weeks of age, were fed with SD (n=15) or HFD (n=15) for 2, 4 or 6 weeks. Afterwards, animals were euthanized 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, 8 weeks of age, with a HFD for 13 weeks, phosphate buffered saline (WT-PBS; n=5, KO-PBS; n=5) or αGC (0.1μg/g body weight; Kirin Brewery Company, Ltd., Tokyo, Japan; WT-αGC; n=5, KO-αGC; 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.

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.

Quantification of NKT cells by Vα14/Jα18 gene expression confirmed that NKT cell infiltration was significantly enhanced into adipose tissue from HFD mice and, importantly, was completely abolished in KO groups (**Figure 1A**).

HFD did not affect fasting plasma levels of glucose, insulin, and homeostasis model assessment of insulin resistance (HOMA-IR) in WT-HFD and KO-HFD compared to WT-SD (**Table I**). 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**). Area under the curve (AUC) values of plasma glucose 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 were also significantly increased by HFD, which, however, were not altered in KO-HFD. Plasma adiponectin level did not change in WT-HFD compared to WT-SD, but significantly increased in KO-HFD. Plasma glucagon level tended to be lower in KO-SD compared to WT-SD, which, however, did not reach statistical significance (**Table I**). HFD significantly increased the weight of visceral adipose tissue compared to groups fed with SD. HFD significantly increased the weight of visceral adipose tissue compared to groups fed with SD. In parallel to visceral adipose tissue weight, adipocyte size measured by morphometric

analysis was significantly increased in WT-HFD than in WT-SD. However, these increases were not altered in KO-HFD.

In parallel to the glucose intolerance, the infiltration of F4/80 positive macrophages by immunohistochemical staining was significantly increased in visceral adipose tissues from WT-HFD than WT-SD and this increase was significantly ameliorated in KO-HFD (**Figure 2 A and B**). MHC class II, CD11c, and arginase gene expression measured by using real time RT-PCR (a quantitative index of macrophage activation, M1macrophage, and M2 macrophage, respectively) demonstrated that infiltrating macrophages possess predominantly M1 phenotype in WT-HFD mice and M2 phenotype in KO-HFD mice (**Figure 2 C, D and E**). Taken together, these data indicated that M1 macrophage infiltration was enhanced in adipose tissue from WT-HFD and this increase was significantly ameliorated in KO-HFD accompanied by phenotypical 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/Jα18 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 of time in visceral adipose tissues (**Supplemental Figure 1 A 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 of time (**Supplemental Figure 1 C and D**). Importantly, macrophages were significantly increased at 13 weeks of HFD feeding. Combining the data during 2 to 6 weeks (**Supplemental Figure 1**) with those that at 13 weeks (**Figure 2B**), the infiltration of NKT cells preceded that of macrophages in obese adipose tissues. Therefore, 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 (PEPCK) and glucose-6-phosphatase (G6Pase) gene expression were measured in the hepatic tissues. Hepatic gluconeogenesis tended to be suppressed in KO-SD compared to WT-SD, which, however, did not reach statistical significance (**Supplemental Figure 2**).

**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, αGC was injected intraperitoneally in WT mice fed HFD for 13 weeks.

αGC did not affect body weight, visceral adipose tissue weight, and adipocyte size in HFD mice 9 days after injection (**Supplemental Table**).

Quantification of NKT cells by Vα14/Jα18 gene expression confirmed αGC significantly enhanced NKT cell infiltration into adipose tissue (**Figure 3A**). Plasma glucose levels during ipGTT were significantly increased by αGC (15 min: 330±11 vs. 296±11 mg/dL, $p<0.05$ and 30 min: 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 αGC (**Figure 4 A and B**). These changes of adipose tissue macrophages by the immunohistochemical analysis were also confirmed by MHC class II and CD11c gene expression (**Figure 4 C and D**). In parallel to macrophage infiltration into the visceral adipose tissue, the injection of αGC significantly increased the expression of MCP-1, TNF-α, IFN-γ, and RANTES genes in HFD mice (**Figure 5 A-D**).

To examine the temporal relationship between infiltrating NKT cells and macrophages, WT mice, 8 weeks of age, were injected PBS or αGC

intraperitoneally and adipose tissues were dissected 1 day, 4 days and 7 days after the injection. NKT cells and macrophages tended to increase at 4 and 7 days after αGC administration in WT mice (**Supplemental Figure 3**).

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

To confirm the specificity of αGC treatment for activating NKT cells, αGC 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 $MHC\text{-}classII^{-}CD8^{-}B220^{-}$ lymphocytes (NKT-enriched cells) were co-cultured with or without αGC for 48 hours. Macrophages conditioned with activated NKT cells by αGC secreted significantly larger amount of MCP-1 into the co-culture media compared to non-conditioned 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 αGC exacerbated glucose intolerance and adipose tissue inflammation including macrophage infiltration as well as 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 as well as a reduction in inflammatory gene expression.[2] 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 stroma-vascular fraction of white adipose tissues.[7] However, they have not examined the changes of these cells by HFD feeding and even their roles in HFD-induced metabolic disorders. 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 as well as inflammatory cytokine gene expression during the development of HFD-induced glucose intolerance. However, the present results were not consistent with the previous study by Elinav *et al* that NKT cells ameliorated glucose intolerance in leptin-deficient *ob/ob* mice.[13] In their study, 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 $T_H2$ bias, whereas αGC injection stimulated NKT cells toward the $T_H1$ slant in the present study. Therefore, the discrepancy between these studies might be due to the differences in the methods to modulate NKT cells and the resultant 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 be also 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 as well as the development of glucose intolerance before the recruitment of macrophages.[4] A recent elegant study by Nishimura *et al*. elucidated the role of T lymphocytes in adipose tissue inflammation in obesity.[14] In their study, large numbers of $CD8^+$ effector T cells were found to infiltrate into obese epididymal adipose tissue, preceding macrophage infiltration, in HFD-induced obese mice and initiate the inflammatory cascade that leads to insulin resistance in adipocytes. We could not completely exclude the possibility that T-lymphocytes are involved also in our model because $\beta_2$ microglobulin KO mice used in the present study lack not only NKT cells but also CD8-positive T lymphocytes.[15] However, the development of both glucose

intolerance and adipose tissue inflammation induced by HFD were significantly exacerbated by the specific activation of NKT cells by using αGC, an activator of NKT cells but not T cells (**Figures 3, 4, and 5**). Based on these results, we consider that NKT cells are critically involved in glucose intolerance and adipose tissue inflammation in obese mice.

NKT cells are specialized lineage of T cells that recognize glycolipid antigens presented by the MHC class I-like molecule CD1d.[16] NKT cells mediate various functions rapidly via producing a mixture of $T_H1$ and $T_H2$ cytokines such as IFN-γ and IL-4 in shaping subsequent adaptive immune responses.[6] The present study demonstrated that accumulated macrophages in adipose tissues in αGC treated mice were classically activated ($M_1$) macrophages, one of the distinct subsets of macrophages that are categorized as $M_1$ by CD11c (**Figure 4**).[17, 18] In agreement with these findings, the activation of NKT cells was associated with the increased gene expression $T_H1$-cytokine IFN-γ and MCP-1 in HFD-fed mice (**Figure 5**). IFN-γ can also promote the recruitment of monocytes by inducing MCP-1 secretion from periadipocytes, and it could activate other cells like 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. 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,[19, 20] 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.[21, 22] Therefore, these reagents may not be suitable to investigate 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 induce both inflammation and insulin resistance.[23] 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 $T_H1$ slant.[5] However, the HFD feeding has been shown to suppress $T_H1$ responses in B6 mice by inhibiting Toll-like receptor-mediated maturation and proinflammatory cytokine production in dendritic cells.[24] The discrepancy between these studies might be due to the differences in the tissues studied (visceral adipose tissue lymphocytes vs. splenic lymphocytes). Importantly, the contribution of NKT cells is not mediated by the modulation of the adipose tissue weight or adipocyte size because they did not differ between HFD-fed groups (**Table I and Supplemental Table**) even though adipocyte cell size has been shown to be an independent predictor of glucose intolerance.[25]

Activated macrophages secrete TNF-α, which can inhibit the insulin signal transduction.[26] Obesity itself can trigger adipose tissue inflammation which leads to the desensitization of insulin action.[27] 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.[10] Some of the inflammatory processes involved in atherogenesis shown in our previous study resemble adipose tissue inflammation in the present study. Therefore, NKT cells are considered to mediate chronic inflammation in the vascular tissue as well as adipose tissue

and can represent a direct and common soil for the development of atherosclerotic cardiovascular disease and diabetes mellitus. 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 elucidate the pathogenetic role of NKT cells.[28] Therefore, we employed 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 the insulin sensitivity.[1] Fasting plasma glucose level and HOMA-IR were significantly lower in KO-SD than in WT-SD (**Table I**). KO-SD mice tended to have lower plasma glucose levels and AUC values during ipGTT compared to WT-SD (**Figure 1 B and C**), which, however, did not reach statistical significance. These data suggested that the absence of NKT cells could improve glucose metabolism in normal 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. NKT cell infiltration preceded macrophage infiltration in obese visceral adipose tissues and may play 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 also in this setting. In contrast, the activation of NKT cells has been reported to be protective against type 1 diabetes mellitus, systemic lupus erythematodes, and infections.[29] Therefore, the inhibition of NKT cells as therapeutic strategies to prevent and treat metabolic syndrome and cardiovascular disease for the obese individuals needs to be cautious 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. NKT cells may be the novel therapeutic targets in atherosclerosis as well as in metabolic syndrome and type 2 diabetes.

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**Disclosures**

None.

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**Figure Legends**

**Figure 1** (A) Vα14/Jα18 gene expression, an index of NKT cells, of visceral adipose tissues in Experiment 1. (B) Plasma glucose concentrations and (C) area under the curve (AUC) values during the ipGTT. **p*<0.01 vs. WT-SD, †*p*<0.01 vs. WT-HFD, ‡*p*<0.01 vs. WT-SD at each time, §*p*<0.01 vs. WT-HFD at each time.

**Figure 2** Macrophage infiltration in adipose tissue in Experiment 1. (A) F4/80 immunohistochemistry. Scale bar, 20μm. (B) The number of F4/80 positive macrophages. (C-E) Gene expression of MHC class II, CD11c, and arginase. **p*<0.05, †*p*<0.01 vs. WT-SD. ‡*p*<0.05, §*p*<0.01 vs. WT-HFD.

**Figure 3** (A) Vα14/Jα18 gene expression, an index of NKT cells, of visceral adipose tissues 8 days after injection of PBS or αGC, a specific activator for NKT cells, in Experiment 2. (B) Plasma glucose concentrations during ipGTT 8 days after PBS or αGC injection. * *p*<0.05 vs. PBS.

**Figure 4** Macrophage infiltration in adipose tissue in Experiment 2. (A) Demonstrable figures of F4/80 immunohistochemistry. Scale bar, 20μm. (B) The number of F4/80 positive nuclei from PBS and αGC mice. (C, D) Expression of MHC class II and CD11c genes in visceral adipose tissues. * *p*<0.05 vs. PBS.

**Figure 5** (A-D) Expression of MCP-1, TNF-α, IFN-γ and RANTES genes in visceral adipose tissues from PBS and αGC mice in Experiment 2. Quantitative RT-PCR was performed 9 days after PBS or αGC injection. **p*<0.05 vs. PBS.

**Table I** Animal characteristics in experiment 1

| | WT-SD (n=10) | KO-SD (n=5) | WT-HFD (n=10) | KO-HFD (n=14) |
|---|---|---|---|---|
| Body weight, g | 29.4 ± 0.5 | 29.4 ± 1.4 | 33.2 ± 0.6† | 31.4 ± 0.7 |
| Blood chemistry | | | | |
| Fasting plasma glucose, mg/dL | 78 ± 8 | 50 ± 8* | 82 ± 8 | 55 ± 4§ |
| Insulin, ng/mL | 0.49 ± 0.09 | 0.27 ± 0.06 | 0.77 ± 0.22 | 0.90 ± 0.13 |
| HOMA-IR | 2.10 ± 0.22 | 0.79 ± 0.21† | 3.29 ± 0.66 | 3.23 ± 0.68 |
| Total cholesterol, mg/dL | 102 ± 2 | 103 ± 3 | 181 ± 10† | 193 ± 6† |
| Leptin, ng/mL | 2.2 ± 0.5 | 2.4 ± 0.9 | 12.9 ± 2.5† | 16.0 ± 4.0† |
| TNF-α, pg/mL | 37 ± 5 | 13 ± 4† | 85 ± 40 | 29 ± 6‡ |
| Adiponectin, μg/mL | 22 ± 2 | 30 ± 5 | 19 ± 1 | 48 ± 3§ |
| Glucagon, pg/mL | 478 ± 25 | 423 ± 25 | 477 ± 33 | 406 ± 32 |
| Visceral adipose tissue | | | | |
| Visceral adipose tissue weight, mg | 559 ± 57 | 564 ± 77 | 1388 ± 131† | 1331 ± 94† |
| Visceral adipose tissue weight /Body weight, mg/g | 19.7 ± 1.7 | 19.1 ± 2.4 | 42.5 ± 3.8† | 42.0 ± 2.3† |
| Adipocyte size, $\mu m^2$ | 1697 ± 156 | 1492 ± 162 | 2787 ± 324† | 2921 ± 308† |

* $p<0.05$, † $p<0.01$ vs. WT-SD, ‡ $p<0.05$, § $p<0.01$ vs. WT-HFD. WT: C57BL/6J mice, KO: $\beta_2$microalbumin knockout mice, SD: Standard diet, HFD: High fat diet, TNF-α: Tumor necrosis factor-α.

Fig. 1

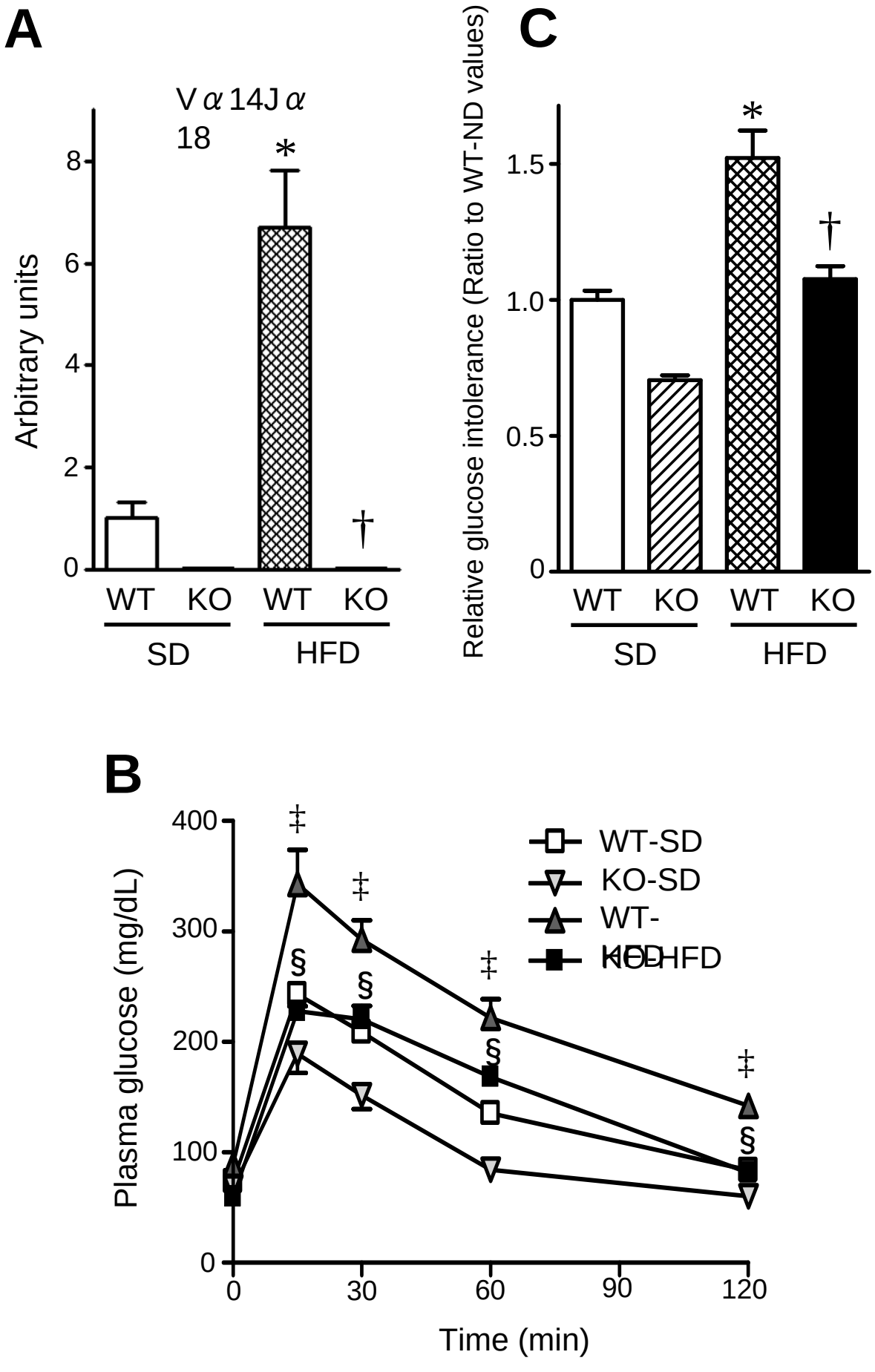

A
Vα14Jα18
Arbitrary units
0
2
4
6
8
*
†
WT
KO
WT
KO
SD
HFD
C
Relative glucose intolerance (Ratio to WT-ND values)
0
0.5
1.0
1.5
*
†
WT
KO
WT
KO
SD
HFD
B
Plasma glucose (mg/dL)
0
100
200
300
400
‡
§
WT-SD
KO-SD
WT-HFD
KO-HFD
0
30
60
90
120
Time (min)

Fig. 2

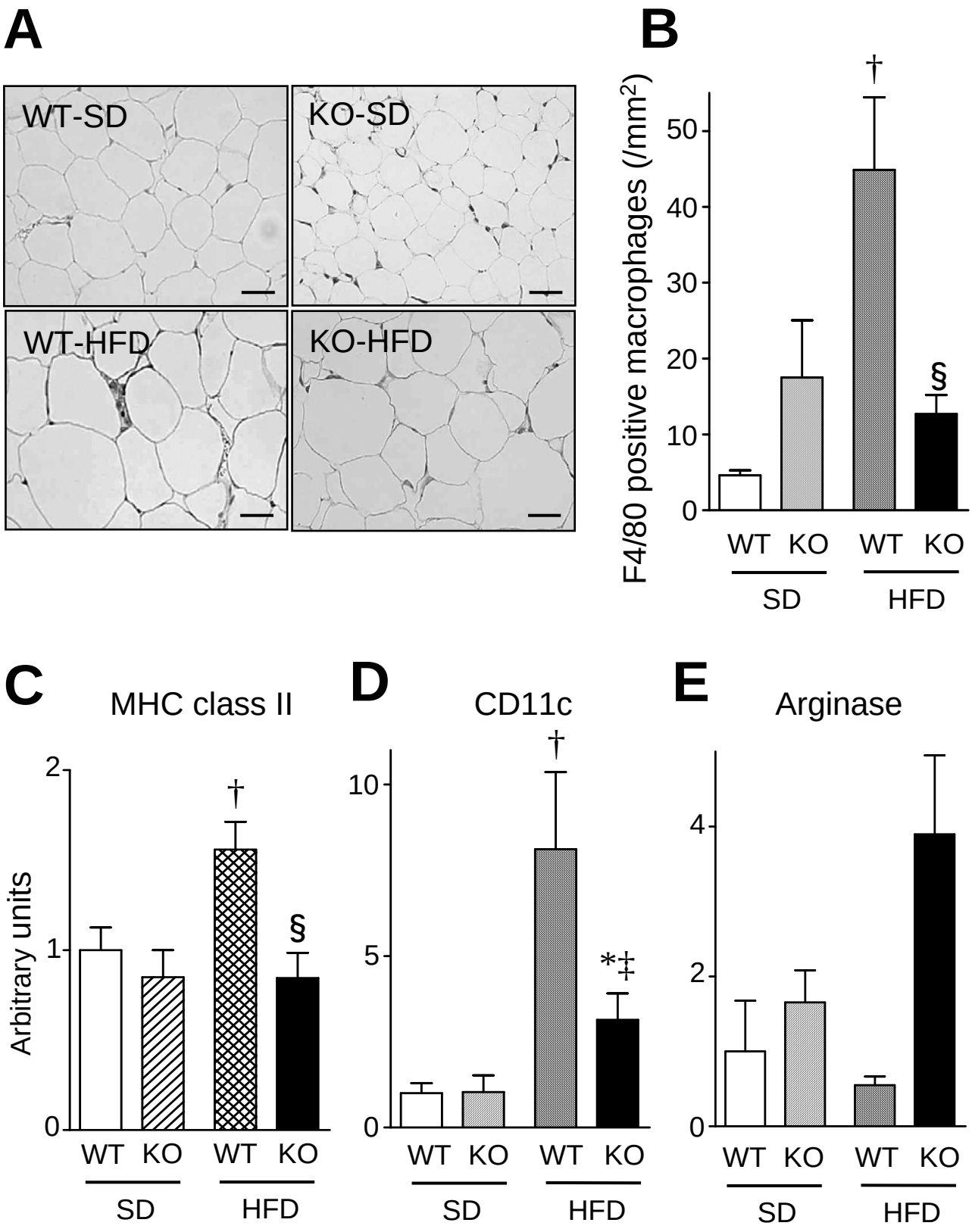

A
WT-SD
KO-SD
WT-HFD
KO-HFD
B
F4/80 positive macrophages (/mm2)
0
10
20
30
40
50
†
§
WT KO WT KO
SD HFD
C
MHC class II
Arbitrary units
0
1
2
†
§
WT KO WT KO
SD HFD
D
CD11c
0
5
10
†
*‡
WT KO WT KO
SD HFD
E
Arginase
0
2
4
WT KO WT KO
SD HFD

Fig. 3

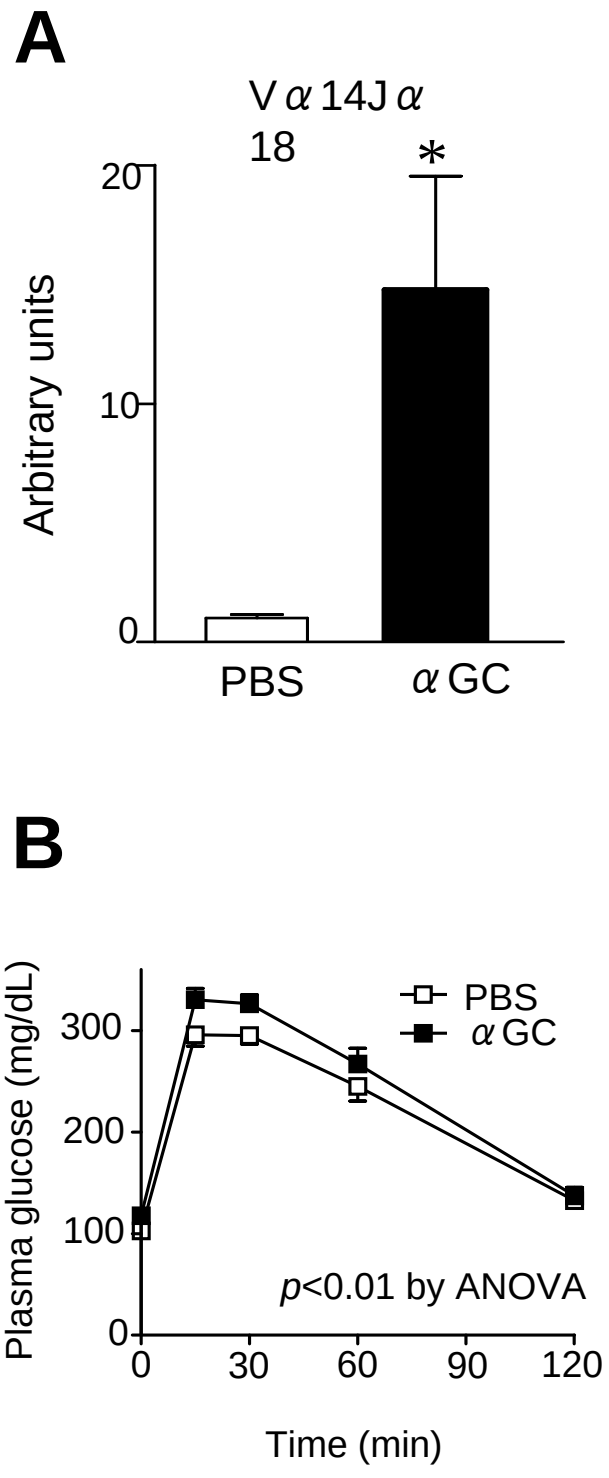

A
Vα14Jα18
*
20
10
0
Arbitrary units
PBS
αGC
B
Plasma glucose (mg/dL)
300
200
100
0
PBS
αGC
p<0.01 by ANOVA
0
30
60
90
120
Time (min)

Fig. 4

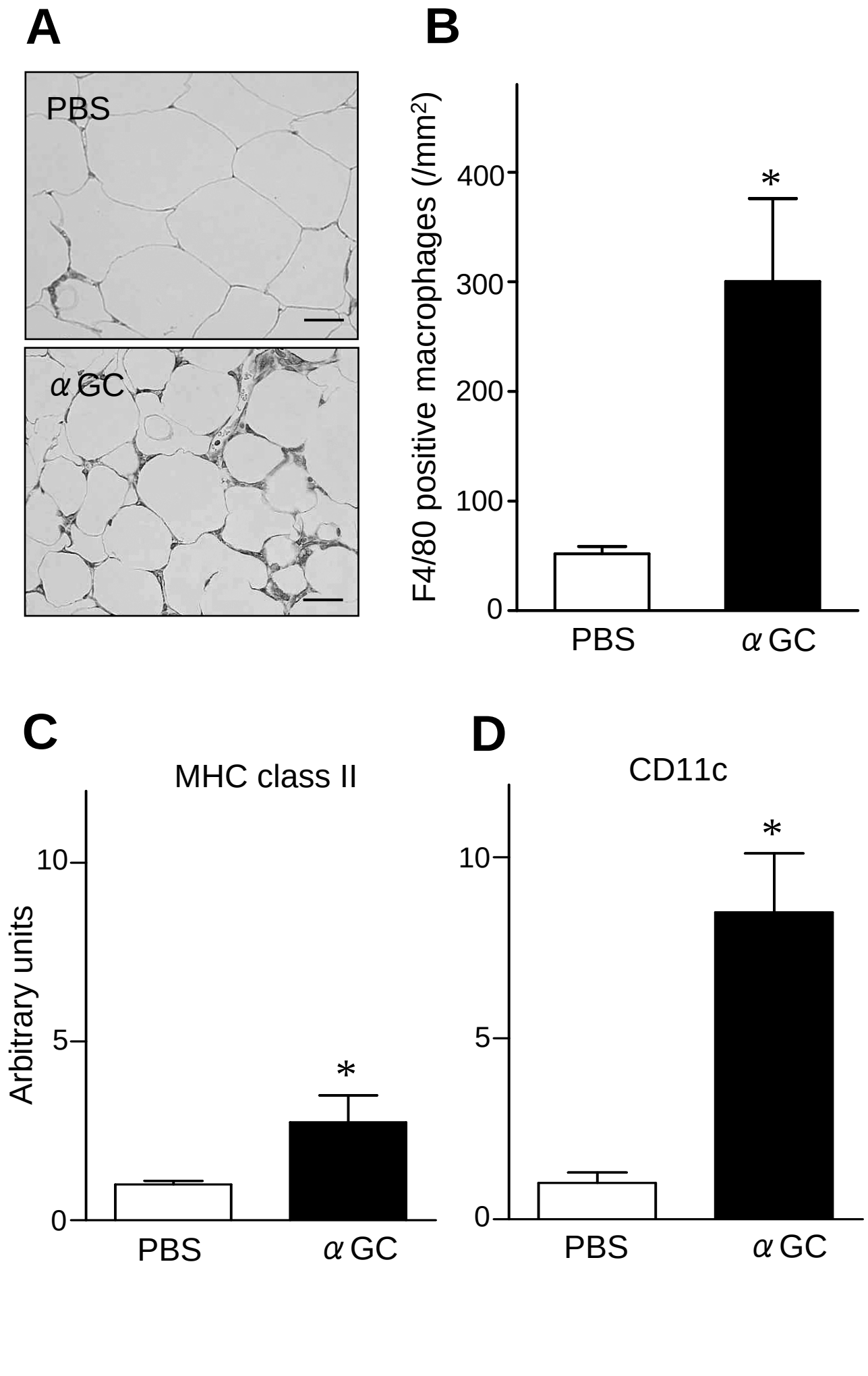
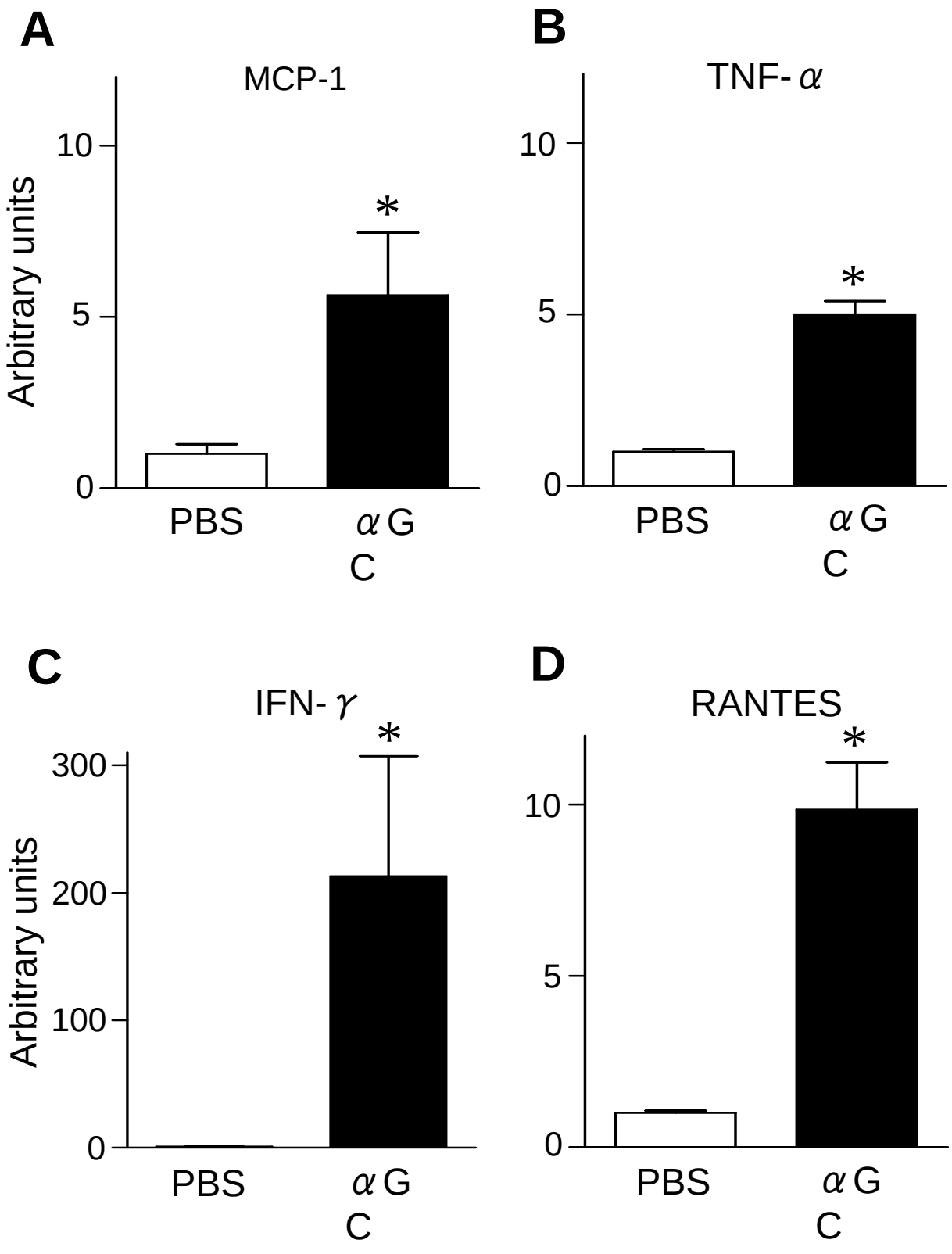

A
PBS
αGC
B
F4/80 positive macrophages (/mm²)
400
300
200
100
0
*
PBS
αGC
C
MHC class II
Arbitrary units
10
5
0
*
PBS
αGC
D
CD11c
10
5
0
*
PBS
αGC

Fig. 5


A
MCP-1
Arbitrary units
10
5
0
*
PBS
αGC
B
TNF-α
10
5
0
*
PBS
αGC
C
IFN-γ
Arbitrary units
300
200
100
0
*
PBS
αGC
D
RANTES
10
5
0
*
PBS
αGC