Immunology Letters 168 (2015) 300–305

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

In vivo and in vitro analyses of α-galactosylceramide uptake by conventional dendritic cell subsets using its fluorescence-labeled derivative

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ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form 12 October 2015
Accepted 12 October 2015
Available online 19 October 2015

Keywords:
α-Galactosylceramide
Dendritic cells
IFN-γ
iNKT cells
Subsets

ABSTRACT

Conventional dendritic cells (cDCs) present α-galactosylceramide (αGC) to invariant natural killer T (iNKT) cells through CD1d. Among cDC subsets, CD8$^+$ DCs efficiently induce IFN-γ production in iNKT cells. Using fluorescence-labeled αGC, we showed that CD8$^+$ DCs incorporated larger amounts of αGC and kept it intact longer than CD8$^-$ DCs. Histological analyses revealed that Langerin$^+$CD8$^+$ DCs in the splenic marginal zone, which was the unique equipment to capture blood-borne antigens, preferably incorporated αGC, and the depletion of Langerin$^+$ cells decreased IFN-γ and IL-12 production in response to αGC. Furthermore, splenic Langerin$^+$CD8$^+$ DCs expressed more membrane-bound CXCL16, which possibly anchored iNKT cells in the marginal zone, than CD8$^-$ DCs. Collectively, it is suggested that the cellular properties and localization of CD8$^+$ DCs are important for stimulation of iNKT cells by αGC.

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Introduction

Dendritic cells (DCs) are antigen-presenting cells (APCs) that exist widely in the body and survey pathogen invasions [1]. Upon recognition of pathogens, DCs present peptide antigens via major histocompatibility complex (MHC) molecules to the T cells. DCs also present lipid antigen via nonclassical MHC CD1 to natural killer T cells (NKT cells) [2], inducing robust cytokine production.

DCs are primarily divided into two populations: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [3]. cDCs are further subdivided into two groups, CD8$^+$ and CD8$^-$ DCs, by the expression of CD8α [4]. NKT cells are divided into two subsets by the pattern of T cell antigen receptor (TCR) expression. Type I NKT cells express invariant TCR, so they are designated as invariant NKT (iNKT) cells [5]. iNKT cells recognize marine sponge α-galactosylceramide (αGC) on the CD1d molecule of cDCs, inducing antitumor activity via the robust production of IFN-γ. During interaction with cDCs, iNKT cells express CD40 ligand (CD40L), which stimulates cDCs via their CD40, subsequently inducing IL-12 production from CD8$^+$ DCs. In turn, IL-12 stimulates iNKT cells, resulting in the strong production of IFN-γ [6,7] Furthermore, using Langerin-eGFP-diphtheria toxin receptor (DTR) mice, depletion of Langerin$^+$ cDCs decreased IL-12 and IFN-γ production after stimulation with injection of αGC [8], suggesting that Langerin$^+$CD8$^+$ DCs are involved in IFN-γ production of iNKT cells *in vivo*. However, it is unclear why CD8$^+$ DCs induce higher IFN-γ production of iNKT cells than CD8$^-$ DCs.

After *i.v.*injection, antigens in the blood are captured by the splenic marginal zone (MZ) cells, causing a rapid immune response [9]. MZ consists of SIGNR1/ER-TR9$^+$ macrophages, MZB cells, and cDCs, forming special equipment to sense/capture antigens in the blood flowing into the sinus between the MZ and marginal CD169/SER4$^+$ metallophil zone. Interestingly, Langerin$^+$CD8$^+$ DCs were observed in the MZ, and iNKT cells migrated to the MZ after injection of αGC [10].

Abbreviations: αGC, α-galactosylceramide; cDCs, conventional dendritic cells; Cy5-αGC, Cy5-conjugated αGC; iNKT, cells invariant natural killer T cells.

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http://dx.doi.org/10.1016/j.imlet.2015.10.008
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Here, we compared αGC uptake and degradation between CD8⁻ and CD8⁺ DC subsets using Cy5-conjugated αGC (Cy5-αGC). We also performed histological analyses to observe the uptake of Cy5-αGC in the spleen. Our results suggest that both cellular properties and localization of CD8⁺ DCs enable efficient stimulation of iNKT cells in response to αGC.

Materials and methods

Mice

C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan), Jα18 knock out (KO) mice (B6 background) were provided from Dr. Taniguchi (RIKEN RCAI, Yokohama, Japan), and langerin-diphtheria toxin receptor (DTR) mice (B6 background) were provided by Dr. Kabashima (Kyoto University, Kyoto, Japan). All mice were housed under specific pathogen-free conditions and used at 7–12 week of age. All experiments were conducted according to our institutional guidelines.

Reagents and antibodies

αGC (Funakoshi Co., Ltd., Tokyo, Japan) was dissolved at 200 μg/ml in 0.5% Tween-20 in PBS. Cy5-αGC was provided from Dr. Watarai (The University of Tokyo, Tokyo, Japan). Diphtheria toxin (DT) and 30% bovine serum albumin (BSA) solution were purchased from Sigma-Aldrich (St Louis, MO). 7-Aminoactinomycin D (7-AAD) was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). RPMI 1640 was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan).

Fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (N418), phycoerythrin (PE)-conjugated anti-CD8α (53-6.7) and anti-CD11c mAbs were obtained from eBioscience (San Diego, CA, USA). FITC-conjugated anti-CD31 (MEC13.3) and allophycocyanin (APC)-conjugated anti-CD8α (53-6.7) mAb were obtained from BD Biosciences (San Diego, CA, USA). Anti-Langerin (L31) and anti-CXCL16 (12-18) [11] mAbs were provided by Dr. Steinman (Rockferor University, New York, USA) and Dr. Yonehara (Kyoto University, Kyoto, Japan), respectively. Anti-CXCL16 mAb was biotinylated in our laboratory. Anti-SER4 mAb was purified from culture supernatant of hybridoma and biotinylated in our laboratory. Streptavidin-PE was purchased from BD Biosciences. Cy3-conjugated anti-rat IgG was purchased from Jackson ImmunoResearch Europe Ltd. (Cambridgeshire, UK).

Hybridomas producing anti-CD16/32 mAb (2.4G2) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and culture supernatants were produced in our laboratory.

MACS microbeads conjugated with anti-mouse CD11c and FITC were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Cell preparation

cDCs were prepared as follows: spleens were treated with 400 U/ml collagenase D (Roche, Mannheim, Germany) for 45 min at 37 °C, and suspended. Subsequently, low density cells were obtained by centrifugation at 800 × *g* for 30 min using 30% BSA. CD11c⁺ cells were purified from the cells using anti-CD11c microbeads following the manufacturer's protocol. To check depletion of CD8⁺ DCs by DT treatment, whole splenocytes after the collagenase digestion were subjected to flow cytometry. For measurement of CXCL16 on the cell surface, the spleen was dispersed in 5 mM EDTA in PBS and whole splenocytes were analyzed using flow cytometry.

Flow cytometry of cell populations and Cy5-αGC uptake

Fc receptors on cells were blocked with anti-CD16/32 mAb in staining buffer for 30 min on ice, and stained for 30 min on ice with mAbs. Data were collected on FACSCalibur and were analyzed with FlowJo (TreeStar, Ashland, OR). Net MFI was obtained by subtraction of the isotype mAb MFI from that of specific mAb.

For the *in vivo* assay of Cy5-αGC uptake, Jα18 KO mice were injected *i.v.*with 5 μg Cy5-αGC in 200 μL PBS. At various time points, splenocytes were stained with anti-CD11c-FITC, anti-CD8-PE, and Cy5-αGC incorporation of cDCs was measured by flow cytometry. The net uptake of Cy5-αGC was calculated by subtracting MFI of Cy5 fluorescence of untreated mouse cDCs from that of treated mouse cDCs.

For the *in vitro* assay of Cy5-αGC uptake, cDCs stained with anti-CD11c-FITC and anti-CD8-PE were cultured in the presence of graded doses of Cy5-αGC for 1 h, or of 100 ng/mL αGC for various time points. After incubation, Cy5-fluorescence intensity was measured gating on CD11c⁺CD8⁺ and CD11c⁺CD8⁻ by flow cytometry. Autofluorescence intensity and background binding of Cy5-αGC by cDCs were calculated by subtraction of fluorescence intensity (MFI) of cDCs cultured with Cy5-αGC at 4 °C from that of cDCs cultured at 37 °C. In pulse and chase experiments, net MFI was calculated by subtraction of autofluorescence intensity of cDCs from MFI of cDCs cultured with Cy5-αGC at 37 °C. The% reduction was calculated using the following formula: [MFI of Cy5-αGC at the time point/that at the initial time point × 100]. In some cases, NH_4Cl (20 mM) was added in the culture.

Cytokine assay

To observe cytokines in sera, mice were injected *i.v.* with 5 μg αGC. After 2 or 4 h, blood was collected. After clotting, IL-12p70, IL-4, and IFN-γ in the sera were assessed by a cytometric bead array (CBA, BD Bioscience). In some cases, langerin-DTR mice or B6 mice were treated two times, 48 h apart, with 350 ng of DT or vehicle by *i.p.*injection to deplete Langerin⁺ cells.

Immunostaining

Spleens were frozen in Tissue-Tek OCT-compound (SAKURA SEIKI Co., Ltd, Tokyo, Japan) at −20 °C. Cryostat sections (10 μm in thickness) were dried, fixed in acetone for 10 min and then dried again. After Fc blocking with anti-CD16/32 mAb, sections were stained for 1 h with antibodies in staining buffer (1% FCS, 0.02% NaN_3, 5 mM EDTA in PBS). Images were obtained with a fluorescence microscope BX51 (OLYMPUS, Tokyo, Japan). In some cases, mice were pulsed *i.v.*with Cy5-αGC, and spleen sections were prepared after 15 min. To observe the distribution of Langerin⁺ DCs in the spleen, BALB/c mice were used, because of the low expression of Langerin in B6 mice.

Statistical analysis

Data are expressed as the mean ± SD of triplicate cultures or three mice in each group. Statistical significances were determined by the Student's *t*-test. Differences were considered significant for *p* values <0.05. All experiments were performed at least two times and representative results are shown.

Results and discussion

CD8⁺ DCs effectively uptake more Cy-5-αGC than CD8⁻ DCs in vivo

We first confirmed previous results showing higher presentations of αGC and induction of IFN-γ production from iNKT cells by

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