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Ubiquitous versus restricted expression of the two mouse dendritic cell C-type lectin receptors, DCIR1 and DCAR2, among myeloid cells

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ABSTRACT

Dendritic cell inhibitory receptor 1 (DCIR1, also known as DCIR and Clec4a2) and dendritic cell activating receptor 2 (DCAR2, also known as DCAR and Clec4b1) are mouse lectin receptors expressed on antigen presenting cells. They have structurally similar C-type lectin domains, of which amino acid sequences show 90.5% identity, and commercially available antibodies against them cross-react each other. Here we have established novel antibodies against DCIR1 and DCAR2 that can unambiguously discriminate DCIR1 and DCAR2 and examined their distribution among various immune cells. While DCIR1 was ubiquitously expressed on myeloid cells, including conventional DCs (cDCs), macrophages, neutrophils and eosinophils, in various immune organs, significant expression of DCAR2 was detected only on subpopulations of cDCs from bone marrow and skin-draining lymph nodes. Interestingly, in FITC-painted mice, DCAR2 was expressed on all of the FITC$^+$ cDCs, which had migrated from the skin after FITC painting, suggesting that DCAR2 can be a marker of migratory cDCs in skin-draining lymph nodes. Our findings provide a basis to investigate *in vivo* function of DCIR1 and DCAR2.

1. Introduction

Dendritic cells (DCs[1]) play important roles in immune system as professional antigen presenting cells and as sensors for invading pathogens and danger signals [1,2]. However, it is not fully understood how functions of DCs are regulated by various receptors. DCs are widely distributed in various organs and are classified into subsets according to their functions and expression of marker molecules [3,4].

C-type lectin receptors (CLRs), many of which are encoded in the NK gene complex, are expressed on antigen-presenting cells such as monocytes, macrophages and DCs. CLRs recognize carbohydrate structure on pathogens and self antigens often in a Ca^{2+}-dependent manner and are involved in the innate immunity [5–7].

Mouse dendritic cell immunoreceptor (DCIR) family consists of four dendritic cell inhibitory receptors (DCIR1 ~ 4) and two activating receptors (DCAR1, 2) [5]. Among these, dendritic cell inhibitory receptor 1 (DCIR1, also known as DCIR, Clec4a2, Clec4a, Clecsf6) and dendritic cell activating receptor 2 (DCAR2, also known as DCAR, Clec4b1, Aplra2, DCARbeta) share high similarity in their C-type lectin domains with 90.5% identity in the amino acid sequences. DCIR1 contains an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic domain and transmits inhibitory signal [5,8,9]. Although expression analysis of mouse DCIR1 on spleen cells using rabbit polyclonal antibodies (Abs) showed that DCIR1 is expressed on CD11c$^+$ DCs, CD11b$^+$ monocytes/macrophages (MΦs) and CD19$^+$ B cells [10], analysis of DCIR1 expression using a highly specific monoclonal antibody (mAb) has not been reported. Aged DCIR1-deficient mice spontaneously develops autoimmunity, suggesting essential role of DCIR1 to maintain immune homeostasis [11]. DCAR2 associates with the adaptor molecule Fc receptor γ chain, which has an immunoreceptor tyrosine-based activating motif in its cytoplasmic region, and transmits activation signal [9]. Due to the high similarity between DCIR1 and DCAR2, commercially available anti-DCIR1 and anti-DCAR2 mAbs cross-react with DCAR2 and DCIR1, respectively, which hampered detailed analysis of the expression of

Abbreviations used in this article: Ab, antibody; APC, Allophycocyanin; cDCs, conventional dendritic cells; CLRs, C-type lectin receptors; DCAR2, dendritic cell activating receptor 2; DCIR, dendritic cell immunoreceptor; DCIR1, dendritic cell inhibitory receptor 1; DCs, dendritic cells; FITC, fluorescein isothiocyanate; LNs, lymph nodes; mAb, monoclonal antibody; MΦs, macrophages; pDCs, plasmacytoid dendritic cells; PE, R-phycoerythrin; pMXs-IG, pMXs-IRES-EGFP; SA, streptavidin.

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DCIR1 and DCAR2 with flow cytometry.

In this study, we established novel mAbs that can discriminate DCIR1 and DCAR2 unambiguously and analyzed expression of them on various immune cells. Our results provide a basis to investigate physiological function of DCIR1 and DCAR2.

2. Material and methods

2.1. cDNA cloning of DCIR1 and DCAR2

cDNAs of mouse DCIR1 and DCAR2 were isolated from a cDNA library derived from a C57BL/6J mouse spleen using specific primers, (5′-CCTTAGAGAAGGAACAAGGCTC-3′) and (5′-GCCCATGAAGAATGAGTG-3′) for DCIR1 (NCBI accession: NM_011999), (5′-CGAAGGACATTTTCTGGAGCC-3′) and (5′-GAGTGATTCATAAGTTTATTTTCTTCATCTG-3′) for DCAR2 (NCBI accession: NM_027218). Sequences of the obtained cDNAs were identical to those with the above accession numbers.

2.2. Cell lines and culture

BWZ.36 cells were provided by N. Shastri (University of California Berkeley, Berkeley, CA, USA). Mouse IL-6-transfected X63 cells and PAI cells were obtained from H. Karasuyama (Tokyo Medical and Dental University, Tokyo, Japan). The above cell lines were cultured in RPMI 1640 medium (Invitrogen) containing 10% heat-inactivated fetal calf serum (FCS, Invitrogen), 2 mM glutamine (Wako, Osaka, Japan), 50 μM 2-mercaptoethanol (Sigma–Aldrich, St Louis, MO), 100 U ml^{-1} of penicillin G (Sigma–Aldrich) and 100 μg ml^{-1} of streptomycin sulfate (Sigma–Aldrich) (R10 medium). PAI and established hybridomas were cultured in R10 medium supplemented with 1 mM sodium pyruvate (Wako) and 1 × non-essential amino acid (Sigma–Aldrich). The retroviral vector pMXs-IRES-EGFP (pMXs-IG) and the retrovirus packaging cell line Plat-E were provided by T. Kitamura (the University of Tokyo, Tokyo, Japan). Plat-E cells were maintained in DMEM medium (Invitrogen) containing 10% heat-inactivated FCS, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U ml^{-1} of penicillin G, 100 μg ml^{-1} of streptomycin, 10 μg ml^{-1} of blasticidin S HCl (Invitrogen) and 1 μg ml^{-1} of puromycin (Sigma–Aldrich). All the cell lines were cultured at 37 °C under 5% CO_2.

2.3. Construction of DCIR1 and DCAR2 reporter cells

DNA constructs encoding DCIR1 and DCAR2 chimeric receptors that consist of the intracellular domain of the mouse CD3ζ chain (Arg52 through Arg164), the transmembrane region of mouse Ly49A (Ser40 through Ile66) and the extracellular domains of DCIR1 (Phe69 through Ile238) and DCAR2 (Thr40 through Ile209), respectively, were created by PCR, then, cloned into the retrovirus vector pMXs-IG to construct the DCIR1-pMXs-IG and DCAR2-pMXs-IG vectors. Plat-E cells were transfected with the DCIR1-pMXs-IG, DCAR2-pMXs-IG and the empty pMXs-IG vectors using LipofectAMINE™ (Invitrogen) for packaging, then, BWZ.36 cells were transduced with the retroviruses in the presence of 8 μg/ml of Polybrene (Sigma–Aldrich) to establish the DCIR1, DCAR2 and mock reporter cells, respectively. Establishment of DCIR2, DCIR3, DCIR4 and DCAR1 reporter cells will be published elsewhere.

2.4. Establishment of mAbs against DCIR1 and DCAR2

8–12 weeks old female LEW/SsN Slc rats (Japan SLC, Shizuoka, Japan) were immunized with the DCIR1 or DCAR2 reporter cells three times every two weeks. The first and the second immunizations were performed with Freund's complete and incomplete adjuvants (Difco Laboratories, BD Biosciences, Franklin Lakes, NJ), respectively. The last immunization was carried out without adjuvant. After 3 days from the last immunization, lymph node cells obtained from the rats were fused with PAI myeloma cells using Polyethylene Glycol 1500 (Roche Diagnostics GmbH, Mannheim, Germany) and hybridomas were selected in a standard method. The supernatants from the hybridomas were assayed for the capacity to bind DCIR1 reporter or DCAR2 reporter cells by flow cytometry or reporter cell assays as described below. mAbs produced by the hybridomas were captured on ELISA plates (high protein binding, Greiner Bio-One GmbH, Frickenhausen, Germany) that had been coated with 5 μg/mL of goat anti-mouse IgG (H + L) Ab (Jackson Immuno-Research, West Grove, PA) by incubating hybridoma supernatants at 37 °C for 30 min. The DCIR1, DCAR2 or mock reporter cells were cultured in the ELISA plates at 37 °C under 5% CO_2 for 16 h. After the culture, the cells were washed twice with PBS, then, β-galactosidase activities of the cells were determined by colorimetric assays using chlorophenol red-β-D-galactopyranoside (Wako) as a substrate. Absorbance at 570 nm with reference at 630 nm was measured using a Multiskan JX plate reader (Thermo Fisher Scientific, Waltham, MA).

To screen specific mAbs, we examined lack of the cross reactivity of anti-DCIR1 and anti-DCAR2 mAbs to DCAR2 and DCIR1, respectively, by flow cytometry. We successfully established an anti-DCIR1 mAb, TKKT-1 (IgG2a, κ-light chain), and anti-DCAR2 mAbs, 7D2D3 (IgG2b, κ-light chain), 1E3E3 (IgG2b, κ-light chain) and 8C1H10 (IgG2b, κ-light chain).

2.5. Antibodies and flow cytometric assays

mAbs against DCIR1 (320507) and DCAR2 (349214) were obtained from R&D systems (Minneapolis, MN). Fluorescein isothiocyanate (FITC)-conjugated mAbs against mouse IA-IE (M5/114.15.2), CD45RA/B220 (RA3-6B2), CD8α (53-6.7), CD3ε (145-2C11), CD103 (2E7) and Ly6G (1A8) and R-phycoerythrin (PE)-conjugated mAbs against mouse CD11c (N418) and NK1.1 (PK136) were obtained from BioLegend (San Diego, CA). mAbs against CD19 (1D3), CD11b (M1/70) and Fcγ receptors (2.4G2) were purified from supernatants of hybridomas obtained from ATCC (Manassas, VA) with affinity chromatography using Hi-Trap Protein G HP columns (GE Healthcare Japan Corporation, Tokyo, Japan), then labeled with FITC (Sigma–Aldrich). Anti-DCIR1 and anti-DCAR2 mAbs established in this study were also purified from the culture supernatants of hybridomas and then biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo-Fischer Scientific) or labeled with DyLight 649 NHS Ester (Thermo-Fischer Scientific).

For staining of the reporter cells, the cells were stained with hybridoma supernatants or the purified mAbs and then stained with PE-conjugated goat $F(ab')_2$ anti-mouse IgG (H + L) (Beckman Coulter, Fullerton, CA). For staining of immune cells from mice, the single cell suspensions prepared from various immune organs were incubated with anti-mouse Fcγ receptors mAb (2.4G2) before the addition of fluorochrome-conjugated and biotinylated mAbs to block Fc receptors. Biotinylated anti-DCIR1 (TKKT-1) and anti-DCAR2 (7D2D3) mAbs were used in combination with Allophycocyanin (APC) or PE-conjugated streptavidin (SA) (BioLegend). Propidium iodide (Sigma–Aldrich) was added at the final concentration of 1 μg/ml immediately before flow cytometric acquisition to exclude dead cells from analysis. The data were acquired with a FACS Calibur system (BD Biosciences) and were analyzed with the FlowJo software (TreeStar, San Carlos, CA, USA). Background staining was estimated using fluorochrome-conjugated isotype control mAbs: rat IgG2a control mAb (3G72, anti-human AICL mAb) prepared in our laboratory or rat IgG2b control mAb (RTK4530) obtained from BioLegend.