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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 β_2 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 version1.40, 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),[17] arginase (a marker for M2 macrophages), Vα14/Jα18 (a specific marker of NKT cells),[6] 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.
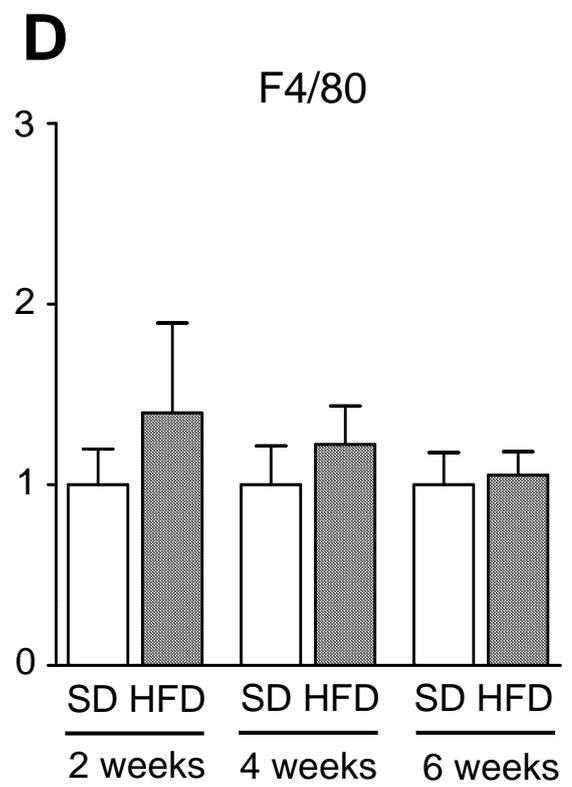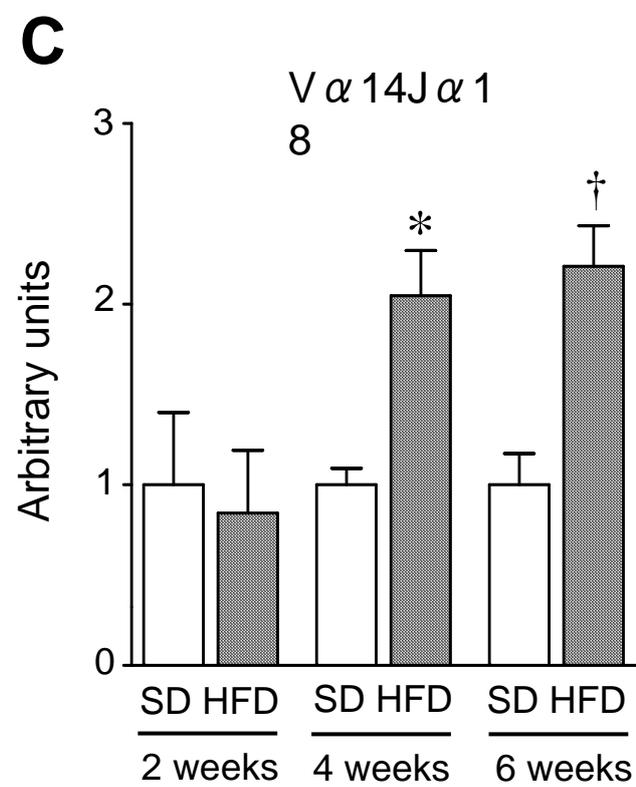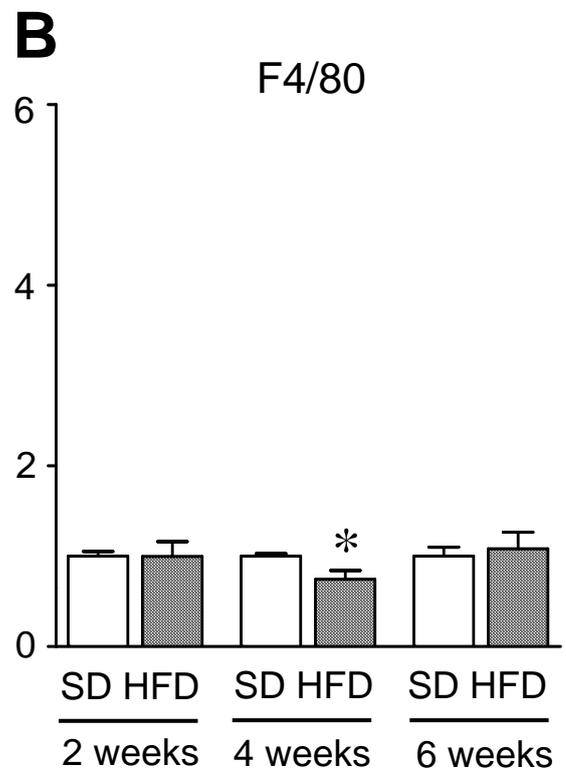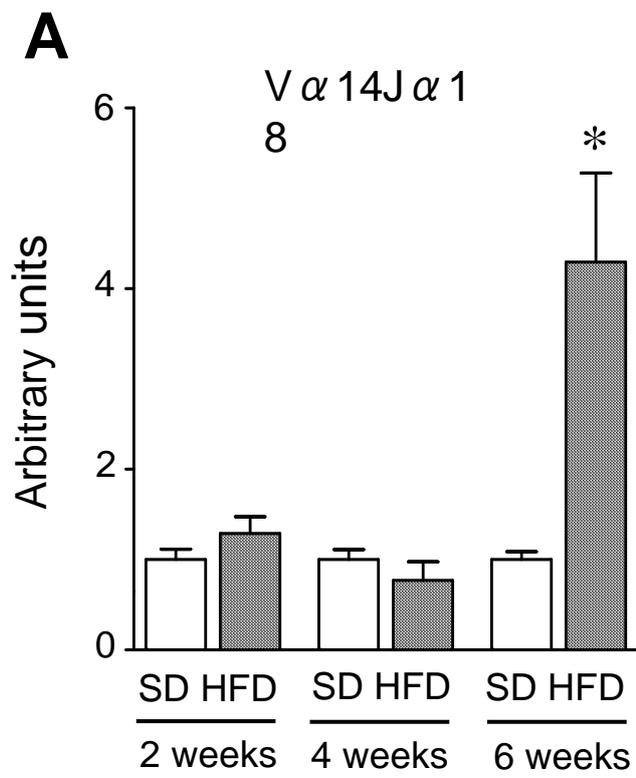
Supplemental Figure 5 Plasma glucose concentrations during ipGTT 7 days after PBS or αGC injection of *ob/ob* mice in Experiment 2.

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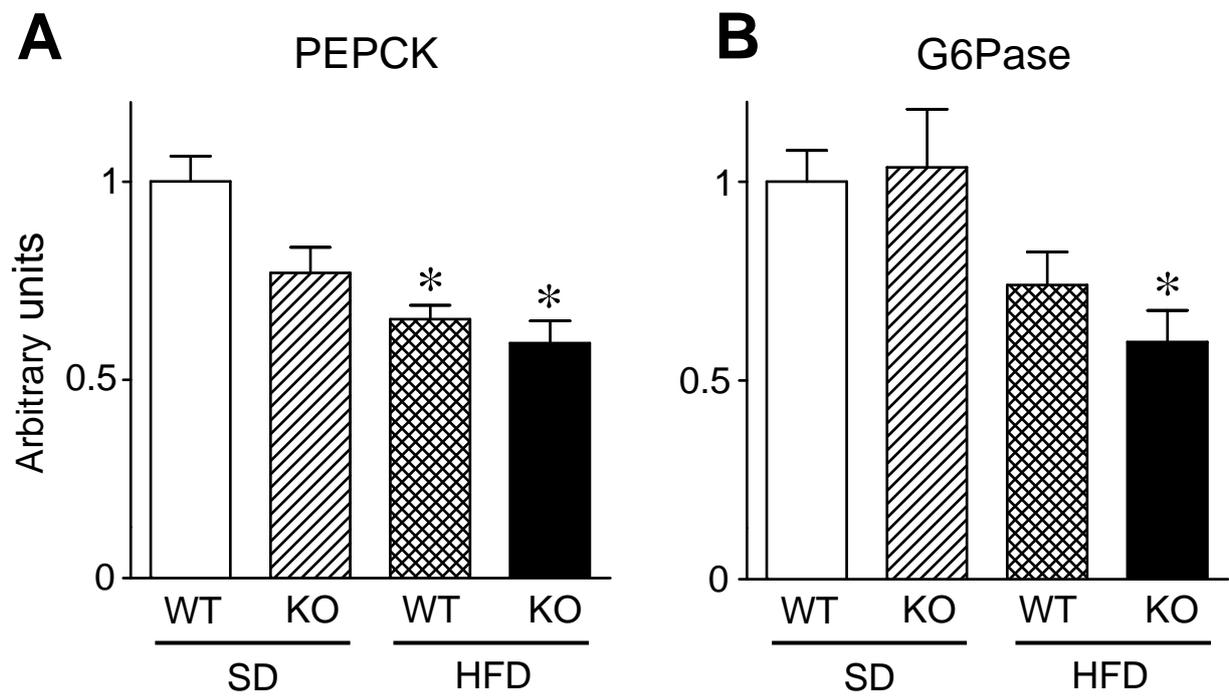
in adipose tissues from KO-PBS (n=5) and KO-αGC (n=5) mice after feeding a HFD for 13 weeks in Experiment 2. Quantitative RT-PCR was performed 9 days after PBS or αGC injection. (C) Plasma glucose concentrations during ipGTT 8 days after PBS or αGC injection.

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.
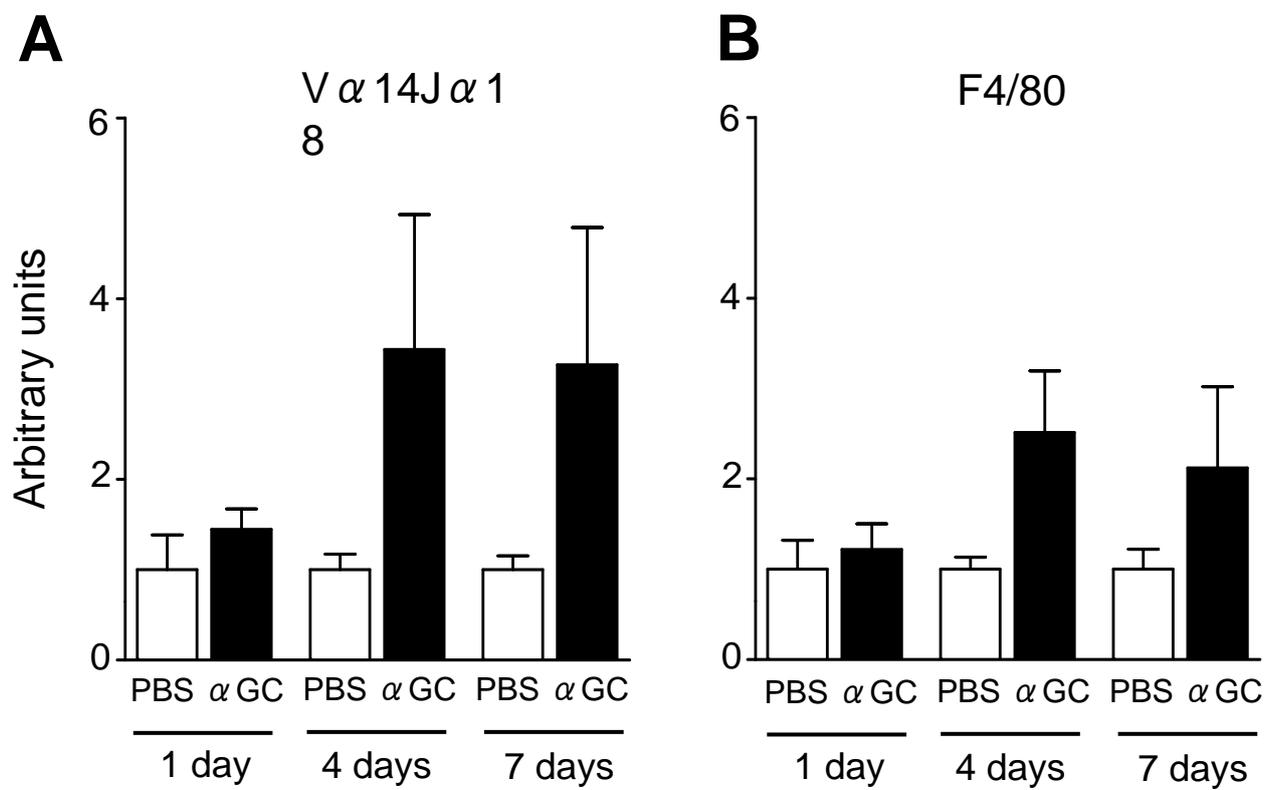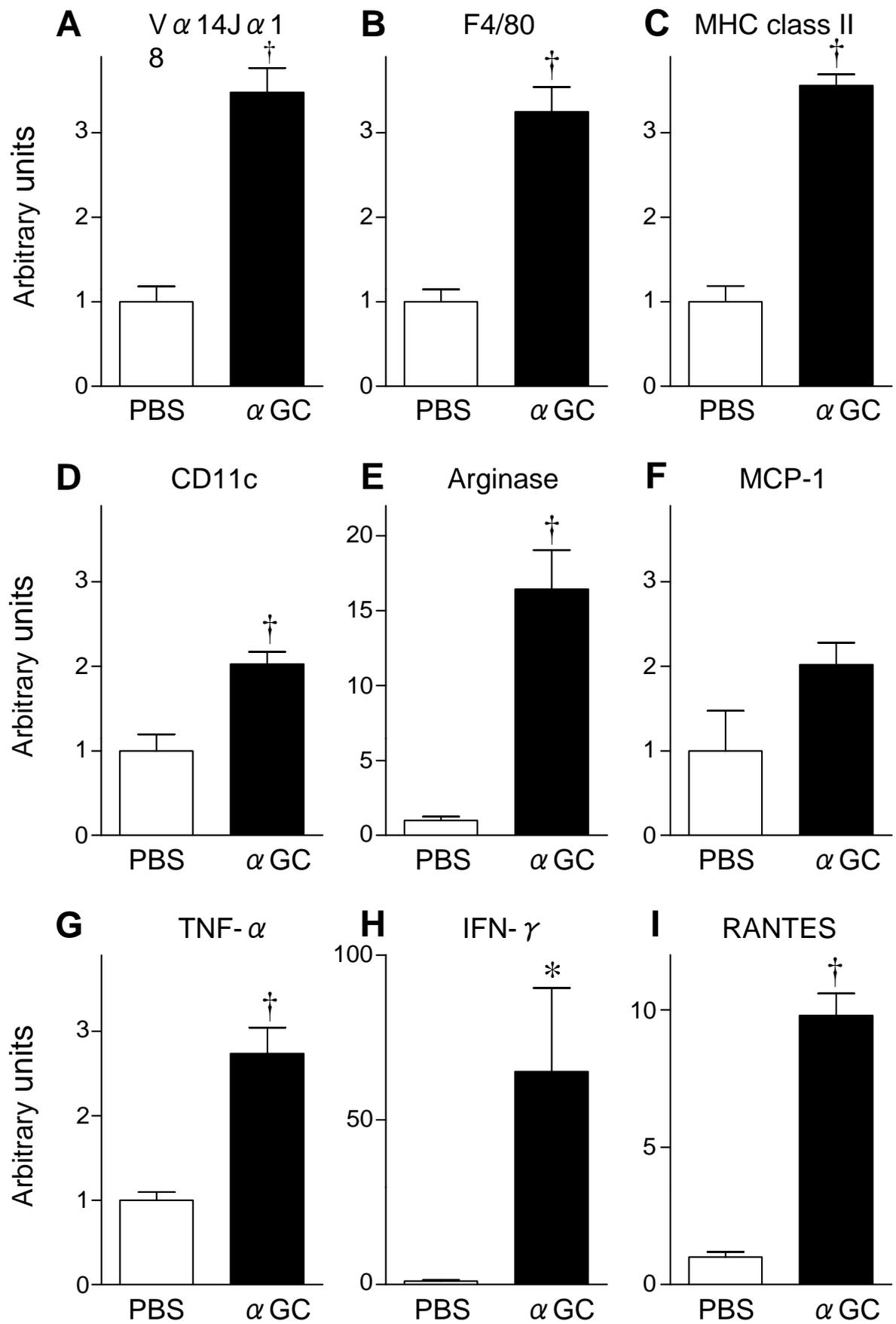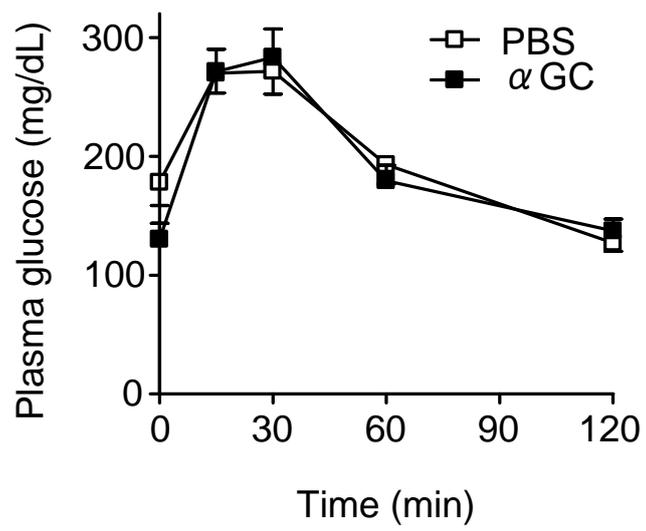
Supplemental Fig. 1

Supplemental Fig. 2
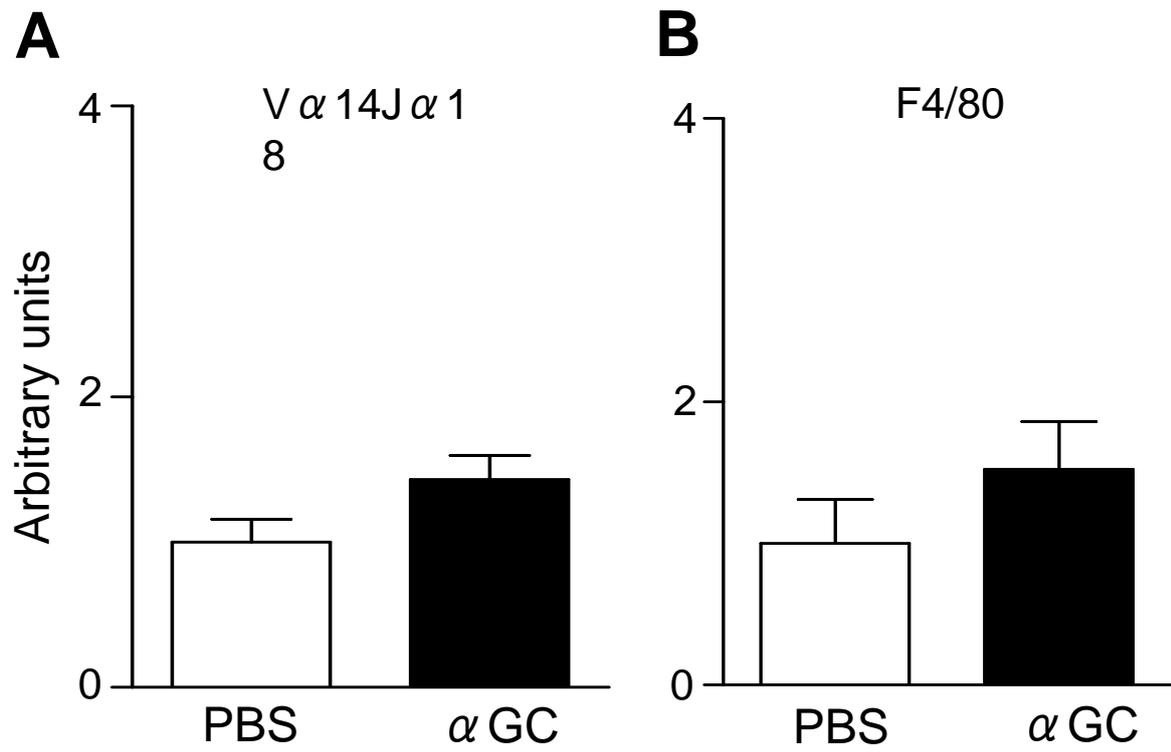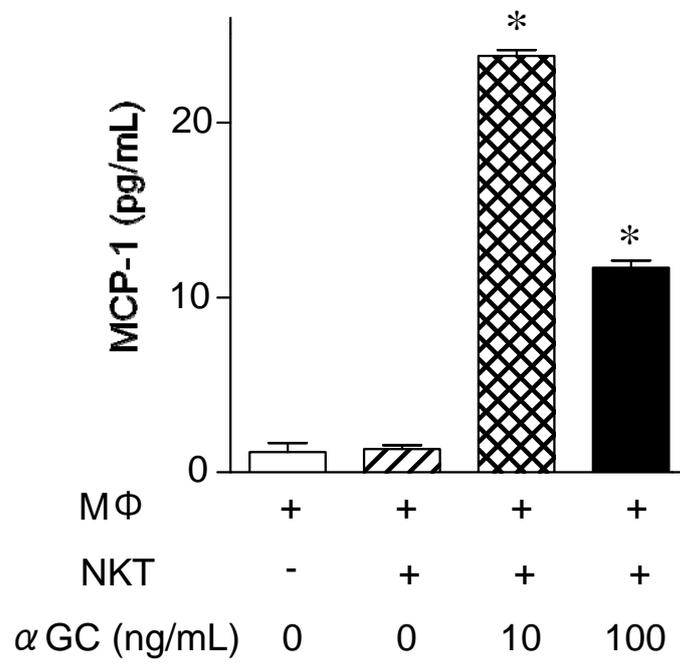
Supplemental Fig. 3

Supplemental Fig. 4

A Vα14Jα18   **B** F4/80   **C** MHC class II

D CD11c   **E** Arginase   **F** MCP-1

G TNF-α   **H** IFN-γ   **I** RANTES

Supplemental Fig. 5

Supplemental Fig. 6

A

Vα14Jα1
8

Arbitrary units

4

2

0

PBS   αGC

B

F4/80

4

2

0

PBS   αGC

C

Plasma glucose (mg/dL)

400

300

200

100

0

□ PBS
■ αGC

0   30   60   90   120

Time (min)
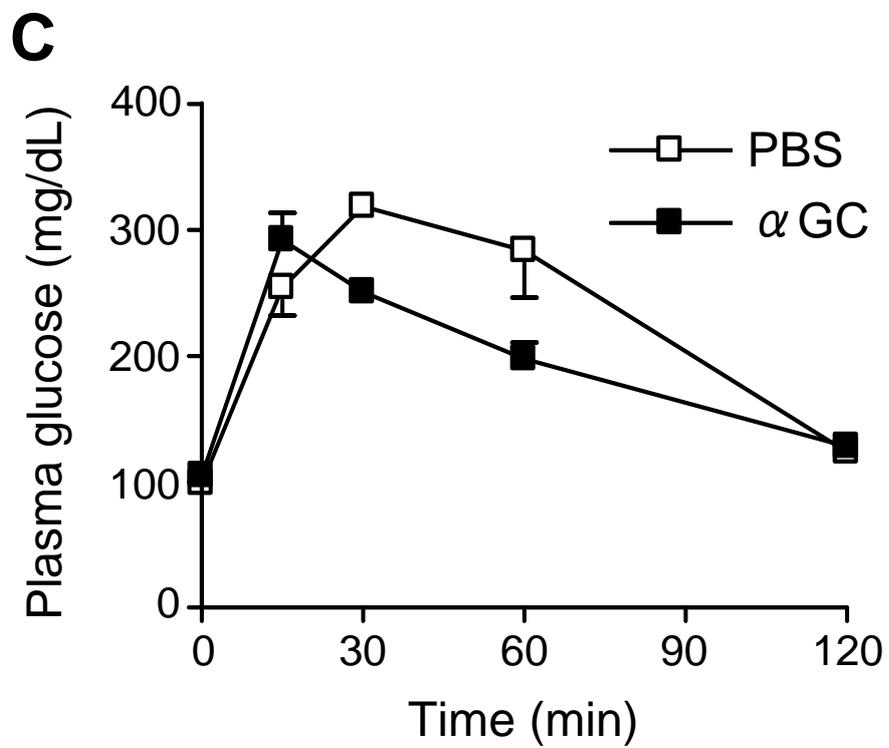
Supplemental Fig. 7

Online Supplemental Table Animal characteristics in experiment 2

	PBS (n=5)	αGC (n=5)
Body weight, g	34.9 ± 1.1	32.8 ± 1.2
Visceral adipose tissue		
Visceral adipose tissue weight, mg	1720 ± 98	1782 ± 330
Visceral adipose tissue weight/Body weight, mg/g	49.2 ± 1.8	54.2 ± 10.0
Adipocyte size, μm^2	3398 ± 338	3863 ± 681

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