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Characterization of the immature dendritic cells and cytotoxic cells both expanded after activation of invariant NKT cells with α-galactosylceramide *in vivo*

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Abstract

Invariant natural killer T (iNKT) cells can perform multiple functions characteristic of both innate and acquired immunity. Activation of iNKT cells *in vivo* by repeated α-GalCer injections can induce immune tolerance, but the mechanisms responsible for such immunoregulation remain unclear. We prepared α-GalCer-liposomes, a single injection of which into mice resulted in the expansion of splenic $CD11c^{low}CD45RB^{high}$ cells, which consists of two populations, $CD180^+$ and $CD49b^+$. Expansion of these cells was not observed in α-GalCer-liposome-treated mice deficient in IL-10 or iNKT cells. MHC and co-stimulatory molecules were down-regulated in $CD11c^{low}CD180^+$ cells compared with conventional dendritic cells (cDCs), suggesting that the former possess characteristics of immature DCs. Meanwhile, the $CD11c^{low}CD49b^+$ cells expressed *IL-10* and *Ctla4*, and possessed greater lytic activity than resting NK cells. These observations suggest that both immature DCs ($CD11c^{low}CD180^+$) and cytotoxic cells ($CD11c^{low}CD49b^+$) might be expanded by α-GalCer-activated iNKT cells and could therefore be involved in immune tolerance.
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Keywords: α-Galactosylceramide; Cytotoxic cells; Immature DCs; Immune tolerance; iNKT cells; IL-10; Liposome; Regulatory DCs

Natural killer T (NKT) cells were identified as a distinct subpopulation which possesses both T cell receptor (TCR) αβ-chain and NK receptors. Invariant NKT (iNKT) cells express a unique invariant antigen receptor encoded by the *Vα14-Jα281* gene. α-Galactosylceramide (α-GalCer) is a glycolipid presented by CD1d molecules of antigen presenting cells (APCs), which activates iNKT cells [1]. iNKT cell activation leads to rapid downstream activation of other immunoregulatory cells, such as dendritic cells (DCs) and regulatory T cells [1,2]. iNKT cells activated by α-GalCer are effective in host defense responses against tumor cells, viral infections, and various other pathogens [1]. iNKT cells are also involved in immune regulation, such as transplantation tolerance and autoimmune diseases [1]. Therefore, it has been proposed that iNKT cells contribute to immune stimulatory or regulatory mechanisms, by cooperating with other immunoregulatory cells.

Dendritic cells (DCs) are professional APCs, which have a pivotal role in the regulation of innate and adaptive immune responses. After encountering pathogen, DCs process antigens for presentation in association with major histocompatibility complex (MHC) molecules at the cell surface. DCs are derived from multiple lineages, have distinct stages of cell development, activation, and maturation, and have the potential to induce both immunity and tolerance [3,4]. Regulatory (tolerogenic) DCs have been shown to suppress several experimental autoimmune diseases. Regulatory DCs were reported to have immature phenotypes, to be maturation-resistant, express surface MHC molecules, have low expression of co-stimulatory molecules and higher levels of inhibitory molecules and have an impaired ability to produce Th1 cytokines. Cells

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with characteristics of both NK cells and DCs have also been reported to exert regulatory function and to be involved in the prevention of autoimmune diabetes by CD40 ligand blockade. Some agents, targeted to DCs, induce differentiation from bone marrow cells by various mechanisms, including production of IL-10 and TGF-β1, and prevention of NF-κB signaling, which is required for the differentiation of regulatory DCs [4–7]. Several reports indicated that regulatory DCs contribute to the induction and activation of regulatory T cells; however, the mechanisms have yet to be determined.

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that eliminate foreign pathogens or tumor cells [8]. Although NK cells were originally defined by their capacity to lyse target cells and produce IFN-γ, recent studies found that they also possess potent regulatory function. It is believed that NK cell regulatory activities are mediated by release of cytokines [9,10], and by interaction with other immunoregulatory cells, such as DCs and regulatory T cells [11,12]. Recently, a distinct population possessing characteristics of both NK cells and DCs was identified in mice, termed "interferon-producing killer dendritic cells" (IKDCs) or "natural killer dendritic cells" (NKDCs) [13,14]. From their lytic and antigen-presenting activity, it is suggested that they play an important role in innate and adaptive immunity, but their physiological functions remain unclear.

Previous research on iNKT cells has demonstrated that repeated α-GalCer injections, but not a single injection, can induce immune tolerance [2]. Instead of using repeated injections, we prepared liposomes containing α-GalCer in a lipid monolayer, predicting that this would have adjuvant-like effects. We found that the iNKT cell-induced $CD11c^{low}CD45RB^{high}$ population, already reported to act as regulatory DCs, contains both immature DCs ($CD11c^{low}CD180^{+}$) and cytotoxic cells ($CD11c^{low}CD49b^{+}$). Both cell types were expanded by α-GalCer-liposome administration in an IL-10-dependent manner. These results suggest that expansion of both $CD11c^{low}CD180^{+}$ cells and $CD11c^{low}CD49b^{+}$ cells mediated by α-GalCer-activated iNKT cells might be involved in immune tolerance.

Materials and methods

Mice. BALB/c and BDF1 mice were purchased from Charles River Japan, Inc. IL-10-deficient mice on a C57B/6 background and iNKT-deficient Jα281-/- mice on a BALB/c background were kindly provided by Dr. R. Nakagawa (RIKEN, RCAI).

Materials and antibodies. α-GalCer was synthesized at RIKEN, RCAI. The α-GalCer-liposomes were prepared as described previously [15]. Biotinylated monoclonal antibodies (mAbs) to CD3e, CD19, CD49b, Ly-6G, and TER119, and FITC-, PE-Texas Red-conjugated streptavidin (all from BD Pharmingen) were used for depletion of lineage-positive cells in splenocytes. FITC-conjugated mAbs to CD3e and CD45RB, PE-conjugated mAbs to CD1d, CD45R/B220, CD45RB, CD80, CD86, CD210/IL-10 receptor, NK1.1, I-Ab, and I-Ad, PerCP-Cy5.5-conjugated anti-CD11b mAb, and APC-conjugated anti-CD11c mAb were purchased from BD Pharmingen. PE-conjugated anti-CD180 mAb and PE-Cy7, APC-conjugated anti-CD49b mAbs were purchased from eBioscience. Anti-CD180 mAb was labeled using the Alexa Fluor 488 monoclonal antibody labeling kit (Invitrogen).

Cell preparation. To prepare splenic DCs, spleens were swollen by infusion of Hank's balanced saline solution (HBSS) containing 10 mM HEPES (pH 7.0), 1 mg/ml collagenase D (Roche Diagnostics) and 200 μg/ml DNase I (Sigma–Aldrich). Spleens were minced and incubated for 45 min at 37 °C. The cells were put through a 100 μm cell strainer (BD Bioscience) and suspended in 4 ml of 14.1% Histodenz (Sigma–Aldrich). Three ml of X-VIVO 15 medium (BioWhittaker) containing 50 μM 2-mercaptoethanol was gently added to make a separate layer and centrifuged at 3000 rpm for 15 min. The cell suspension was separated into low-density and high-density cell fractions; DCs were purified from the low-density cell fraction using appropriate antibodies [16]. To prepare NK cells, spleens from normal mice were homogenized and red cells removed by hypotonic lysis. $CD3^{-}CD49b^{+}$ cells were FACS-sorted as resting NK cells. T and B cells used for RT-PCR analysis were purified using microbead-conjugated anti-CD3 and CD19 mAbs (Miltenyi Biotech). iNKT cells were isolated using α-GalCer-loaded CD1d:Ig recombinant fusion protein (DimerX; BD Bioscience) and anti-mouse IgG1 mAb-conjugated microbeads (Miltenyi Biotech).

Flow cytometry. Cells were pre-treated with anti-CD16/CD32 mAb (BD Pharmingen) to prevent non-specific binding of Abs to their Fcγ receptors. After washing with PBS containing 2% FCS and 0.09% sodium azide, the cells were incubated for 30 min at 4 °C with fluorochrome-labeled mAbs. Flow cytometry and cell sorting were performed using FACSCalibur or FACSAria, respectively (BD Bioscience).

Microarray analysis. Microarray analysis was performed using Mouse Genome 430 2.0 GeneChips (Affymetrix) as described previously [17]. Gene expression was analyzed by reference to the RefDIC (Reference Database of Immune Cells), developed by RIKEN, RCAI [17].

RT-PCR. Total RNA was isolated using RNeasy Mini kits (Qiagen), and cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Sequences of gene-specific primers (Operon Biotechnologies) for semi-quantitative RT-PCR are given in Supplementary Table. Quantitative real-time RT-PCR was performed using TaqMan gene expression assays (Applied Biosystems).

Cytotoxicity assay. Target cells (Yac-1) were labeled with 10 μg/ml calcein-AM (Dojindo Laboratories) for 30 min. Effector cells were co-cultured with 8×10^3 labeled Yac-1 cells for 4 h. Minimum release (non-effector cells) and maximum release (2% NP-40) controls were also prepared. Fluorescence intensity was measured on a Terascan VPS (Minerva Tech). Percent cell viability was calculated by the following formula: 100 − (experimental release − minimum release) × 100/(maximum release − minimum release).

Results

Characterization of expanded $CD11c^{low}CD45RB^{high}$ splenic cells following a single injection of α-GalCer-liposomes

First, we established that a single α-GalCer-liposome injection does induce expansion of $CD11c^{low}CD45RB^{high}$ cells, already reported as regulatory DCs [5], by a mechanism involving activation of iNKT cells. $CD11c^{low}CD45RB^{high}$ cells were markedly expanded following injection of α-GalCer-liposomes but not saline or aqueous α-GalCer (Fig. 1A). This expansion of $CD11c^{low}CD45RB^{high}$ cells was significantly reduced in iNKT cell-deficient mice, implying that α-GalCer-liposomes preferentially induce $CD11c^{low}CD45RB^{high}$ cells in an iNKT cell-dependent fashion. In order to characterize the $CD11c^{low}CD45RB^{high}$ cells, we investigated global gene expression by microarray analysis. NK-related genes (*NK receptors*, *CD49b*), apopto-

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