Expression of human DEC-205 (CD205) multilectin receptor on leukocytes

Masato Kato¹, Kylie J. McDonald¹, Seema Khan¹, Ian L. Ross², Slavica Vuckovic¹, Ke Chen³, David Munster¹, Kelli P. A. MacDonald¹ and Derek N. J. Hart¹,⁴

¹Mater Medical Research Institute, Dendritic Cell Laboratory, Aubigny Place, Raymond Terrace, Queensland 4101, Australia
²Cooperative Research Centre for Chronic Inflammatory Diseases, Institute for Molecular Biosciences, University of Queensland, St Lucia, Queensland 4067, Australia
³Queensland Institute of Medical Research, Leukaemia Foundation Laboratory and ⁴School of Medicine, University of Queensland, Herston Road, Queensland 4006, Australia

Abstract

DEC-205 (CD205) belongs to the macrophage mannose receptor family of C-type lectin endocytic receptors and behaves as an antigen uptake/processing receptor for dendritic cells (DC). To investigate DEC-205 tissue distribution in human leukocytes, we generated a series of anti-human DEC-205 monoclonal antibodies (MMRI-5, 6 and 7), which recognized epitopes within the C-type lectin-like domains 1 and 2, and the MMRI-7 immunoprecipitated a single ~200 kDa band, identified as DEC-205 by mass spectrometry. MMRI-7 and another DEC-205 mAb (MG38), which recognized the epitope within the DEC-205 cysteine-rich and fibronectin type II domain, were used to examine DEC-205 expression by human leukocytes. Unlike mouse DEC-205, which is reported to have predominant expression on DC, human DEC-205 was detected by flow cytometry at relatively high levels on myeloid blood DC and monocytes, at moderate levels on B lymphocytes and at low levels on NK cells, plasmacytoid blood DC and T lymphocytes. MMRI-7 F(ab’)² also labeled monocytes, B lymphocytes and NK cells similarly excluding reactivity due to non-specific binding of the mAb to FcγR. Tonsil mononuclear cells showed a similar distribution of DEC-205 staining on the leukocytes. DEC-205-specific semiquantitative immunoprecipitation/western blot and quantitative reverse transcriptase-PCR analysis established that these leukocyte populations expressed DEC-205 protein and the cognate mRNA. Thus, human DEC-205 is expressed on more leukocyte populations than that were previously assumed based on mouse DEC-205 tissue localization studies. The broader DEC-205 tissue expression in man is relevant to clinical DC targeting strategies and DEC-205 functional studies.

Introduction

Dendritic cells (DC) are specialized antigen presenting cells with unique capacities for migrating, antigen uptake/processing and presenting to T cells, and stimulating and activating B and NK cells (1, 2). DC are equipped with an array of C-type lectin receptors, including classical [Ca²⁺-dependent sugar binding proteins, e.g. DC-SIGN, macrophage mannose receptor (MMR)] and non-classical C-type lectins (e.g. β-glucan receptor), which appear to play major roles in both innate and adaptive immunity (3, 4).

DEC-205 (CD205) is a putative antigen uptake and processing receptor, cloned first in mouse (5). The anti-mouse DEC-205 mAb NLDC-145 stains interdigitating DC, Langerhans cells and thymic epithelia (6), and has become a “gold standard” DC marker for mouse DC research and has been used extensively to localize mouse DC in tissues and to phenotype mouse DC subsets. The mouse DEC-205 cDNA analysis revealed that DEC-205 belongs to a family of type I transmembrane multilectin receptors, including the MMR (7, 8), phospholipase A₂ receptor (PLA₂R) (9) and recently described Endo180 (10). The extracellular domain of these molecules contains a cysteine-rich domain (CR), a fibronectin type II domain (FN) and multiple C-type lectin-like domains (CTLD; 8 for MMR, PLA₂R and Endo180, and 10 for DEC-205). The relatively short cytoplasmic domains (CP) contain motifs...
for Tyr- and/or di-hydrophobic amino acid-based endocytosis (11). We and others cloned human DEC-205, which exhibits ~80% protein identity to the mouse homolog (12, 13), suggesting that its function is conserved between species. The ligand specificity for DEC-205 has not been established, nor has function on thymic epithelia been described, though it was suggested recently that DEC-205 plays a role in clearance of apoptotic thymocytes by thymic epithelial cells (14).

Recently Mahnke et al. showed that DEC-205 CR contains a cluster of acidic amino acids (an acidic triad, EDE in one letter amino acid code), which mediates efficient transport of endocytosed DEC-205 to late endosomes/MHC class II compartments. When compared with MMR CR, which lacks an acidic triad, DEC-205 CR increased antigen processing and presentation to CD4 T lymphocytes by artificial antigen-presenting cells 100-fold (15). More recent publications indicated that NLDC-145 genetically or chemically conjugated to test antigens could target DC in vivo, and elicit antigen-specific CD4 and CD8 T lymphocyte responses when injected into mice (11, 16–18). Depending on the presence or absence of an additional activation signal (i.e. CD40 agonist), it resulted in either immunostimulatory or immunoregulatory effects, respectively. Therefore, DEC-205 is a very attractive target for therapeutic antigen delivery and this possibility prompted further studies on human DEC-205 tissue distribution and function.

We produced the DEC-205 mAb MMRI-5, 6 and 7, which recognized human DEC-205 specifically. Using the MMRI-7 and the independently derived DEC-205 mAb MG38 for staining and quantitative reverse transcriptase (RT)–PCR, we undertook a comprehensive analysis of DEC-205 expression on human leukocytes. Unexpectedly, we discovered that human DEC-205 is present at various levels on more leucocyte populations than anticipated.

**Methods**

**Cell lines, antibodies and other reagents**

Hodgkin’s Reed-Sternberg (HRS) cell line L428 was provided by V. Diehl (Klinik fur Innere Medizin, Cologne, Germany) (19). The other HRS cell line KM-H2 (20) was obtained from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). Mouse myeloma cell line NS-1 and the independently derived DEC-205 mAb MG38 for tissue distribution and function.

Splenocytes from Balb/C mice immunized with a mixture of DEC-205-Ig fusion proteins were purified using HiTrap protein A column chromatography (Amersham Pharmacia Biotec) and their purity was analyzed by SDS-PAGE.

**Production of DEC-205-Ig fusion proteins**

The production of DEC-205 CR/FN-Ig was described previously (23). To express the DEC-205 internal domains (CTLD1-10) lacking signal peptides as Ig fusion proteins, a 1.4 kb HindIII–NcoI fragment (containing human IgG1 Fc) from pIG-1 vector (24) was subcloned into the HindIII–NcoI sites of the pSecTagB vector (Invitrogen) to construct the pSecFbc vector. PCR was performed using Expand polymerase (Roche Applied Science) and DEC-205 gene-specific primers (Table 1) to amplify fragments containing consecutive two DEC-205 extracellular domains (i.e. CTLD1/2, CTLD3/4, CTLD5/6, CTLD7/8 and CTLD9/10) from the L428 cDNA (12). These fragments were cloned into the pSecFbc vector to generate pCTLD1/2-Ig, pCTLD3/4-Ig, pCTLD5/6-Ig, pCTLD7/8-Ig and pCTLD9/10-Ig, respectively. These constructs were transiently transfected into COS-7 cells using FuGene 6 (Roche Applied Science) and secreted DEC-205-Ig fusion proteins were purified using HiTrap protein A column chromatography (Amersham Pharmacia Biotec) and their purity was analyzed by SDS-PAGE.

**Production of DEC-205 mAb**

Splenocytes from Balb/C mice immunized with a mixture of DEC-205-Ig fusion proteins (CTLD1/2-Ig, CTLD3/4-Ig, CTLD5/6-Ig, CTLD7/8-Ig and CTLD9/10-Ig) were fused to mouse myeloma cell line NS-1 using a conventional polyethylene glycol fusion protocol. IgG-producing hybridomas were selected by dot blot analysis using a HRP-conjugated goat anti-mouse IgG (γ-chain-specific), and the signal detected by enhanced chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA) on a AGFA Curix Ortho HT-G film.
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Table 1. Primers used in this study

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Gene-specific DNA sequences are shown in capital letters. Engineered restriction enzyme recognition sites (i.e. gagtc for BamHI; gaattc for EcoRI and aagctt for HindIII) are in italic. The splicing donor sequence (24) is underlined.

Characterization of DEC-205 mAb

For ELISA analysis, an ELISA plate coated with DEC-205-Ig fusion proteins and human immunoglobulins (Intragam, CSL, Australia) was incubated with DEC-205 mAbs, and their binding detected with HRP-conjugated goat anti-mouse IgG (γ-chain-specific) and α-phenylenediamine (Sigma).

For immunoprecipitation analysis, PBMC were cell surface biotinylated using sulfo-NHS-LC-biotin (Pierce), and were lysed in the lysis buffer (1% Triton X-100, 0.25% sodium deoxycholate, 0.15 M NaCl, 50 mM Tris·HCl, pH 7.4 and 5 mM EDTA) containing a cocktail of protease inhibitors [Complete, Roche Applied Science, and 1 mM phenylmethylsulphonylfluoride (PMSF)] and the lysate was cleared by centrifugation at 20,000 × g for 10 min at 4°C. The protein concentration was determined by bichinonic acid (BCA) assay (Pierce) using BSA as standards. The extract precleared with protein G Sepharose beads (Amersham Pharmacia) was subjected to immunoprecipitation with the DEC-205 mAbs and isotype control mAb 401.21 then protein G Sepharose beads. The protein G-bound proteins were fractionated using a 4–12% Nu-PAGE gel with MOPS buffer (Invitrogen). The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (PVDF-Plus, Osmonics, Westborough, MA, USA) and the signals detected with HRP-streptavidin (1:5000 dilution, Roche Applied Science) and enhanced chemiluminescence.

For DEC-205 mAb epitope mapping, KM-H2 cells were preincubated with unconjugated DEC-205 mAbs (10 μg ml⁻¹) on ice, washed and stained with 5 μg ml⁻¹ FITC-conjugated MMRI-7 or M335. The cells were subjected to flow cytometry.

Identification of DEC-205 protein by mass spectrometry

L428 cells (4 × 10⁷ cells) were lysed with 1 ml of the lysis buffer with a cocktail of protease inhibitors and precleared with the isotype control mAb (401.21, 25 μg) and protein G Sepharose twice. The precleared lysate was subjected to immunoprecipitation with 25 μg MMRI-7 or 401.21 as described above and fractionated with a 4–12% Nu-PAGE gel under reducing conditions. The gel was stained with Coomassie Brilliant Blue R250 (CBB R250) and the MMRI-7-specific ~200 kDa band was excised and subjected to mass spectrometry as described below.

The MMRI-7-specific band was reduced with 10 mM di-thiothreitol (DTT) for 10 min and alkylated with 100 mM iodoacetamide for 1 h in the dark. The band was dehydrated with methanol and then washed three times in ultrapure water. The washed band was dried in a vacuum centrifuge (Speedvac) and rehydrated with 20 μl of a solution containing protein sequencing grade trypsin (2 μg ml⁻¹, Promega) in 50 mM NaHCO₃, pH 8. Digestion was allowed to proceed overnight at 37°C. The resultant peptides were extracted from the gel with 0.1% (v/v) formic acid and fractionated by C₁₈ HPLC using a 5–60% acetonitrile gradient in 0.1% formic acid. The column output was analyzed using a quadrupole time of flight tandem mass spectrometer (QTOF, ABI QStar) operating in independent data acquisition (IDA) mode. Mascot (Matrix Science, Boston, MA, USA) was used to identify peptides and confirm protein identity (25).

Biosensor assay

For biosensor analysis, DEC-205-Ig fusion proteins and human immunoglobulins (Intragam) were immobilized onto a CMS sensor chip surface (BIACORE, Box Hill, VIC, Australia) using an amine coupling method to obtain 3000–4000 response units using a BIAcore 2000. For the binding kinetic experiments, the antibody concentrations ranged from 1 to 35 nM in six serial dilutions were passed over the sensor chip surface, and the results and kinetic parameters were evaluated using Global Analysis in the BIAevaluation software 3.1.
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**F(ab')2 purification**

The F(ab')2 of MMRI-7 and 401.21 were purified using an ImmunoPure IgG1 Fab and F(ab')2 Preparation Kit (Pierce). Briefly, ~1 mg of mAb was digested with immobilized ficin in the presence of 1 mM cysteine at 37°C overnight and subjected to a protein A column chromatography to remove the Fc fraction and undigested mAb. The F(ab')2 was concentrated using a Centricon (YM30, Millipore, North Ryde, NSW, Australia) and their quality was assessed by SDS-PAGE.

**DEC-205 expression analysis by flow cytometry**

Blood was obtained from volunteer donors and "inflamed" palatine tonsils were obtained at routine tonsillecotony with appropriate informed consent, according to the Mater Hospital Human Research Ethical Committee guidelines. Buffy coat was obtained from Australian Red Cross. The flow cytometers were calibrated daily using fluorescent calibration beads (Calibrite, BD Bioscience). The data were analyzed using CellQuest (BD Bioscience). For flow cytometry analysis, PBMC were suspended in cold PBS with 2 mM EDTA, 0.5% (w/v) BSA (MACS buffer) and stained for 20–30 min on ice with FITC-MMRI-7 or MG38 in combination with fluorochrome-conjugated lineage antibodies. Cells were washed with excess volume of cold PBS, fixed with 2% (w/v) PFA and subjected to flow cytometry using a FACS Calibur (BD Bioscience). Appropriate isotype control mAbs were used for negative controls. For staining with the F(ab')2 of MMRI-7 or 401.21, PBMC were stained with the F(ab')2, washed and stained with FITC-conjugated F(ab')2 sheep anti-mouse IgG, that were F(ab')2-specific (Jackson Laboratory). The cells were blocked with 10% mouse serum in PBS, and stained with fluorochrome-conjugated lineage antibodies. A strict gating strategy was applied for detecting DEC-205 expression on a leukocyte population without a possible contamination of other leukocyte populations as below (26): T lymphocytes were defined as CD3+/CD11c− HLA-DR−; B lymphocytes were CD20+/HLA-DR+/CD11c−; NK cells were CD56+/HLA-DR−; monocytes were CD14+/HLA-DR+/CD19−; and dendritic cells (BDC) were HLA-DR+/Lin− and BDCA2+/BDCA2−. The purity of these leukocyte populations was >98%.

To isolate T lymphocytes, PBMC or tonsil MNC were incubated with the unconjugated anti-CD3 then goat anti-mouse-conjugated MACS beads (Miltenyi Biotech) and subjected to an AutoMACS (Miltenyi Biotech) to purify CD3+ T lymphocytes. To remove the contaminating CD11c+ cells and/or HLA-DR+ cells (presumably monocytes, B lymphocytes and CD11c+ DC) in the AutoMACS preparation, the cells were further stained with FITC-CD3, PerCP-HLA-DR and APC-CD11c and subjected to FACS (FACSVantage, BD) for CD3+/CD11c+/HLA-DR− T lymphocytes. To isolate B lymphocytes, CD19+ B lymphocytes isolated from tonsil MNC by MACS were stained with FITC-CD3, PE-CD20 and APC-CD11c and subjected to FACS for CD20+/CD3−/CD11c− B lymphocytes. To isolate NK cells, NK cells were enriched by negative selection using non-NK cell surface markers (CD3, CD14, CD19, CD20, CD34, CD11c, HLA-DR and CD235a) by an AutoMACS. The cell preparation was stained with PE-CD16, PE-CD56 and FITC-SAM and subjected to FACS for CD16+/CD56− NK cells. CD14+ monocytes were isolated from PBMC by MACS and checked its purity by flow cytometry for CD14+/CD3−/CD20−.

To isolate blood DC subsets, PBMC were negatively selected for CD3−/CD14+/CD19−/CD20−/CD34−/CD56+/CD235a− (Lin−) using an AutoMACS. The Lin− PBMC were stained with FITC-SAM, PE-CD4 and APC-CD11c, and subjected to FACS for Lin−/CD4+/CD11c+ (myeloid blood DC) and Lin−/CD4+/CD11c− (plasmacytoid DC).

**Semi-quantitative immunoprecipitation/western blot analysis**

Five to ten million cells were lysed with 1 ml of the lysis buffer with a cocktail of protease inhibitors and the protein concentration in the lysate was determined by BCA assay. Serially diluted cell lysate (final protein concentration: 200, 100 and 50 μg ml−1) was subjected to immunoprecipitation using the rabbit antisera against DEC-205 CP peptide (anti-DEC-205 CP, 5 μl sample−1) and protein A Sepharose as described previously (27). The bound proteins were eluted with 2 × NuPAGE sample buffer, fractionated in 4–12% Nu-PAGE gels in a non-reducing condition and transferred to a PVDF membrane. DEC-205 was detected by incubating the membrane with a mixture of DEC-205 mAbs (MMRI-7 and M335, 5 μg ml−1 each) followed with HRP-conjugated goat anti-mouse IgG (γ-chain-specific) and enhanced chemiluminescence as above.

**Quantitative RT-PCR**

Total RNA purified from FACS-purified cell populations (1.2 × 10^6 to 1 × 10^7 cells/preparation) using a Trizol (Invitrogen) was treated with DNase I to remove contaminating genomic DNA, and subjected to cDNA synthesis with an oligo dT<sub>17</sub> primer using Expand reverse transcriptase (Roche Applied Science).
For quantitative RT-PCR analysis, the cDNA was combined with DEC-205 gene-specific forward and reverse primers (see Table 1) and SYBR green master mix (QuantiTect SYBR PCR kit, Qiagen, Clifton Hill, VIC, Australia), and subjected to real-time PCR using a Rotorgene 3000 thermal cycler (Corbett Research, Mortlake, NSW, Australia). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of cDNA input. The standard curves were generated using serially diluted gene-specific amplicons. The thermal cycling conditions were initial denaturation at 95°C for 15 min, 45–50 cycles of 95°C for 20 s, 61°C for 20 s and 72°C for 20 s, followed by melting temperature analysis (72°C to 99°C with 1°C increments). Data analysis was performed using Rotorgene 5.0 software (Corbett Research). The amplification was specific as judged by melting temperature analysis, agarose gel analysis and DNA sequencing of the amplicons. The experiments were performed in duplicates and repeated at least three times.

Results

Production of mAb against human DEC-205

We constructed a series of eukaryotic expression vectors for DEC-205-Ig fusion proteins, each of which contained consecutive pairs of human DEC-205 extracellular domains (Fig. 1A). These Ig fusion proteins were transiently expressed in COS-7 cells, and the secreted fusion proteins were purified by protein A column chromatography (Fig. 1B). Mice were immunized with a mixture of the human DEC-205-Ig fusion proteins and DEC-205 CR/FN fusion proteins, each of which contained consecutive pairs of human DEC-205 extracellular domains (Fig. 1A). We obtained three human DEC-205 mAbs termed MMRI-5, 6 and 7 (all IgG1 isotype). These mAbs bind to the CTLD1/2-Ig fusion proteins by ELISA (Fig. 1C), whereas the previously established DEC-205 mAbs M335 (28) and MG38 (21) bind to the CR/FN-Ig. Although the immunogen for M335 is unknown, the reactivity of MG38 to the CR/FN-Ig was consistent with the fact that a DEC-205 CR/FN fusion protein was used to produce M335 (~80%) and MMRI-7 (~20%), but not by MMRI-5, 6 or MG38. These data suggested that the epitopes for MMRI-7 and M335 were in close proximity on the structurally intact native molecule.

Human DEC-205 is expressed on DC in blood

We examined DEC-205 expression on the two main blood DC subsets, myeloid (lin<sup>−</sup>DR<sup>+</sup>CD11c<sup>+</sup>) and plasmacytoid DC (lin<sup>−</sup>DR<sup>−</sup>CD11c<sup>−</sup> and BDCA2<sup>+</sup>) using FITC-conjugated MMRI-7 and MG38, fresh blood preparations and four-color flow cytometry (Fig. 3A). Both mAb detected relatively high levels of DEC-205 expression on CD11c<sup>+</sup>Lin<sup>−</sup>HLA-DR<sup>+</sup> myeloid blood DC, but in some donors the myeloid DC population was split into at least two subpopulations according to DEC-205 expression levels, which correlated with the HLA-DR expression levels (data not shown). Low levels of DEC-205 expression were detected on CD11c<sup>+</sup>Lin<sup>−</sup>HLA-DR<sup>+</sup> or DR<sup>−</sup>CD11c<sup>−</sup>BDC2a<sup>+</sup> plasmacytoid DC.

DEC-205 is expressed on T lymphocytes, B lymphocytes, NK cells and monocytes in PBMC

The analysis of DEC-205 expression on blood DC suggested that other leukocytes also expressed substantial levels of cell surface DEC-205. Therefore, we investigated the DEC-205 expression on other leukocytes, including T lymphocytes, B lymphocytes, NK cells and monocytes using FITC-conjugated MMRI-7 and MG38 by flow cytometry (Fig. 3B). Cells were stained with DEC-205 mAbs combined with lineage antibodies and stringent gating strategies used to assess pure leukocyte populations for DEC-205 expression. This minimized the possible contamination of myeloid cells (monocytes and myeloid DC) and B lymphocytes expressing relatively high levels of DEC-205 as aggregates (26).

As shown in Fig. 3(B), both MMRI-7 and MG38 detected cell surface DEC-205 on CD14<sup>+</sup>CD19<sup>−</sup>DR<sup>+</sup> monocytes at relatively high levels. It was noted that DEC-205 expression on these cell types was similar to that on CD11c<sup>+</sup> myeloid blood...
Fig. 1. Characterization of monoclonal antibodies against human DEC-205. (A) A schematic structure of human DEC-205. Distinct domain structures are shown as boxes. Bold lines indicate positions of DEC-205 domains included in DEC-205-Ig fusion proteins. SP, signal peptide; CR, cysteine-rich domain; FN, fibronectin type II domain; CTLD, C-type lectin-like domain; TM, transmembrane domain; CP, CP. (B) SDS-PAGE analysis of DEC-205-Ig fusion proteins. The fusion proteins were fractionated in 10% SDS-PAGE under reducing conditions and stained with CBB R250. Positions for molecular mass standards are indicated on the left. (C) Characterization of DEC-205 mAb by ELISA. An ELISA plate was coated with DEC-205 fusion proteins and human IgG and the mAb binding to these coated proteins were detected with HRP-conjugated goat anti-mouse IgG and o-phenylenediamine. The DEC-205 mAb M335 and the isotype control mAb 401.21 (Cont IgG1) were used as positive and negative control, respectively. (D) DEC-205 immunoprecipitation from PBMC cell lysate using DEC-205 mAb. Cell surface-biotinylated PBMC lysate was immunoprecipitated with DEC-205 mAbs and protein G Sepharose. The precipitate was subjected to western blot analysis and DEC-205 was detected.
DC (see Fig. 3A). Human CD20⁺CD11c⁻DR⁺ B lymphocytes expressed moderate levels of DEC-205. Furthermore, DEC-205 was also expressed on CD3⁺CD11c⁺HLA-DR⁺ T lymphocytes at low levels and on CD56⁺HLA-DR⁺ NK cells.

**MMRI-7 F(ab')₂ confirms DEC-205 surface expression on monocytes and lymphocytes**

Mouse DEC-205 is present at high levels on DC and at low levels on Gr-1⁺ granulocytes, Thy-1⁺ T lymphocytes, B220⁺ B lymphocytes and thioglycollate-elicted peritoneal macrophages, but not on NK cells or monocytes (30). Therefore, it was puzzling to detect DEC-205 on the anticipated DEC-205 negative and/or weakly positive leukocyte populations in human PBMC at high (monocytes) and moderate/low levels (B lymphocytes and NK cells). It was possible that MMRI-7 staining on monocytes, B lymphocytes and NK cells was due to the binding of MMRI-7 to FcγR expressed on these cells. To eliminate this possibility, F(ab')₂ of MMRI-7 and the isotype control mAb 401.21 were prepared to detect DEC-205 on PBMC (Fig. 4). SDS-PAGE analysis confirmed that the MMRI-7 F(ab')₂ preparation contained a major ~100 kDa band under non-reducing conditions, which reduced to ~25 kDa, indicating that the Fc portion of MMRI-7 had been removed (Fig. 4A). The isotype control mAb SDS-PAGE profile included a slightly smaller band upon reduction, which was likely derived from an excess ficin digestion.

The F(ab')₂ confirmed that the staining of the B lymphocytes, NK cells and monocytes for surface DEC-205 and established that it was not due to the non-specific MMRI-7 binding to FcγR expressed on these cells (Fig. 4B). The majority of T lymphocytes were also stained with MMRI-7 F(ab')₂, consistent with the results obtained with the intact MMRI-7. The MMRI-7 F(ab')₂ staining on CD11c⁺ myeloid BDC and CD11c⁺BDCA₂⁺ plasmacytoid BDC was essentially consistent with the staining obtained with the intact MMRI-7 (Fig. 3).

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**Fig. 2.** (A) Titration of FITC-conjugated DEC-205 mAbs. KM-H2 cells were stained with serially diluted FITC-conjugated DEC-205 mAb and their binding was determined by flow cytometry. (B) Inhibition of FITC-conjugated MMRI-7 and M335 binding to KM-H2 cells by unconjugated DEC-205 mAb. KM-H2 cells were preincubated with unconjugated DEC-205 mAb (10 µg ml⁻¹), stained with FITC-conjugated MMRI-7 (left panel) or M335 (right panel) and their binding detected by flow cytometry. The isotype control mAb 401.21 (Cont IgG1) were used as negative control.
Fig. 3. Expression of DEC-205 on human BDC and other leukocytes. (A) DEC-205 expression on CD11c⁺ myeloid and CD11⁻ plasmacytoid BDC.
PBMC were stained with PE-conjugated lineage mAb (CD3, CD14, CD19, CD20 and CD56), PerCP-conjugated HLA-DR, APC-CD11c and FITC-
MMRI-7 (left panel) or MG38 (right panel), and subjected to four-color flow cytometry. (B) Expression of DEC-205 on lineage-positive leukocytes.
PBMC were stained with FITC-MMRI-7 (left panel) or MG38 (right panel) in combination with PE-conjugated lineage markers (CD3, CD20, CD56 or
CD14) and other cell surface markers as described in Methods. The bold line and gray fill indicate DEC-205 mAb staining and an isotype control
staining, respectively.
We further investigated the DEC-205 expression on tonsil-derived MNC using FITC-conjugated MMRI-7 by flow cytometry and immunofluorescent microscopy (Fig. 5). MMRI-7 indicated higher levels of DEC-205 expression on tonsil myeloid (Lin−DR+CD11c+) and plasmacytoid DC (BDCA2+) compared with the BDC subsets (Fig. 5A). Tonsil monocytes/macrophages (CD14+CD19−DR+) and NK cells (CD56−DR+) also expressed higher levels of DEC-205 than did PBMC monocytes and NK cells. Tonsil T (CD3+CD11c−DR−) and B lymphocytes (CD20+CD11c−DR+) expressed similar levels of cell surface DEC-205 to those on PBMC T and B lymphocytes. Staining with MMRI-7 revealed that the strongly DEC-205+ cells were scattered in the T cell area and the B cell area throughout the tonsil, but at higher density in the T cell area. These DEC-205+ cells were large cells with dendritic morphology and also strongly CD11c+, suggesting that they were myeloid DC. A similar DEC-205 staining pattern was also obtained with MG38. There was also weak DEC-205+ staining present on both T and B lymphocytes, but background issues meant this was better determined by flow cytometry.

**Semiquantitative immunoprecipitation/western blot analysis detects a 200 kDa DEC-205 band in FACS-purified leukocyte cell lysate**

As further confirmation of the presence of DEC-205 on the various leukocyte populations, we performed semiquantitative immunoprecipitation/western blot analysis to detect DEC-205 protein in monocyte-derived DC (MoDC), macrophage and lineage-positive cell preparations. The serially diluted cell lysate of highly purified leukocytes was subjected to immunoprecipitation with the rabbit anti-DEC-205 CP antibody and protein A agarose. The immunoprecipitated proteins were fractionated on SDS-PAGE and subjected to western blot analysis for DEC-205 (Fig. 6) (27). The DEC-205 expressing cell line L428 was used as a positive control for immunoprecipitation and to normalize the two PAGE gels required for the analysis.

We detected a single ~200 kDa band in all cell preparations tested, including T lymphocytes, B lymphocytes, NK cells, monocytes, macrophages and MoDC. Detection of the DEC-205 band was specific because there was no band in L428 lysate immunoprecipitated with the pre-immune rabbit serum (lane 2). Interestingly, the similar amount of DEC-205 protein (less than two-fold difference) was detected in the non-activated cell lysate tested when normalized by cellular proteins. We also detected a 180 kDa band in cell lysates from T lymphocytes, B lymphocytes, NK cells and monocytes, but not in other cell types. The nature of this 180 kDa band is unknown but it may correspond to a splice variant of DEC-205 mRNA we described recently, encoding a truncated CTLD10 (27). Activation of macrophages and MoDC using LPS increased the ~200 kDa band markedly, LPS-activated MoDC, in particular, increased DEC-205 protein expression at least 30-fold.
Quantitative RT-PCR detects DEC-205 mRNA in DC, lymphocytes, monocytes, granulocytes and macrophages

To investigate whether the surface DEC-205 was expressed by a wide range of leukocyte populations endogenously or was simply adsorbed on their surface, we performed quantitative RT-PCR on DC, granulocytes, macrophages, T lymphocytes, B lymphocytes and NK cells purified by flow cytometry (purity >98%) (Fig. 7). GAPDH was used to normalize cDNA input.

We detected relatively high levels of DEC-205 mRNA in MoDC, blood DC, macrophages and granulocytes as expected. The mRNA levels in the MoDC increased markedly upon maturation of MoDC by LPS as shown previously (23). Despite relatively high levels of cell surface DEC-205 expression (Figs. 3 and 4), both B lymphocytes and monocytes express comparatively low levels of DEC-205 mRNA. On the other hand, T lymphocytes and NK cells, which express
low levels of cell surface DEC-205 (Figs. 3 and 4), expressed moderate levels of DEC-205 mRNA. Overall, the amount of DEC-205 mRNA levels in these leukocyte populations correlated to the DEC-205 protein levels normalized by cellular protein (see Fig. 6), suggesting that DEC-205 expression is transcriptionally regulated.

Taken together, these data demonstrate that human DEC-205 is expressed not only by the expected cell types based on the published mouse DEC-205 expression (i.e. DC, B lymphocytes and T lymphocytes) but also by monocytes, macrophages and NK cells.

Discussion

We developed a series of mAbs against human DEC-205 and performed a comprehensive analysis of DEC-205 expression on human leukocytes. This established that DEC-205 is expressed on many more leukocyte populations, including DC, B lymphocytes, T lymphocytes, NK cells and monocytes, at higher levels than had been hitherto realized. The rat mAb NLDC-145, which first identified mouse DEC-205, has been used extensively to identify DC based on the original description of its reactivity as being restricted to interdigitating DC, LC and thymic epithelia (6). Thus, it has been used to define murine DC subsets and this apparent specificity exploited in functional studies (11, 16). It has been assumed that the distribution of the human ortholog might be similarly restricted. The high affinity rabbit polyclonal antibody against mouse DEC-205, however, revealed that mouse DEC-205 was also expressed on Gr-1+ granulocytes, Thy-1+ T lymphocytes, B220+ B lymphocytes and thioglycollate-elicited peritoneal macrophages but their expression levels were much lower (>1 log) than those on DC. Mac-1+Gr-1+ monocytes did not express DEC-205 (30). The polyclonal antibody also detected DEC-205 in mouse non-lymphatic tissues, including capillary endothelial cells in brain, the stroma of bone marrow
and the epithelial cells in pulmonary airways and gastrointestinal tract (31). Therefore, mouse DEC-205 is predominantly expressed by DC.

Several mAbs against human DEC-205 have been described to date, including MR6 (13, 32), M335 (28) and MG38 (21). In addition to these, we developed a series of DEC-205 mAb termed MMRI-5, 6 and 7. These DEC-205 mAb recognized the CRD1/2-Ig (Fig. 1C) and immunoprecipitated a single ~200 kDa band (Fig. 1D). Furthermore, we subjected the ~200 kDa band immunoprecipitated with MMRI-7 to internal sequencing by mass spectrometry and confirmed its identity as DEC-205 (Fig. 1E and F). We utilized two mAbs MG38 and MMRI-7, which recognize distinct DEC-205 domains (CR-FN domain for MG38 and CTLD1/2 domain for MMRI-7) (Fig. 1C), to analyze DEC-205 expression in man (Figs. 3 and 4). Further, expression of DEC-205 in the human leukocyte populations was confirmed by semiquantitative immunoprecipitation/western blot analysis (Fig. 6) and DEC-205-specific quantitative RT–PCR (Fig. 7). Our results showed that DEC-205 was expressed at high levels on myeloid DC and monocytes, at moderate levels on B lymphocytes and at low levels on NK cells, plasmacytoid DC and T leukocytes, indicating that human DEC-205 expression on leukocytes differed from the mouse ortholog.

DEC-205 has been proposed to be an antigen uptake/processing receptor on DC because (a) DEC-205 is structurally similar to MMR, a prototype antigen uptake receptor expressed on macrophages and MoDC (33), (b) DEC-205 is highly expressed on DC (5, 30) and (c) DEC-205 CP contains an acidic triad motif for efficient targeting of late endosomes and antigen processing (15). In fact, it was shown recently that chemically or genetically modified NLDG-145 conjugated with test antigens can target lymph node DC when intradermally injected, and elicit antigen-specific CD4+ and CD8+ T lymphocyte responses with high efficiency (11, 16). Although DEC-205 appears to be an attractive target to load antigens into DC and the mouse data argue that there is a functional specificity, our results suggest that clinical studies will need to take account of its broad tissue distribution and the affinity of any candidate mAb-based therapeutics. Ultimately, anti-human DEC-205 clinical trials will be required to test functional DC targeting specificity.

Acknowledgements

We thank Ken Field and Dalia Khalil for assistance in flow cytometry analysis and cell sorting. We also thank Prof. Andrew Boyd for access to the biosensor facility. This project was supported by National Health and Medical Research Council of Australia.

Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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<tr>
<td>BDC</td>
<td>blood dendritic cells</td>
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<tr>
<td>CBB R250</td>
<td>Coomassie Brilliant Blue R250</td>
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<tr>
<td>CP</td>
<td>cytoplasmic domain</td>
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<td>CR</td>
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<td>CTLD</td>
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<td>DC</td>
<td>dendritic cells</td>
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<td>FN</td>
<td>fibronectin type II domain</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>Lin+</td>
<td>CD3+CD14+CD19+CD20+CD34+CD56+CD235a+</td>
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<td>MMR</td>
<td>macrophage mannose receptor</td>
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<td>MoDC</td>
<td>monocyte-derived DC</td>
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<td>PLA_R</td>
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References


