



Therefore, it appears that the balance between CD4⁺ versus regulatory (presumably DN) NKT cells is determined or imprinted early in life but might be influenced by exposure to specific types of infections, particularly those that can affect NKT cells. In our studies, H3N1 infection in 2-week-old pups activated the immature NKT cells and preferentially expanded a DN NKT cell subset. In addition, our studies suggest that α -C-GalCer and glycolipids from *H. pylori* can profoundly affect this NKT cell subpopulation, which may explain epidemiological studies showing an association of *H. pylori* infection with protection against asthma (2, 3). Although these studies were performed in mice, which mature from neonates to adults in only 35 days versus many years in humans, taken together, our results suggest that infection with certain microorganisms can prevent the subsequent development of asthma and allergy by expanding the relative proportion of a specific subset of NKT cells, thus providing an immunological mechanism for the hygiene hypothesis. Finally, these results predict that treatment of children with compounds such as α -C-GalCer and others derived from microorganisms (e.g., *H. pylori*) might expand this regulatory NKT cell subset and be effective in preventing the development of asthma.

Methods

Mice. WT BALB/c ByJ and *Tbet*^{-/-} (C.129S6-Tbx21tm1Glm/J) mice were purchased from The Jackson Laboratory. *J α 18*^{-/-} mice were gifts from M. Taniguchi and T. Nakayama (Chiba University, Chiba, Japan). *Tlr7*^{-/-} mice were generated by Shizuo Akira, and the V α 14 Tg mice were provided by Albert Bendelac (University of Chicago, Chicago, Illinois, USA). These strains were backcrossed to BALB/c for more than 10 generations. DO11.10 X *Rag*^{-/-} mice were provided by Abul Abbas (UCSF, San Francisco, California, USA). For studies in suckling mice, BALB/c, *Tlr7*^{-/-}, and *Tbet*^{-/-} mice were bred, and the offspring were infected at 2 weeks of age, then weaned at 3 weeks. The Animal Care and Use Committee at Children's Hospital Boston approved all animal protocols.

Influenza A infection. Two-week-old pups (suckling mice) or 8-week-old adult mice were anesthetized with 3% isoflurane and inoculated intranasally (i.n.) with influenza A virus (strain Mem/71 [H3N1]) in 20 μ l PBS for suckling mice or 50 μ l PBS for adult mice. The virus is a reassortant influenza virus strain carrying the hemagglutinin of A/Memphis/1/71 (H3) and the neuraminidase of A/Bellamy/42 (N1). The virus was grown and harvested from 10-day embryonated chicken eggs as previously described (59). The dose of virus used (1.2×10^4 PFU/mouse) causes nonlethal pneumonia of both suckling and adult mice, with complete virus clearance around day 7 after infection. Control (mock-infected) mice were treated with i.n. allantoic fluid (AF) diluted 1:500 in PBS.

Reagents. α -GalCer and PBS30 (31) were synthesized by P.B. Savage (Brigham Young University, Provo, Utah, USA). *H. pylori* glycolipids were extracted and purified as described in the Supplemental Methods. The *H. pylori* glycolipid PI57 (cholesteryl 6-O-tetradecanoyl- α -D-glucopyranoside) was synthesized based on ¹H, ¹³C NMR spectrometry, TLC analysis, ES-mass spectrometry of lipids from *H. pylori* SS1 and human *H. pylori* S strains (Supplemental Figure 4 and Supplemental Methods), and data reported for purified *H. pylori* glycolipids (30). An analog of α -C-GalCer, called "GCK151", which has activity with mouse and human NKT cells (27), was synthesized by Richard W. Franck (Hunter College of CUNY).

PI57-loaded CD1d tetramers. To generate PI57-loaded mCD1d monomers, a 10-fold molar excess of PI57 in DMSO at 2 mg/ml was incubated with biotinylated-mCD1d (from the NIH Tetramer facility) in 2 mM CHAPS and 20 mM Tris pH 7.0 overnight at room temperature. The mCD1d monomers were tetramerized by adding SA-PE (S868; Invitrogen) to the lipid-loaded monomers as previously described (60).

Induction of AHR and measurement of airway responsiveness in the OVA model. To induce AHR, BALB/c mice were sensitized with 100 μ g of OVA (Sigma-Aldrich) in alum administered i.p. (on day 0). After sensitization, mice were exposed to i.n. antigen (50 μ g OVA/day) or normal saline for 1 day (day 7; single-dose challenge protocol) or for 3 consecutive days (days 7–9). AHR was assessed on the day after last OVA challenge. Control mice received i.p. injection of PBS and i.n. administrations of normal saline.

Collection and analysis of bronchoalveolar lavage. Immediately after the AHR measurement, mice were euthanized and the lungs were lavaged twice with 0.5 ml of PBS, and the fluid was pooled. Cells in bronchoalveolar lavage (BAL) were counted and analyzed as previously described (20). The relative number of different types of leukocytes was determined from slide preparations of BAL stained with Diff-Quik solution (Dade Behring).

Adoptive transfer of NKT cells. NKT cells were purified from splenocytes of WT BALB/c, influenza virus-infected BALB/c, influenza virus-infected *Tlr7*^{-/-}, influenza virus-infected *Tbet*^{-/-}, V α 14 TCR transgenic mice, PI57-treated BALB/c, and α -GalCer-treated BALB/c mice using magnetic cell sorting (MACS), as previously described (20). Splenic NKT cells were labeled with PE-conjugated CD1d tetramer, followed by anti-PE microbeads (Miltenyi Biotec) and then sorted with AutoMACS according to the manufacturer's instruction. Purity of NKT cells was approximately 93% (Supplemental Figure 2A), and there was no detectable Treg cell contamination (Supplemental Figure 2B). Purified NKT cells were adoptively transferred into immunized recipient mice by intravenous injection (10^6 for *J α 18*^{-/-}; 5×10^5 for BALB/c) 1 hour before the first challenge of OVA (day 7). For the OVA-specific Treg cell experiment, 5×10^4 DO11.10 CD4⁺ T cells (from DO11.10 \times *Rag*^{-/-} mice) were adoptively transferred into recipient mice 5 hours before sensitization with OVA/alum (day 0). The recipients later received NKT cells 1 hour before the first challenge of OVA (day 7).

ELISA. Mouse or human IL-4 and IFN- γ levels were measured by ELISA, as previously described (20). Mean values of triplicate cultures were shown. Data are representative of 2 or 3 independent experiments.

Statistics. Differences between groups with parametric distributions were analyzed using the Student's 2-tailed *t* test. Otherwise, the Mann-Whitney *U* test was used. Data represent mean \pm SEM. *P* values of 0.05 or less were considered statistically significant.

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In vitro differentiation of lineage-negative bone marrow cells into microglia-like cells

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Abstract

Microglia are believed to be the only resident immune cells in the CNS, originating from hematopoietic-derived myeloid cells and invading the CNS during development. However, the detailed mechanisms of differentiation and transformation of microglial cells are not fully understood. Here, we demonstrate that murine microglial cells show two morphological forms *in vitro*, namely, small round cells expressing CD11b, Iba1, triggering receptor expressing on myeloid cells-2 (TREM2), and weakly expressing major histocompatibility complex class II and large flat cells expressing only CD11b and Iba1. Moreover, lineage-negative bone marrow (LN) cells cultured with primary mixed glial culture cells could differentiate into only the small round microglia-like cells, despite the absence of CCR2 and Gr-1 expression. Addition of macrophage colony stimulating factor (M-CSF) to LN cell culture allowed the proliferation and expression of TREM2 in LN cells, and the addition of neutralizing anti-M-CSF antibodies suppressed the proliferation of LN cells despite the expression of TREM2. When LN cells were cultured with M-CSF, the number of small round cells in the culture was considerably low, indicating that the small round morphology of the immature cells is not maintained in the presence of only M-CSF. On the other hand, when LN cells were grown in the presence of astrocytes, the small round cells were maintained at a concentration of approximately 30% of the total population. Therefore, cell–cell contact with glial cells, especially astrocytes, may be necessary to maintain the small round shape of the immature cells expressing TREM2.

Introduction

Microglia are believed to be the only resident immune cells in the CNS; they develop from hematopoietic-derived myeloid cells and invade the CNS during development (Ling & Wong, 1993). Microglial cells are recognized to play an important role not only in neuroinflammation and neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease, but also in neuroprotective and anti-neuroinflammatory processes (Akiyama & McGeer, 2004; Sanders & De Keyser, 2007; Takahashi *et al.*, 2007). In a recent study, it has been reported that physiological microglial phagocytosis induced the efficient removal of apoptotic cells and cellular debris without inflammatory processes; this process is expected to be a novel, attractive target for protection from neuroinflammation or neurodegeneration (Takahashi *et al.*, 2005, 2007; Neumann & Takahashi, 2007). The detailed mechanisms underlying the differentiation and transformation of microglial cells, however, are not fully understood. Even in adulthood, hematopoietic-derived cells develop into resident perivascular macrophages and microglia (Hickey & Kimura, 1988; Priller *et al.*, 2001; Simard & Rivest, 2004). Although the exact

cellular subtype of myeloid precursors that develop into microglia is unknown, it has been reported that only Ly-6C^{high} CCR2⁺ monocytes can invade and differentiate into perivascular microglia (Mildner *et al.*, 2007).

The bone marrow produces new blood cells, including all cell types of the myeloid lineage, some of which may differentiate into microglia. Lineage-negative bone marrow (LN) cells are defined by the absence of surface markers, such as CD3, CD4, CD5, CD8 α , CD11b/MAC-1 α , B220, Gr-1 and TER-119, and are considered to comprise many hematopoietic precursors, including microglia precursors. Thus, LN cells might represent microglial precursors and may serve as a natural vehicle for CNS cells in gene therapy.

In this study, we show that murine microglial cells are present *in vitro* in two morphological forms, namely, as small round cells expressing CD11b, Iba1, triggering receptor expressing on myeloid cells-2 (TREM2), and weakly expressing major histocompatibility complex (MHC) class II and as large flat cells expressing only CD11b and Iba1. We found that LN cells could differentiate into the small round-type but not the large flat-type microglia-like cells. Moreover, we concluded that not only macrophage colony-stimulating factor (M-CSF) but also cell–cell contacts with astrocytes play an important role in microglial differentiation.

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Materials and methods

Isolation of LN cells from adult green fluorescence protein (GFP) mice bone marrow

Bone marrow cells were collected from 8- to 10-week-old C57BL/6 mice (Charles River, Japan) or GFP transgenic mice on a C57BL/6 mice background that were kindly provided by Dr Masaru Okabe (Osaka University, Japan) by flushing out the femora and tibiae of the hind limb under deep anesthesia by the diethyl ether. Erythrocyte removal was performed by lysis with the Mouse Erythrocyte Lysing Kit (R&D, Minneapolis, MN, USA). For eliminating lineage marker-positive cells via negative selection, bone marrow cells were incubated at 4°C for 30 min with eight types of rat monoclonal antibodies against mice lineage markers [CD3, CD4, CD5, CD8 α , CD11b/MAC-1 α , B220, Gr-1 and TER-119 (R&D)]. The cells were then washed and incubated with immunomagnetic beads (Invitrogen, Tokyo, Japan) at 4°C for 30 min. Finally, LN cells were collected by the removal of lineage marker-positive bone marrow cells by using a magnet stand that attracted the lineage marker-positive cells attached to the antibodies.

All experiments were approved by the Ethics Committee of Kanazawa University, and performed in accordance with the guidelines of the local animal care and use committee of Kanazawa University.

Primary mixed glial cell culture

Microglial cells were prepared from the brains of postnatal days 3–5 (P3–P5) C57BL/6 mice under deep anesthesia by the diethyl ether, as previously described (Takahashi *et al.*, 2005). Briefly, meninges were removed mechanically, and the cells were dissociated by trituration and cultured in basal medium Eagle (Invitrogen), 10% fetal calf serum (Invitrogen), 1% glucose (Sigma, Tokyo, Japan), 1% L-glutamine (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) for 14 days to form a confluent glial monolayer. LN cells obtained from GFP mice were added onto the confluent glial monolayer.

Treatment of LN cell culture with mixed glial cell culture supernatant, neutralizing antibodies or cytokines

LN cells were cultured with the mixed glial cell culture supernatant obtained from the Day 14 primary mixed glial culture. LN cells were also cultured with a culture medium containing M-CSF (10 ng/mL; Peprotech, Rocky Hill, NJ, USA), tumor necrosis factor- α (TNF- α ; 10 ng/mL; Peprotech) or vascular endothelial growth factor (VEGF; 50 ng/mL; Peprotech) for 7 days. For the neutralizing assay, anti-M-CSF (2 μ g/mL; R&D), anti-TNF- α (2 μ g/mL; R&D) or anti-VEGF (1 μ g/mL; R&D) antibodies were added in the LN cell culture for 7 days.

Immunohistochemistry

Mixed glial cell cultures with or without GFP-positive (GFP+) LN cells were fixed in 4% paraformaldehyde for 1 h, blocked by Protein Block (Dako, Denmark) for 2 h, and then immunostained with monoclonal rat antibodies directed against CD11b (Serotec, Oxford, UK) and a secondary fluorescence rhodamine-conjugated antibody directed against rat IgG (1 : 200; Millipore, Billerica, MA, USA). To identify the cell type, cells were double-labeled with a purified polyclonal sheep antibody directed against TREM2 (1 : 50, R&D), monoclonal rabbit antibodies directed against Iba1 (Wako, Kanagawa, Japan), and glial fibrillary acidic protein (GFAP; Dako) and

monoclonal mouse antibodies directed against IAb (BD Pharmingen, Tokyo, Japan), followed by a secondary fluorescein isothiocyanate (FITC)-conjugated antibody directed against mouse IgG. Images were collected by fluorescence microscopy with a 20 \times objective (Olympus, Tokyo, Japan). A confocal microscope with a 40 \times objective (Zeiss, Jena, Germany) was used to obtain Z-stack images, and series of optical sections (512 \times 512 pixels, pixel size: 440 nm) were collected at intervals of 380 nm. Images were analysed using the Zeiss LSM Image browser (Zeiss).

To quantify the number of cells, 10 fields under the 20 \times objective were randomly selected and photographed by fluorescence microscopy using a digital camera (Olympus) for each experiment. Total cells, GFP-positive cells and positively immunostained cells were classified and counted according to their morphology.

Isolation of microglia and splenic macrophages

Microglial cells and GFP+ LN cells-derived microglial cells in primary mixed glial culture were obtained by shaking the flasks at 0.25 g for 2 h. Adult CNS microglia and splenic macrophages were obtained from GFP mice. The cortex and spinal cord of GFP mice were isolated and homogenized. Homogenates were incubated with 0.3 Wunsch units/mL Liberase Blendzyme 3 (Roche, Tokyo, Japan) and 0.1 mg/mL DNaseI (Roche) in RPMI 1640 medium at 37°C for 30 min. Microglia were separated through a density gradient. The cells were suspended in 27% Percoll (GE Healthcare, Tokyo, Japan) and overlaid with a 72% gradient. The density gradient was centrifuged at 1500 g for 30 min at 4°C. Myelin collected in the 27% Percoll layer was removed. The majority of microglia were found in the interface of the 27 and 72% Percoll layers. Cells were obtained from this interface and washed from the Percoll with phosphate-buffered saline. The spleen was isolated and cut into small fragments. Cells were incubated with 0.3 Wunsch units/mL Liberase Blendzyme 3 (Roche) and 0.1 mg/mL DNaseI (Roche) in RPMI 1640 medium at 37°C for 45 min. Erythrocyte removal was performed using ACK solution.

Flow cytometry analysis of LN cells, microglia and splenic macrophages

For flow cytometry analysis, cells were first incubated for Fc-receptor blockade by CD16/CD32 antibody (BD Pharmingen) and then stained with phycoerythrin (PE)-conjugated anti-IAb antibody and anti-CD45 antibody (BD Pharmingen), PerCP-Cy5.5-conjugated anti-CD11b antibody (BD Pharmingen), APC-conjugated anti-TREM2 antibody (R&D) and anti-F4/80 antibody (eBioscience, San Diego, CA, USA), or purified anti-CCR2 antibody (Abcam, Tokyo, Japan) followed by rhodamine-conjugated anti-Goat IgG (Millipore). Analysis was performed with a FACSCalibur flow cytometer (BD Biosciences, Tokyo, Japan).

Proliferation assay

Microglial cells and GFP+ LN cells-derived microglia-like cells in primary mixed glial culture, adult CNS microglia and splenic macrophages were cultured with various concentrations of M-CSF (0–100 ng/mL), and cultures were pulsed with 1 mCi of [3 H]TdR (MP Biomedicals, Tokyo, Japan) for the last 16 h of the incubation. Cell incorporation of [3 H]TdR was counted with a Topcount (Perkin Elmer, Boston, MA, USA). The mean cpm of triplicate cultures was calculated.

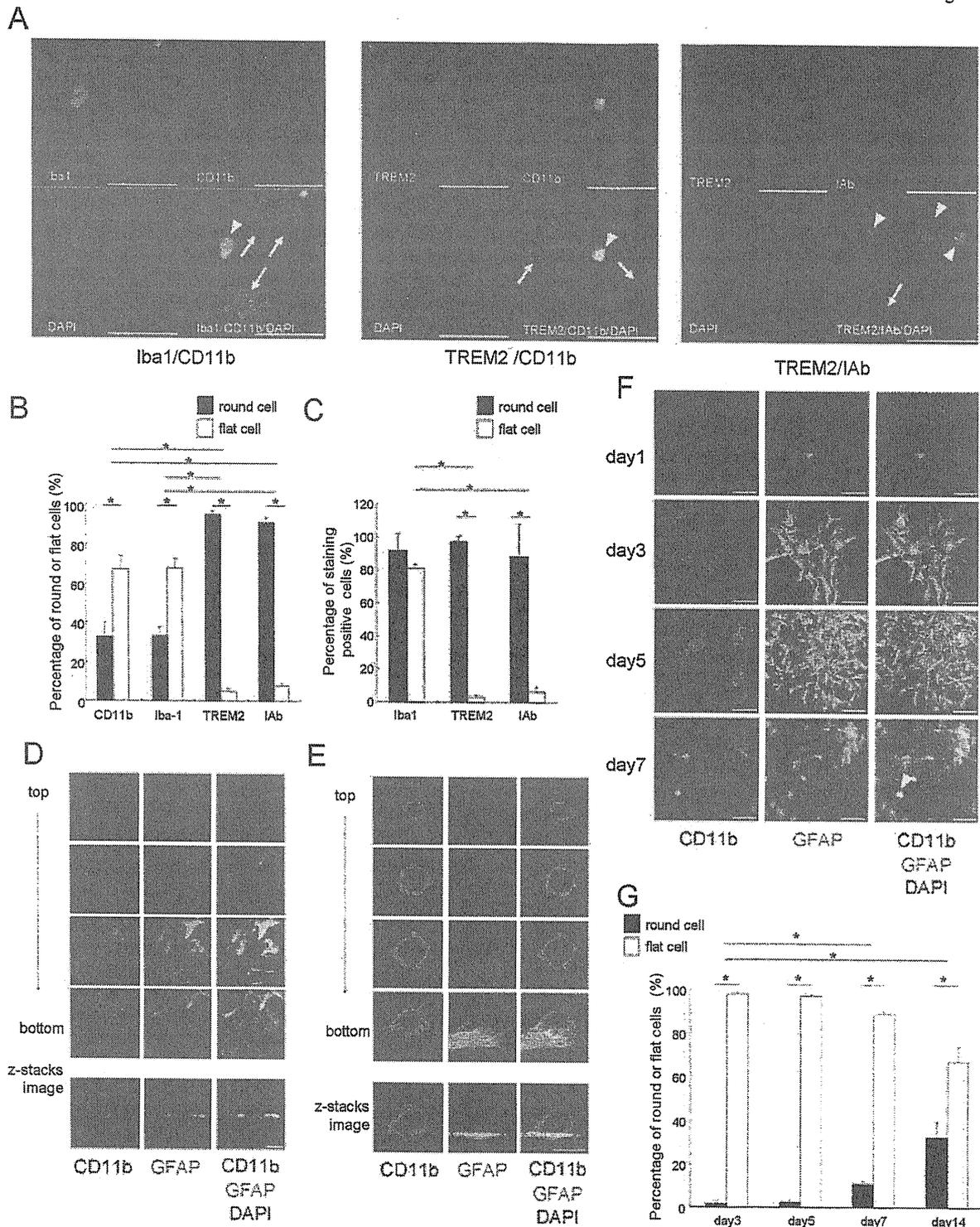


FIG. 1. Morphology of microglia in primary mixed glial culture. (A) Immunocytochemistry of primary mixed glial culture stained with anti-Iba1, anti-triggering receptor expressing on myeloid cells-2 (TREM2) anti-IAb, anti-CD11b and 4',6-diamidino-2-phenylindole (DAPI). Arrowheads indicate small round cells. Arrows indicate large flat cells. Scale bar: 50 μ m. (B) The percentage of round cells or flat cells among staining-positive cells was quantified by microscopic analysis. Data are presented as mean \pm standard deviation (SD). (C) The percentage of Iba-1-, TREM2- or IAb-positive cells among CD11b-positive (CD11b+) round or flat cells was quantified by microscopic analysis. Data are presented as mean \pm SD. (D) Z-stack immunofluorescence confocal microscopy of primary mixed glial culture stained with anti-CD11b (red), anti-glial fibrillary acidic protein (GFAP; green) and DAPI (blue). Scale bar: 10 μ m. (E) Z-stack immunofluorescence confocal microscopy of primary mixed glial culture stained with anti-CD11b (red), anti-GFAP (green) and DAPI (blue). Scale bar: 10 μ m. (F) Immunocytochemistry of primary mixed glial culture. Cultures were fixed at Days 1, 3, 5 and 7 of the culture and stained with primary anti-CD11b and anti-GFAP antibodies followed by rhodamine- or FITC-conjugated secondary antibody and DAPI. Arrowheads indicate small round cells. Scale bar: 50 μ m. (G) The percentage of CD11b+ round or flat cells was quantified by microscopic analysis. Data are presented as mean \pm SD; * P \leq 0.05. Data are representative of three independent experiments.

Stimulation by anti-TREM2 antibody

Small round cells were added to culture dishes coated with the anti-TREM2 monoclonal antibody (R&D) or control antibody, and centrifuged at 400 *g* for 5 min. After 60 min, the cells were fixed at a final concentration of 4% formaldehyde for 10 min at 37°C. Following centrifugation, the supernatant was removed, and the cells were resuspended in 90% ice-cold methanol and incubated for 30 min at 4°C. Cells were washed and stained with Alexa 647-conjugated anti-phospho-ERK1/2 antibody (Cell Signaling Technology, Tokyo, Japan) for 30 min. Analysis was performed using a FACSCalibur flow cytometer.

Bio-Plex cytokine assay system

Culture supernatant samples were analysed simultaneously for 17 different cytokines and chemokines (IL-1b, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN- γ , TNF- α , IL-15, IL-18, FGF-basic, LIF, M-CSF, MIG, MIP-2, PDGF-BB and VEGF) using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Briefly, 50 μ L of each sample and standard (Bio-Rad) was added to 50 μ L of antibody-conjugated beads (Bio-Rad) in a 96-well filter plate (Millipore). After 30-min incubation, the plate was washed, and 25 μ L of a biotinylated antibody solution (Bio-Rad) was added to each well, followed by 30-min incubation again. The plate was washed, and 50 μ L of streptavidin-conjugated PE (Bio-Rad) was added to each well and incubated for 10 min. After a final wash, the contents of each well were resuspended in 125 μ L of assay buffer (Bio-Rad) and analysed using a Bio-Plex Array Reader (Bio-Rad). The lower detection limit for each cytokine or chemokine was 2 pg/mL.

Statistical analysis

Data are presented as mean \pm SD of at least three independent experiments. Data were analysed by the Mann-Whitney *U*-test to determine significant differences.

Results

Characterization of microglia in the primary mixed glial culture

To characterize microglial cells in the mixed glial culture, we stained several microglia markers such as Iba1, CD11b, TREM2 and IAb (MHC class II molecule of C57/BL6 mice) on the cultured microglial cells (Fig. 1A). CD11b-positive (CD11b⁺) cells in the primary mixed glial culture showed two major morphological forms, namely, small round-shaped cells ($32.8 \pm 6.9\%$ SD of CD11b⁺ cells) expressing Iba1, TREM2 and IAb (Iba1⁺ cells, $91.9 \pm 10.2\%$ SD; TREM2⁺ cells, $97.5 \pm 3.5\%$ SD; IAb⁺ cells; $88.9 \pm 19.2\%$ SD), and large flat cells ($67.2 \pm 6.9\%$ SD of CD11b⁺ cells) expressing only Iba1 (Iba1⁺ cells, $81.1 \pm 2.6\%$ SD; TREM2⁺ cells, $3.3 \pm 1.1\%$ SD; IAb⁺ cells, $6.4 \pm 2.8\%$ SD; Iba1 vs. TREM2, $P = 0.0495$; Iba1 vs. IAb, $P = 0.0495$; Fig. 1B and C). Z-sectioned scans by confocal microscopy revealed that small, spherically shaped cells lay above the astrocytes, and large flat cells lay under the astrocytes (Fig. 1D). Moreover, the spherical cells appeared to have extended processes toward the astrocytes (Fig. 1E).

To investigate the time point at which both the forms of the microglial cells appeared in the primary mixed glial culture, we performed a kinetic study. At Day 1 after the culture of mixed glial cells, CD11b⁺ cells showed a small amoeboid shape (Fig. 1F). At Day

3, the CD11b⁺ cells assumed a larger flat shape. Small round CD11b⁺ cells were very few in number until Day 5 (Day 3, $1.9 \pm 1.1\%$ SD; Day 5, $2.7 \pm 0.9\%$ SD), but increased greatly after Day 7 (Day 7, $10.9 \pm 1.1\%$ SD vs. Day 3, $P = 0.0495$; Day 14, $32.8 \pm 6.9\%$ SD vs. Day 3, $P = 0.0495$; Fig. 1F and G).

Characteristics of LN cells

LN cells were isolated by negative selection using magnet beads. The purity of LN cells after negative selection by flow cytometry was consistently above 90% on several examinations (Fig. 2A). LN cells expressed neither the microglia marker TREM2 nor CD11b, which is one of the lineage markers for negative selection (Fig. 2B). No expression of CCR2 or Gr-1 (lineage markers for negative selection) was detected on the LN cells, which have previously been described as

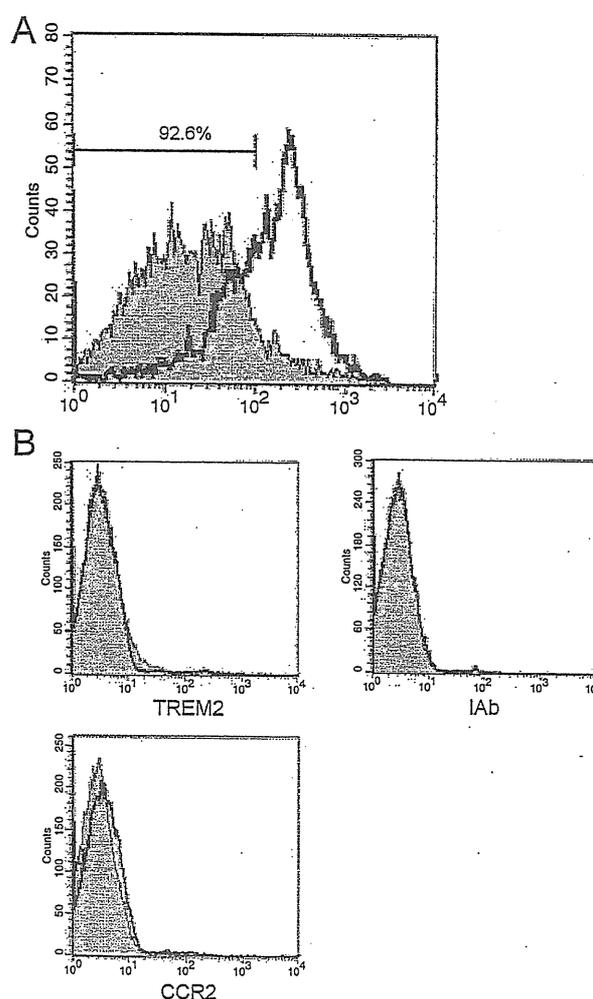


Fig. 2. Flow cytometry analysis of LN cells. (A) Flow cytometry analysis of LN cells (filled histogram) and bone marrow cells (open histogram) stained with rat monoclonal antibodies against mice lineage markers (CD3, CD4, CD5, CD8 α , CD11b/MAC-1 α , B220, Gr-1 and TER-119). Numbers above the lines indicate the percentage of LN cells. (B) Flow cytometry analysis of LN cells. Filled histograms, staining with antibodies to markers below plots; open histograms, isotype-matched control antibody. Data are representative of three independent experiments. TREM2, triggering receptor expressing on myeloid cells-2.

markers of microglial precursors (Fig. 2B). IAb, which is an MHC class II antigen, was negative on the surface of the LN cells.

Differentiation of LN cells into microglia-like cells

LN cells obtained from GFP mice were co-cultured with primary mixed glial cells for 2 weeks. Two weeks after the co-culture, the cells were stained by anti-TREM2, anti-Iba1, anti-MHC class II and anti-CD11b antibodies, followed by rhodamine- or Cy3-conjugated secondary antibodies (Fig. 3A). GFP+ cells showed two major morphologies, i.e. small and round cells with bright nuclei

($27.7 \pm 7.9\%$ SD in GFP+ cells) and flat cells with dark nuclei ($69.9 \pm 6.9\%$ SD in GFP+ cells), similar to the microglia in the primary mixed glial cells seen in Fig. 1. Most of the small round cells were TREM2-, Iba1-, CD11b- and IAb-positive (TREM2+ cells, $92.7 \pm 1.9\%$ SD; Iba1+ cells, $93.6 \pm 1.6\%$ SD; CD11b+ cells, $96.5 \pm 3.1\%$ SD; IAb+ cells, $84.6 \pm 13.4\%$ SD; Fig. 3B), and were spherically shaped along the z-axis (Fig. 3C). On the other hand, flat cells expressed none of the following: CD11b, Iba1, TREM2 or IAb (TREM2+ cells, $6.5 \pm 3.9\%$ SD; Iba1+ cells, $3.5 \pm 1.0\%$ SD; CD11b+ cells, $9.3 \pm 3.7\%$ SD; IAb+ cells, $3.9 \pm 1.9\%$ SD). Because TREM2/DAP12 signaling is known to induce extracellular signaling-regulated kinases (ERK) phosphorylation in immature dendritic

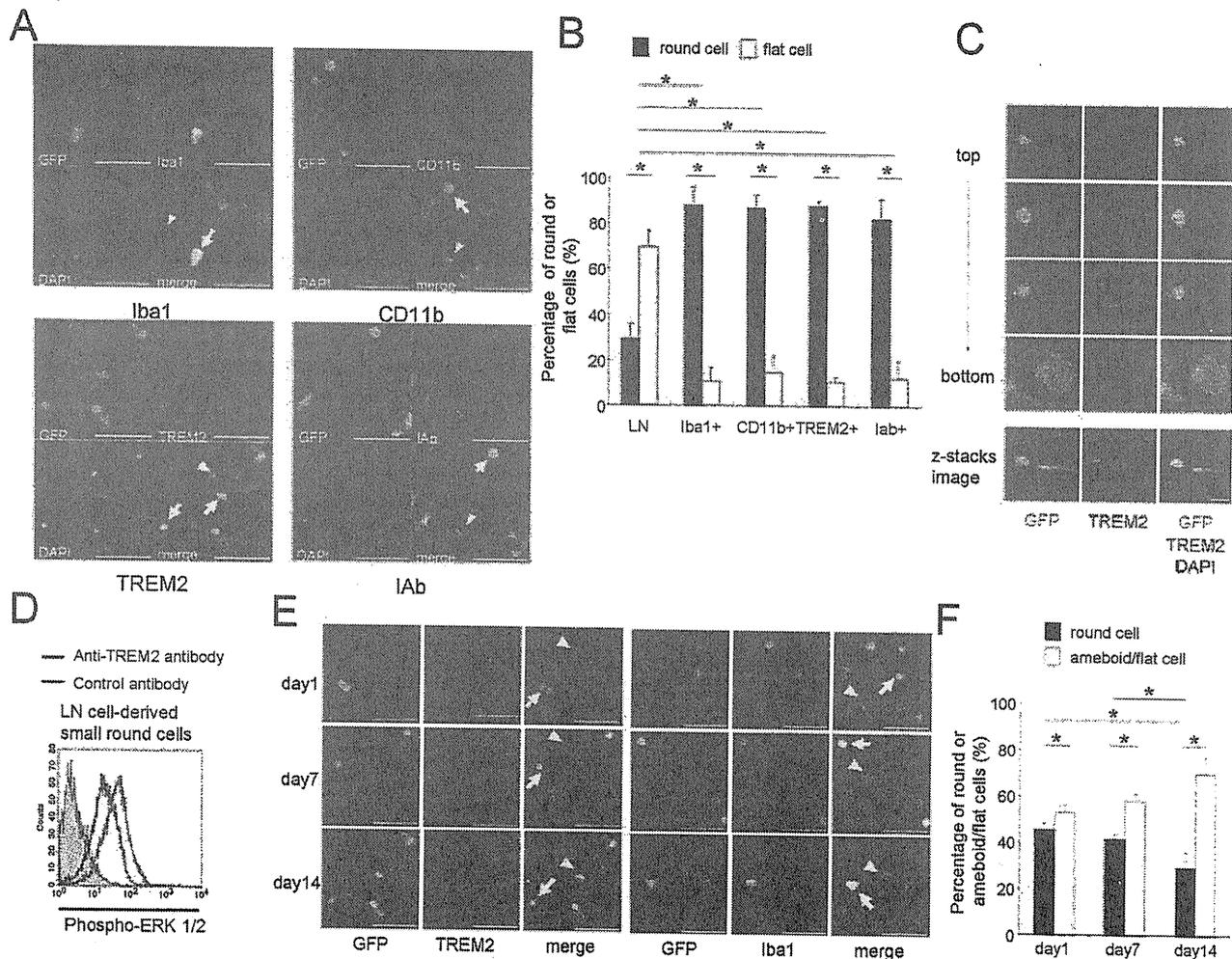


FIG. 3. Lineage-negative bone marrow (LN) cells co-cultured with primary mixed glial culture. (A) Immunocytochemistry of LN cells derived from green fluorescence protein (GFP) mice co-cultured with primary mixed glial culture. Cultures were stained with primary anti-Iba1, anti-triggering receptor expressing on myeloid cells-2 (TREM2), anti-IAb and anti-CD11b antibodies followed by rhodamine- or Cy3-conjugated secondary antibody and 4',6-diamidino-2-phenylindole (DAPI). Arrows indicate double-positive cells. White arrowheads indicate GFP-negative rhodamine- or Cy3-positive cells. Red arrowheads indicate GFP-positive (GFP+) rhodamine- or Cy3-negative cells. Scale bar: $50 \mu\text{m}$. (B) Percentage of round or flat cells among GFP+ cells or double-positive cells. Data are presented as mean \pm SD. (C) Z-stack immunofluorescence confocal microscopy of LN cells derived from GFP mice co-cultured with primary mixed glial culture stained with anti-TREM2 (red) and DAPI (blue). GFP and TREM2 double-positive cells show a spherical shape. Scale bar: $10 \mu\text{m}$. (D) Phosphorylation of ERK after cross-linking stimulation of LN cell-derived small round cells by flow cytometry. Red line histograms, stimulated with anti-TREM2 antibody; blue line histograms, stimulated with control antibody; filled histograms, stained with isotype-matched control antibody. Data are representative of three independent experiments. (E) Immunocytochemistry of LN cells derived from GFP mice co-cultured with primary mixed glial culture. Cells were fixed at Days 1, 7 and 14 after co-culture and stained with primary anti-TREM2 or anti-Iba1 antibodies followed by rhodamine-conjugated secondary antibody and DAPI. Arrows indicate GFP and TREM2/Iba1 double-positive cells. Arrowheads indicate GFP+ and rhodamine-negative cells. Scale bar: $50 \mu\text{m}$. (F) The percentage of round or amoeboid/flat cells of GFP+ cells showed no changes at Days 1 and 7, but the number of small round cells reduced at Day 14. Data are presented as mean \pm SD; * $P \leq 0.05$. Data are representative of three independent experiments.

cells, we analysed the phosphorylation of ERK using flow cytometry after cross-linking stimulation of LN cell-derived small round cells in order to determine whether the TREM2 on small round cells was functional. Stimulation of TREM2 of the LN cell-derived small round cells induced phosphorylation of ERK as demonstrated by a specific antibody recognizing the phosphorylated form of ERK (Fig. 3D).

In the kinetic study, GFP+ LN cells were immunostained Days 1, 7 and 14 after co-culturing with primary mixed glial cells with anti-TREM2 and anti-Iba1 antibodies followed by secondary antibodies. At Day 1, LN cells had already differentiated into two morphological groups – small round cells expressing Iba1 and TREM2, and flat amoeboid cells (Fig. 3E). Interestingly, the ratio of LN cell-derived small round and flat cells remained identical at Days 1 and 7, but reduced at Day 14 (Day 1, $45.7 \pm 2.5\%$ SD; Day 7, $41.7 \pm 1.9\%$ SD vs. Day 1, $P = 0.0495$; Day 14, $29.5 \pm 6.4\%$ SD vs. Day 1, $P = 0.0495$; Fig. 3F).

Analysis of surface cell markers and proliferative capacity of LN cell-derived small round cells

In many publications, not only the expression of CD11b, Iba1 and F4/80, but also low expressions of CD45 and MHC class II have been used as microglial markers. Among them, low expressions of CD45 and MHC class II are one of the most important resting microglial markers. We measured the quantitative expression of CD45 and MHC class II on brain microglia, cultured microglia and LN cell-derived small round cells. As shown in Fig. 4A, LN cell-derived small round cells showed low expressions of CD45 and MHC class II, which was

identical to results for cultured and brain microglia as compared with those for spleen-derived macrophages. LN cell-derived small round cells were also F4/80 positive, which is known to be another microglial marker.

Because microglia can continue to proliferate and differentiate to macrophages, we investigated the proliferative capacity of brain microglia and LN cell-derived small round cells with various concentrations of M-CSF, and compared this capacity to that of splenic macrophages. LN cell-derived small round cells showed increased incorporation of [3 H] thymidine similar to that by brain microglia, but [3 H] thymidine incorporation by splenic macrophages did not increase in a low concentration of M-CSF (1 ng/mL; LN cell-derived small round cells vs. splenic macrophages, $P = 0.0495$; brain microglia vs. splenic macrophages, $P = 0.0495$; Fig. 4B).

These results indicate that small round cells and microglia are similar in terms of their cell surface molecules and the proliferative capacity.

Proliferation and differentiation of LN cells in the presence of M-CSF

To examine the humoral factors necessary for the differentiation of LN cells into microglia, we cultured LN cells with the supernatant of the primary mixed glial culture for 7 days. Most of the LN cells cultured with this supernatant (LN-Sup cells) were flat in shape and expressed CD11b, Iba1, TREM2 and IAb (Fig. 5A).

Next, we measured the concentrations of cytokines in the supernatant of the primary mixed glial culture using the Bio-Plex Cytokine Assay System. Among the assayed cytokines, the concen-

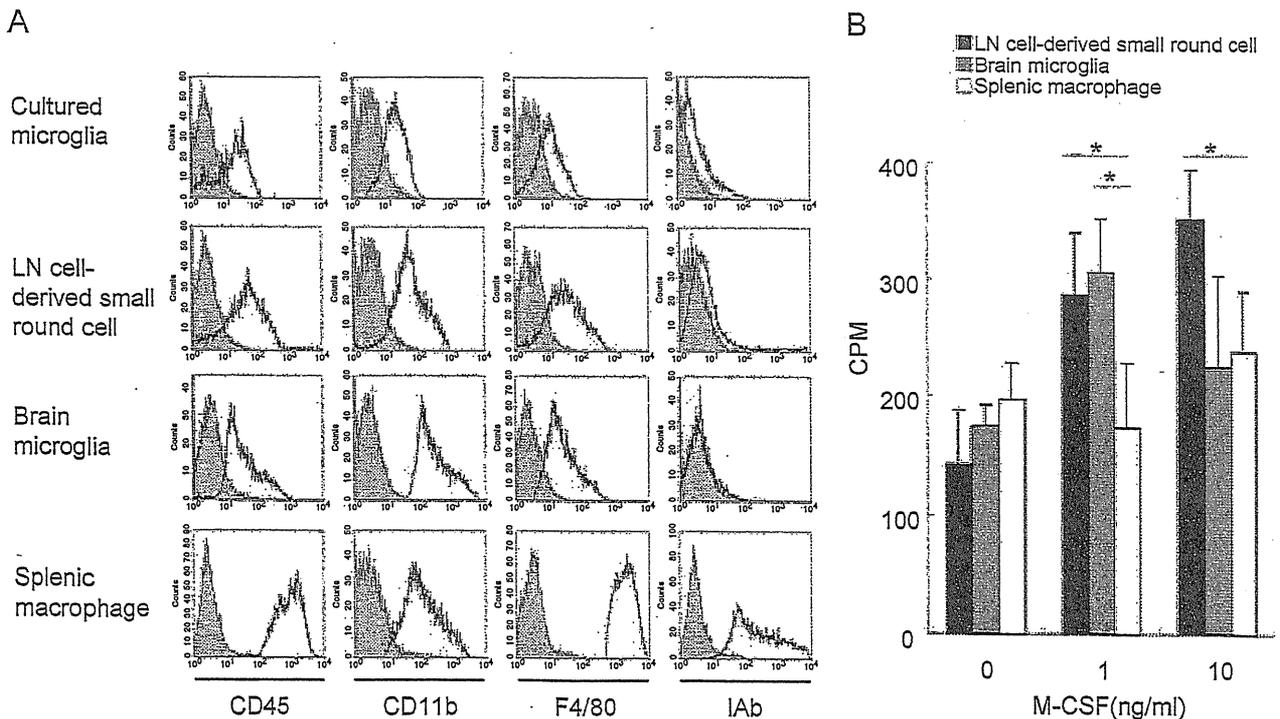


FIG. 4. Surface cell markers and proliferative capacity of lineage-negative bone marrow (LN) cell-derived small round cells. (A) Flow cytometry analysis of cultured microglia, LN cell-derived small round cells, brain microglia and splenic macrophages. Open histograms, staining with antibodies to markers below plots; filled histograms, isotype-matched control antibody. Data are representative of three independent experiments. (B) Proliferative capacity of cultured microglia, LN cell-derived small round cells, brain microglia and splenic macrophages. Proliferation was measured by thymidine incorporation. Data are presented as mean \pm SD; $*P < 0.05$. Data are representative of three independent experiments. M-CSF, macrophage colony stimulating factor.

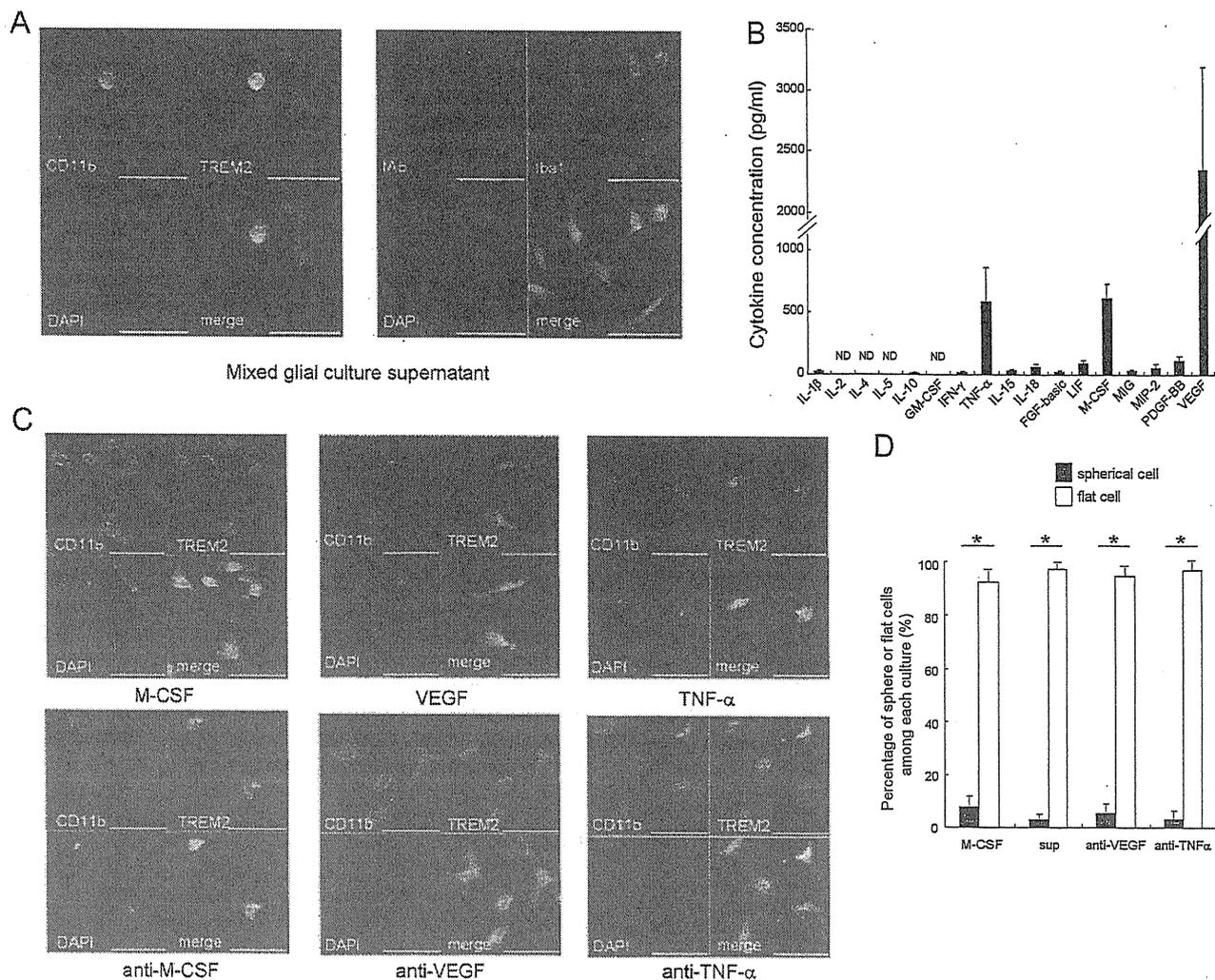


FIG. 5. Proliferation and expression of triggering receptor expressing on myeloid cells-2 (TREM2) on LN cells in the presence of macrophage colony stimulating factor (M-CSF). (A) Immunocytochemistry of LN cells cultured with supernatant of primary mixed glial culture. Cultures were stained with anti-Iba1, anti-TREM2, anti-IAb, anti-CD11b and 4',6-diamidino-2-phenylindole (DAPI). Scale bar: 50 μ m. (B) Analysis of concentration of cytokines [IL-1 β , IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN- γ , tumor necrosis factor- α (TNF- α), IL-15, IL-18, FGF-basic, LIF, M-CSF, MIG, MIP-2, PDGF-BB and vascular endothelial growth factor (VEGF)] in the supernatant of primary mixed glial culture by using the Bio-Plex Cytokine Assay System. ND; not detectable. Data are presented as mean \pm SD of 12 independent experiments. (C) Immunocytochemistry of LN cells cultured with M-CSF-, VEGF- or TNF- α -containing medium, and anti-MCSF, anti-VEGF or anti TNF- α antibody-containing supernatant of mixed glial culture. The cells were stained with anti-TREM2 antibodies, CD11b antibodies and DAPI. LN cells cultured with M-CSF showed no change in their morphology or surface markers as compared with LN cells cultured with the supernatant of primary mixed glial culture; however, the addition of anti-M-CSF antibodies remarkably reduced the cell number. Scale bar: 50 μ m. (D) The percentage of spherical or flat cells was quantified by confocal microscopy. Data are presented as mean \pm SD; * P \leq 0.05. Data are representative of three independent experiments.

trations of M-CSF, VEGF and TNF- α were remarkably high (614.2 ± 121.5 SD pg/mL of M-CSF, 2349.4 ± 845.9 SD pg/mL of VEGF and 585.5 ± 278.2 SD pg/mL of TNF- α) compared with those of the other cytokines (Fig. 5B). IL-2, IL-4, IL-5 and GM-CSF were not detected, and the concentrations of IL-1 β , IL-10, IFN- γ , IL-15, FGF-basic and MIG were very low (24.8 ± 10.7 SD pg/mL of IL-1 β , 12.4 ± 6.9 SD pg/mL of IL-10, 18.0 ± 13.0 SD pg/mL of IFN- γ , 34.4 ± 12.5 SD pg/mL of IL-15 and 21.6 ± 11.9 SD pg/mL of FGF-basic). The concentrations of IL-18, LIF, MIP-2 and PDGF-BB were at an intermediate level (61.9 ± 23.3 SD pg/mL of IL-18, 92.5 ± 23.8 SD pg/mL of LIF, 34.7 ± 11.6 SD pg/mL of MIG, 57.0 ± 31.6 SD pg/mL of MIP-2 and 115.7 ± 36.1 SD pg/mL of PDGF-BB).

To investigate the effect of each cytokine on the differentiation of LN cells, we cultured LN cells with M-CSF, VEGF or TNF- α for 7 days. All the LN cells cultured with M-CSF (LN-MCSF cells) were CD11b- and TREM2-positive (Fig. 5C). Morphologically, most LN-MCSF cells showed flat shapes on confocal microscopy (Fig. 5D). The number of LN cells cultured with TNF- α (LN-TNF cells) or with VEGF (LN-VEGF cells) were very few, although these cells expressed CD11b and TREM2 (Fig. 5C).

Next, LN cells were cultured with the mixed glial culture supernatant containing neutralizing antibodies of M-CSF, TNF- α or VEGF. Addition of the anti-VEGF antibody or anti-TNF- α antibody resulted in no change in the morphology or surface markers as compared with the culture containing the control antibodies; however,

the addition of anti-M-CSF antibodies remarkably reduced the cell number despite the expression of TREM2 (Fig. 5C).

Cell-to-cell contact between microglia and astrocytes

In order to reveal the role of cell-to-cell contact between microglia and other glial cells, especially astrocytes, in the differentiation of LN cells to microglia, we performed immunostaining of the mixed glial culture with LN cells by anti-GFAP antibody followed by secondary rhodamine-conjugated antibody against rabbit-IgG. Analysis by confocal microscopy revealed that LN cell-derived small round cells were positioned above the GFAP-positive astrocytes (Fig. 6). On the other hand, large flat cells lay immediately beneath the GFAP-positive astrocytes.

Discussion

Microglia, the immune cells of the CNS, exist in three distinct forms – amoeboid, ramified and reactive microglia. Ramified microglia are present in the brain parenchyma and constitute approximately 10–20% of the total population of glial cells in an adult (Vaughan & Peters,

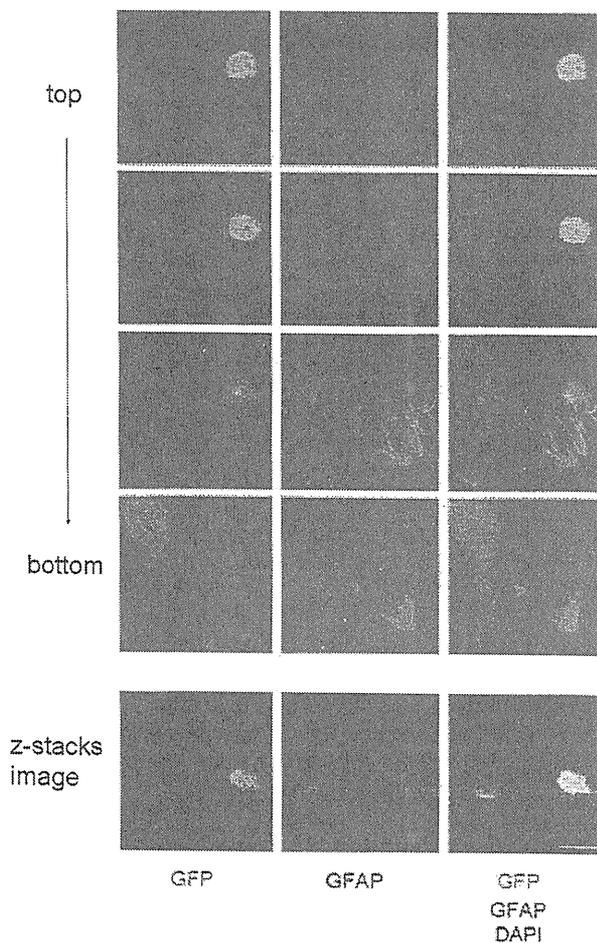


FIG. 6. Z-stack immunofluorescence confocal microscopy of LN cells derived from green fluorescence protein (GFP) mice co-cultured with primary mixed glial culture stained with anti-glial fibrillary acidic protein (GFAP; red) and 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bar: 10 μ m.

1974; Banati, 2003). Ramified microglia are small round cells comprising branching processes and are considered to be functionally inactive. Further, they are known to express TREM2 *in vivo* (Schmid *et al.*, 2002; Sessa *et al.*, 2004), but not MHC class II (Santambrogio *et al.*, 2001; Servet-Delprat *et al.*, 2002). Recently, HSP60 has been identified as the ligand of TREM2, and its interaction with microglia has been demonstrated to stimulate microglial phagocytosis (Stefano *et al.*, 2009). This result indicates that TREM2 might play an important role in physiological phagocytosis as one of the microglia-specific functions. TREM2 expression on small round cells in primary mixed glial culture might indicate that small round cells have greater mobility as microglia than do large flat cells in patrolling the brain environment in order to identify HSP60-expressing cells. Moreover, as shown in Fig. 4A, small round cells weakly expressed MHC class II molecules similar to brain microglia. These results support our hypothesis that small round microglia *in vitro* differentiated to a greater extent than did large flat cells. Consistent with the results of the current study, previous studies have reported two different morphological shapes of microglia in primary mixed glial culture cells derived from mice (Saura *et al.*, 2003) and rats (Tanaka *et al.*, 1999; Kuwabara *et al.*, 2003). In another study, the kinetic analysis of rat primary mixed glial culture demonstrated that 'amoeboid' or 'round' cells appeared during the early stages of the culture, and that the majority of ramified microglia were formed after the complete formation of the astrocyte monolayer (Tanaka *et al.*, 1999). We demonstrated that LN cells directly differentiated into small round microglia-like cells without the intermediary formation of large flat precursors. Although Ly-6C^{high} Gr-1+CCR2+ monocytes in the peripheral blood have been reported to be the precursors of adult murine microglia (Mildner *et al.*, 2007; Getts *et al.*, 2008), the LN cells in our study did not express CCR2 or Gr-1 (the anti-Gr-1 antibodies used in this study also reacted with Ly-6C). The role of CCR2, a crucial chemokine receptor for the chemotactic attraction of monocytes or macrophages during CNS inflammation, has been investigated in animal models of multiple sclerosis, such as experimental autoimmune encephalomyelitis (Fife *et al.*, 2000; Izikson *et al.*, 2000). However, the physiological development and functions of microglia are independent of CCR2 expression (Mildner *et al.*, 2007). Because the absence of CCR2 expression is highly related to the Ly-6C^{high} subpopulation (Mildner *et al.*, 2007), Ly-6C may play a more important role than CCR2 in microglial differentiation. It is unclear why the LN cells differentiated into small round microglia-like cells despite the lack of Ly-6C and Gr-1 expression, but we hypothesize that the blood–brain barrier plays a key role in the differentiation of LN cells. Indeed, Ly-6C is known to regulate endothelial adhesion and the homing of CD8+T cells by activating integrin-dependent adhesion pathways (Hänninen *et al.*, 1997).

On the other hand, the LN cells in the current study did not differentiate into flat cells as the precursors of small round microglia. Since they were first described by del Rio-Hortega (1932), the developmental origin of microglia has not been completely elucidated. It is widely hypothesized that microglia are derived from myeloid lineage precursors and/or hematopoietic precursors during CNS development. The fact that the formation of microglia occurs before the onset of vascularization in the developing brain (Wang *et al.*, 1996) suggests that hematopoietic stem cells, which act as precursors to microglia during development, may be more primitive than precursor LN cells. Therefore, it is likely that the LN cells adopt a different pathway to differentiate into small round microglia-like cells.

The supernatant of the primary mixed glial cultures derived from mice contains many cytokines, such as M-CSF, VEGF and TNF- α . Our study showed that M-CSF plays an important role in the

differentiation and proliferation of LN cells. TREM2 is a unique molecule that is only expressed on microglia, osteoclasts and immature dendritic cells (Colonna, 2003), while other microglia-specific markers such as Iba1 and CD11b are also expressed on macrophages and monocytes. Because TREM2 was not expressed on the mature dendritic cells or the activated microglia (macrophages), it is likely that TREM2 expression may be restricted to the immature stages of myeloid lineage cells. In our study, M-CSF induced the proliferation of LN cells, and the expression of not only CD11b and Iba1 but also TREM2 on LN cells; this indicates that M-CSF determines the differentiation of LN cells into the myeloid lineage, but not into fully differentiated cells. Although the addition of anti-M-CSF antibodies appears not to suppress the expression of TREM2 on the LN cells, these TREM2+ cells may have originated from more mature precursors that are independent of M-CSF. These immature LN cells would eventually be fully differentiated under physiological conditions. When LN cells were cultured with M-CSF, the number of small round cells in the culture was considerably low, indicating that the small round morphology of the immature cells is not maintained in the presence of only M-CSF. On the other hand, when LN cells were grown in the presence of other glial cells (especially astrocytes), the small round cells were maintained at a concentration of approximately 30% of the total population. Therefore, cell-cell contact with glial cells, especially astrocytes, may be necessary to maintain the small round shape of immature cells expressing TREM2.

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Abbreviations

ERK, extracellular signaling-regulated kinases; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; GFP, green fluorescence protein; LN cells, lineage-negative bone marrow cells; M-CSF, macrophage colony stimulating factor; MHC, major histocompatibility complex; PE, phycoerythrin; TNF- α , tumor necrosis factor- α ; TREM2, triggering receptor expressed on myeloid cells-2; VEGF, vascular endothelial growth factor.

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

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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—Male β_2 -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 μ g/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 obesity. (*Arterioscler Thromb Vasc Biol.* 2010;30:193-199.)

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

Obesity, specifically visceral obesity, increases the risk for metabolic disorders, such as type 2 diabetes mellitus, 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 that adipose tissue inflammation plays an important role in the pathogenesis of these metabolic disorders.^{1,2} Macrophages are attracted by chemokines, such as 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 The 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 al⁴ and Rocha et al⁵ demonstrated that CD3-positive T lymphocytes are present in human adipose tissue; and regulated upon activation, normal T cell expressed secreted (RANTES), a T-cell-specific chemokine, and its respective receptor CCR5 are expressed in adipose tissue from obese patients. However, the role of other types of lymphocytes in adipose tissue inflammation is largely unexplored.

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Natural killer T (NKT) cells are innatelike T lymphocytes that recognize glycolipid antigens and are capable of rapidly producing 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

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immune systems. Caspar-Bauguil et al⁷ have reported the presence of significant levels of NKT cells in the stroma-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 atherosclerosis.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 T_H1 cytokines, such as IFN- γ , 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 atherosclerosis. 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 β_2 microglobulin knockout (KO) mice lacking NKT cells. Moreover, we further examined the effects of NKT cell activation by α -galactosylceramide (α 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, were fed with a standard diet (SD) (WT-SD, n=10; and KO-SD, n=5) or an 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, 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 α GC, 0.1 μ g/g body weight (Kirin Brewery Company, Ltd, Tokyo, Japan) (WT- α GC, n=5; and KO- α 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 α GC, 0.1 μ 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.

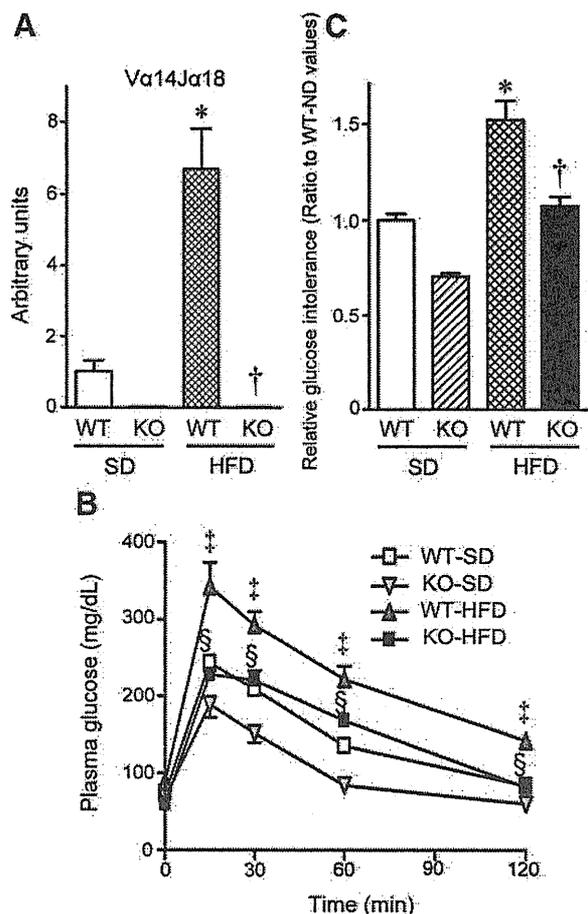


Figure 1. A, V α 14/J α 18 gene expression, an index of natural killer T (NKT) cells, of visceral adipose tissues in experiment 1. B, Plasma glucose concentrations. C, Area under the curve (AUC) values during the intraperitoneal glucose tolerance test (ipGTT). * P <0.01 vs wild type (WT)-standard diet (SD), † P <0.01 vs WT-high-fat diet (HFD), ‡ P <0.01 vs WT-SD at each time, and § P <0.01 vs WT-HFD at each time.

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 α 14/J α 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 homeostasis model assessment of insulin resistance (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 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

Table. Animal Characteristics in Experiment 1

Characteristic	Animal Group			
	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 results				
Fasting plasma glucose level, mg/dL	78 (8)	50 (8)†	82 (8)	55 (4)‡
Insulin level, ng/mL	0.49 (0.09)	0.27 (0.06)	0.77 (0.22)	0.90 (0.13)
HOMA-IR level	2.10 (0.22)	0.79 (0.21)*	3.29 (0.66)	3.23 (0.68)
Total cholesterol level, mg/dL	102 (2)	103 (3)	181 (10)*	193 (6)*
Leptin level, ng/mL	2.2 (0.5)	2.4 (0.9)	12.9 (2.5)*	16.0 (4.0)*
TNF- α level, pg/mL	37 (5)	13 (4)*	85 (40)	29 (6)§
Adiponectin level, μ g/mL	22 (2)	30 (5)	19 (1)	48 (3)‡
Glucagon level, 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, μ m ²	1697 (156)	1492 (162)	2787 (324)*	2921 (308)*

Abbreviations: HFD, high-fat diet; KO, β_2 -microglobulin knockout mice; SD, standard diet; TNF, tumor necrosis factor; WT, wild type (C57BL/6J) mice.

* $P < 0.01$ vs WT-SD.

† $P < 0.05$ vs WT-SD.

‡ $P < 0.01$ vs WT-HFD.

§ $P < 0.05$ vs WT-HFD.

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, but significantly increased in KO-HFD. The plasma glucagon level tended to be lower in KO-SD compared with WT-SD, which did not reach statistical significance (Table). An HFD significantly increased the weight of visceral adipose tissue compared with groups fed with SD. An HFD significantly increased the weight of visceral adipose tissue compared with the weight of 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 2A and B). Major histocompatibility complex (MHC) class II, CD11c, and arginase gene expression, measured by using real-time reverse transcriptase–polymerase chain reaction (a quantitative index of macrophage activation, M1 macrophage, and M2 macrophage, respectively), demonstrated that infiltrating macrophages possess a predominantly M1 phenotype in WT-HFD mice and an M2 phenotype in KO-HFD mice (Figure 2C–E). Taken together, these data indicated that M1 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 V α 14/J α 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 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 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).

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

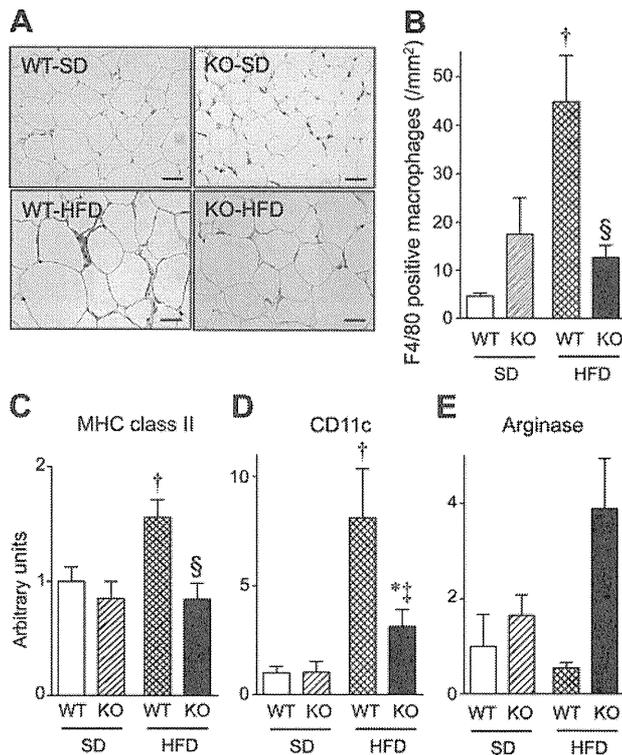


Figure 2. Macrophage infiltration in adipose tissue in experiment 1. A, F4/80 immunohistochemistry. Scale bar, 20 μ m. B, The number of F4/80-positive macrophages. C–E, Gene expression of major histocompatibility complex (MHC) class II, CD11c, and arginase, respectively. * $P < 0.05$ and † $P < 0.01$ vs wild type (WT)–standard diet (SD), and ‡ $P < 0.05$ and § $P < 0.01$ vs WT–high-fat diet (HFD).

adipose tissue inflammation, α GC was injected intraperitoneally in WT mice fed HFD for 13 weeks.

α 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 *V α 14/J α 18* gene expression confirmed α GC significantly enhanced NKT cell infiltration into adipose tissue (Figure 3A). Plasma glucose levels during ipGTT were significantly increased by α GC (15 minutes: 330 [11] vs 296 [11] mg/dL [$P < .05$]; and 30 minutes: 326 [9] vs 295 [8] mg/dL [$P < .05$]) (Figure 3B).

F4/80-positive macrophage infiltration was significantly increased in the adipose tissues for WT mice by α 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 α 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 α 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 α GC administration in WT mice (supplemental Figure 3).

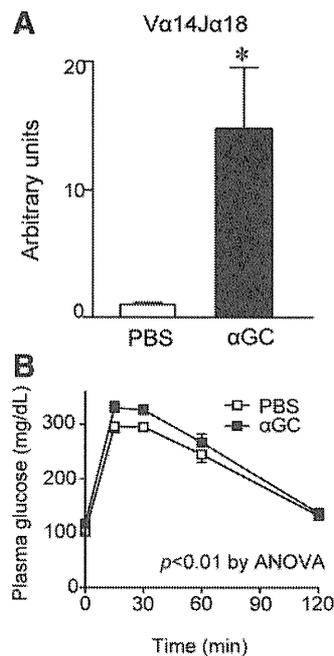


Figure 3. A, *V α 14/J α 18* gene expression, an index of natural killer T (NKT) cells, of visceral adipose tissues 8 days after the injection of phosphate-buffered saline (PBS) or α -galactosylceramide (α GC), a specific activator for NKT cells, in experiment 2. B, Plasma glucose concentrations during the intraperitoneal glucose tolerance test (ipGTT) 8 days after PBS or α GC injection. * $P < 0.05$ vs PBS.

To examine the effects of α GC treatment on the metabolic phenotypes of genetically induced obese mice, α GC was injected intraperitoneally in ob/ob mice. Natural killer T cell and macrophage infiltration were significantly increased in α 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 α GC-treated ob/ob mice (supplemental Figure 4C–E). Similar to diet-induced obese mice, the injection of α 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 α GC-treated ob/ob mice were comparable to those in PBS-treated ob/ob mice (supplemental Figure 5).

To confirm the specificity of α GC treatment for activating NKT cells, α 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 α GC for 48 hours. Macrophages conditioned with activated NKT cells by α 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

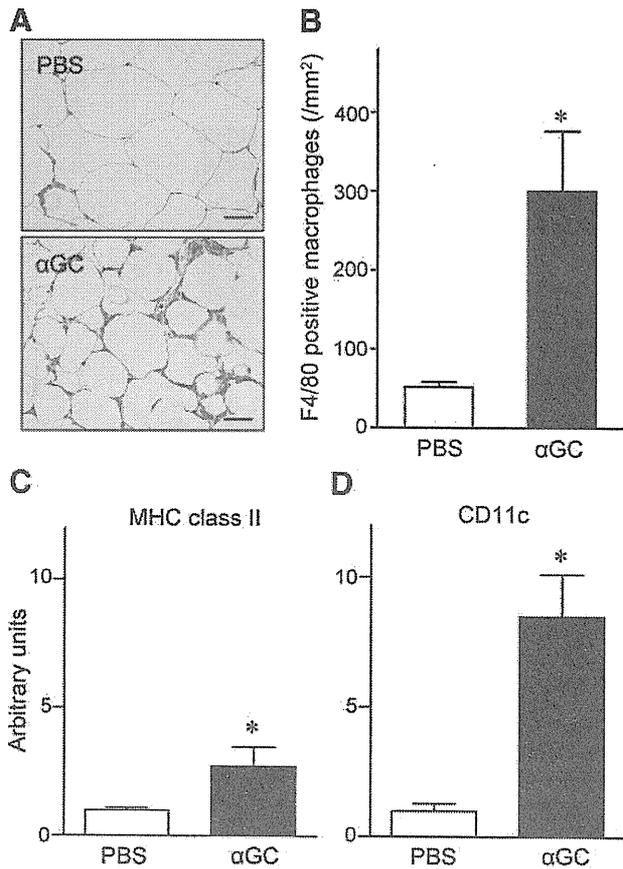


Figure 4. Macrophage infiltration in adipose tissue in experiment 2. A, Demonstrable figures of F4/80 immunohistochemistry. Scale bar, 20 μ m. B, The number of F4/80-positive nuclei from mice given phosphate-buffered saline (PBS) and α -galactosylceramide (α GC). C and D, Expression of major histocompatibility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

macrophages during the development of glucose intolerance in a mouse model of HFD-induced obesity. The depletion of NKT cells in β_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 α 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.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.

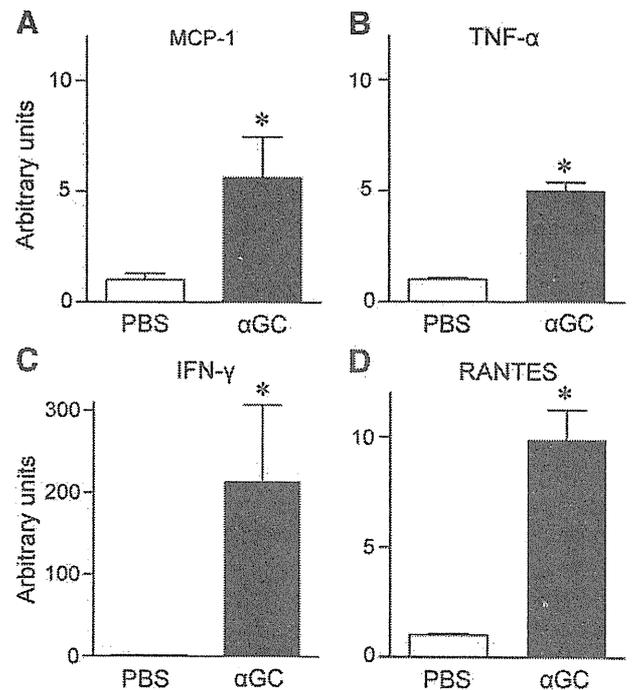


Figure 5. A–D, Expression of monocyte chemoattractant protein (MCP) 1, tumor necrosis factor (TNF) α , interferon (IFN) γ , and regulated upon activation normal T cell expressed secretion (RANTES) genes, respectively, in visceral adipose tissues from mice given phosphate-buffered saline (PBS) and α -galactosylceramide (α GC) in experiment 2. Quantitative reverse transcription (RT)–polymerase chain reaction (PCR) was performed 9 days after PBS or α GC injection. * $P < 0.05$ vs PBS.

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 T_H2 bias, whereas α GC injection stimulated NKT cells toward the T_H1 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 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 α GC 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.⁴ A recent elegant study by Nishimura et al¹⁴ 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 β_2 microglobulin KO mice used in the present study lack not only NKT cells but also CD8⁺ T lymphocytes.¹⁵ 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 α GC, 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.¹⁶ The NKT cells mediate various functions rapidly by 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 α GC-treated mice were classically activated (M1) macrophages, one of the distinct subsets of macrophages categorized as M1 by CD11c (Figure 4).^{17,18} 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 periadipocytes, 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 α GC-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 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 α GC, 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 resis-

tance.²³ In agreement with our results, Rocha et al⁵ 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. 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 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 Supplemental Table); however, adipocyte cell size has been shown to be an independent predictor of glucose intolerance.²⁵

Activated macrophages secrete TNF- α , which can inhibit 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 (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, α GC 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 used α GC 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.¹ 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.²⁹ 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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Disclosures

None.

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Correction

In the article “Natural Killer T Cells Are Involved in Adipose Tissues Inflammation and Glucose Intolerance in Diet-Induced Obese Mice” by Ohmura et al, which appeared in the February 2010 issue of the journal (*Arterioscler Thromb Vasc Biol.* 2010;30:193–199; DOI: 10.1161/ATVBAHA.109.198614), the publisher omitted several important corrections from the final, published version:

1. Page 193, Affiliations, lines 1 and 3: The Department of Cardiovascular Medicine, Hokkaido University Graduate School of Medicine and the Division of Immunobiology, Institute for Genetic Medicine, Hokkaido University are located in Sapporo, Japan.
2. Page 193, abstract, line 1 of Methods and Results: “To determine whether NKT cells are involved in the development of glucose intolerance” should have been removed and the section should have started with “Male β_2 -microglobulin knockout (KO) mice lacking NKT cells. . .”

The online version of the article has been corrected.

The publisher sincerely regrets the errors.

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Dendritic cell differentiation with prostaglandin E₂ results in selective attenuation of the extracellular signal-related kinase pathway and decreased interleukin-23 production

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Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells and have a unique ability to activate naïve T cells.^{1–3} Thus, DCs are primarily responsible for the

Summary

The balance between interleukin (IL)-12 and IL-23 production by dendritic cells (DCs) is crucial for the induction of appropriate immune responses. In the present study, we examined the effect of prostaglandin E₂ (PGE₂) treatment of DCs during differentiation on IL-12 and IL-23 production in response to Toll-like receptor (TLR) stimulation. Bone marrow-derived DCs were generated by culturing murine bone marrow cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) alone (cont-DCs) or GM-CSF plus PGE₂ (PG-DCs). Upon TLR stimulation, IL-23 production by PG-DCs was markedly decreased compared with that by cont-DCs. However, no significant difference was detected in IL-12 production between these types of DC. To examine the mechanism underlying the impaired production of IL-23 by PG-DCs, we analysed the activities of extracellular signal-related kinases (ERKs) 1/2, p38 mitogen-activated protein kinase (MAPK), c-jun N-terminal kinases 1/2, Akt, and nuclear factor (NF)- κ B (p65) in these DCs upon TLR stimulation. The ERK1/2 activity in PG-DCs was significantly lower than that of cont-DCs. No significant differences were detected in the activities of other molecules between cont-DCs and PG-DCs. In addition, treatment of cont-DCs with U0126, a specific inhibitor of the ERK pathway, reduced the TLR-mediated production by the DCs of IL-23 but not IL-12. Thus, DC development in the presence of PGE₂ results in selective attenuation of the ERK pathway. The attenuation of ERK activation appears to be responsible for the decreased IL-23 production by PG-DCs.

Keywords: dendritic cells; extracellular signal-related kinases 1/2; interleukin-23; prostaglandin E₂

initiation and regulation of immune responses to various antigens. Immature DCs are present in almost all tissues as sentinels of the immune system. When they encounter pathogens, immature DCs recognize pathogen-derived components via pattern recognition receptors such as

Abbreviations: Ab, antibody; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; DMSO, dimethyl sulphoxide; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-related kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; JNK, c-jun N-terminal kinase; LPS, lipopolysaccharide; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MHC, major histocompatibility complex; NF, nuclear factor; P3C, Pam3CSK4; PG, prostaglandin; PI3K, phosphatidylinositol 3-kinase; TCR, T-cell receptor; Th1, T helper type 1; TLR, Toll-like receptor; TLR-L, TLR ligand.