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Short communication

# Expression of DNAM-1 (CD226) on inflammatory monocytes

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ABSTRACT

DNAM-1 is an activating receptor expressed on NK cells and T cells and plays an important role in cytotoxicity of these cells against target cells. Although the role of DNAM-1 in the function of T cells and NK cells has been well studied, the expression and function of DNAM-1 on myeloid cells have been incompletely understood. In this study, we investigated expression of DNAM-1 on monocyte subsets in mouse peripheral blood and found that only inflammatory monocytes (iMos), but not patrolling monocytes (pMos), expressed high levels of DNAM-1. In addition, we found that DNAM-1 was highly expressed on iMos, rather than pMos, also in human. Furthermore, we found that DNAM-1 on inflammatory monocytes was involved in cell adhesion to CD155-expressing cells. Therefore, we propose that expression of DNAM-1 on inflammatory monocytes are evolutionally conserved and act as an adhesion molecule on blood inflammatory monocytes.



## 1. Introduction

DNAM-1, also known as CD226, is a member of the immunoglobulin superfamily and is constitutively expressed on the majority of NK cells, $CD8^+$ T cells, $CD4^+$ T cells, monocytes, and platelets in both humans and mice (Shibuya et al., 1996; Tahara-Hanaoka et al., 2005). CD155 (also known as poliovirus receptor (PVR), Necl-5 or Tage4) and CD112 (also known as PRR-2 or nectin-2) are ligands for human and mouse DNAM-1 (Bottino et al., 2003; Tahara-Hanaoka, 2004; Tahara-Hanaoka et al., 2005). CD155 and CD112 are broadly expressed on hematopoietic, epithelial, and endothelial cells in many tissues in humans and mice (Aoki et al., 1997; Bottino et al., 2003; Iwasaki et al., 2002; Lopez et al., 1998; Maier et al., 2007; Morrison and Racaniello, 1992; Ravens et al., 2003; Reymond et al., 2004; Tahara-Hanaoka et al., 2006). Interactions between DNAM-1 on NK cells or $CD8^+$ T cells and CD155 or CD112 on target cells enhances cell-mediated cytotoxicity against target cells and cytokine production (Bottino et al., 2003; Iguchi-Manaka et al., 2008; Martinet and Smyth, 2015; Nabekura et al., 2010; Tahara-Hanaoka, 2004; Verhoeven et al., 2008). Although the role of DNAM-1 as an activating receptor on NK cells and T cells has been well studied, expression and function of DNAM-1 on myeloid cell populations have not yet been well characterized. We previously observed that DNAM-1 is expressed on $CD11b^+$ macrophages/monocytes in mouse spleen (Tahara-Hanaoka et al., 2005) and human $CD14^+$ monocytes in the peripheral blood (Shibuya et al., 1996). However, expression and function on circulating monocyte populations in mouse peripheral blood remains undetermined.

Monocytes are divided into two populations: $CX_3CR1^{int}CCR2^+Ly6C^{hi}$ inflammatory monocytes (iMos) and $CX_3CR1^{hi}CCR2^-Ly6C^{lo}$ patrolling monocytes (pMos) (Geissmann et al., 2003; Gordon and Taylor, 2005). Human counterparts of these subsets are classical $CD14^+CD16^-$ monocytes (iMos) and non-classical $CD14^{lo}CD16^+$ monocytes (pMos) (Geissmann et al., 2003; Gordon and Taylor, 2005; Passlick et al., 1989). iMos are rapidly recruited into the site of infection and plays an important

*Abbreviations:* DNAM, DNAX accessory molecule-1; iMos, inflammatory monocytes; pMos, patrolling monocytes; CCR2, chemokine (C-C motif) receptor 2; $CX_3CR1$, chemokine (C-X3-C Motif) receptor 1; DC, dendritic cells; PBMC, peripheral blood mononuclear cells; mAb, monoclonal antibody; CFSE, carboxyfluorescein succinimidyl ester; MFI, mean fluorescence intensity.

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role in host defense against pathogens; in contrast, pMos are patrolling along the endothelium, migrate into noninflamed tissue and act as a first line of detection of pathogens (Auffray et al., 2007; Geissmann et al., 2003; Ginhoux and Jung, 2014; Soehnlein and Lindbom, 2010). One of the important steps of functions of iMos is to adhere to the blood vessels and migrate into inflamed peripheral tissue (Muller, 2011; Shi and Pamer, 2011).

Here, we found that iMos, but not pMos, express DNAM-1 and it is conserved in mice and human. Furthermore, DNAM-1 contributed to the adhesion of iMos to CD155-expressing cells. These results suggest that DNAM-1 on iMos plays and important role in cell–cell adhesion via interaction with CD155, and may contribute to the migration ability of iMos.

## 2. Materials and methods

### 2.1. Mice

C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). DNAM-1-deficient (*Cd226* $^{-/-}$) mice on the C57BL/6 background were generated as described previously (Iguchi-Manaka et al., 2008). All mice were 8–12-week-old and bred under specific pathogen-free conditions at the Laboratory Animal Resource Center (University of Tsukuba, Japan).

### 2.2. Flow cytometry analysis

Mouse peripheral bloods were collected by cardiac puncture and red blood cells were lysed by using ACK (ammonium–chloride–potassium) buffer. Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors and isolated by Ficoll density gradient following protocol of Lymphoprep (Stemcell Technologies, Vancouver, British Columbia, Canada). Ba/F3 transfectant expressing murine CD155 were generated as described previously (Tahara-Hanaoka et al., 2005).

FITC-conjugated anti-mouse CD11c (HL3) and CD49b/Pan-NK Cells (DX5), PE-conjugated anti-mouse Ly6G (1A8), Siglec-F (E50-2440), CD8 (53-6.7), and anti-human HLA-DR (G46-6), PE-Cy7-conjugated anti-mouse Ly6C (AL-21) and CD4 (RM4-5), APC-Cy7-conjugated anti-mouse CD11b (M1/70) and B220 (RA3-6B2) mAbs, biotin-conjugated isotype-matched control antibodies, and Horizon V450-conjugated streptavidin were purchased from BD Biosciences (San Jose, CA, USA). APC-conjugated anti-mouse CD3 (145-2C11) mAb was purchased from TONBO Biosciences (San Diego, CA, USA). FITC-conjugated anti-human CD16 (VEP13) and APC-conjugated anti-human CD14 (TÜK4) mAbs were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-mouse DNAM-1 (TX42) (Tahara-Hanaoka et al., 2005), CD155 (TX56) (Iguchi-Manaka et al., 2008) and anti-human DNAM-1 (TX25) mAbs were generated in our laboratory by standard method and conjugated with biotin. Propidium iodide was used to identify and exclude dead cells. Sample acquisition was performed by using FACSFortessa and FACSCallibur cell analyzer (BD Biosciences). FlowJo software (Tree Star, Ashland, OR, USA) was used for data analysis.

### 2.3. Adhesion assay

96 well flat-bottom culture plates (Costar, Corning, NY, USA) were pre-coated with Ba/F3 or Ba/F3 transfectants expressing CD155 overnight at 37 °C and 5% $CO_2$ in RPMI medium supplemented with 5% FBS. iMos were purified from mouse peripheral blood by using MACS cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously (Totsuka et al., 2014), labeled with carboxyfluorescein succinimidyl ester (CFSE), plated over the pre-coated transfectants at $2 \times 10^4$ cells/well, and then incubated for 1 h at 37 °C and 5% $CO_2$. The plate was gently washed with PBS once to remove non-adherent cells. For antibody-blocking assay, CFSE-labeled cells were pre-incubated with anti-mouse DNAM-1 mAb (TX42) or isotype-matched control antibody for 20 min at 4 °C, prior to plating. After washing with PBS once, adherent cells were imaged by KEYENCE BZ-X700 fluorescence microscope, and all CFSE positive cells in wells were counted by using BZ-X analyzer software (KEYENCE, Osaka, Japan). Percentages of adherent cells were calculated as (%) = (# adherent cells)/(# cells plated).

### 2.4. Statistical analysis

Statistical analyses were performed by using the unpaired two-sided Student's *t*-test (GraphPad Prism 5, GraphPad Software, La Jolla, CA, USA). *P* values less than 0.05 were considered statistically significant.

### 2.5. Ethics

All animal experiments were performed humanely after receiving approval and in accordance with the guidelines of the Animal Ethics Committee of the Laboratory Animal Resource Center, University of Tsukuba. Peripheral blood was obtained from healthy volunteers after informed consent was obtained; this study was approved by the ethical review boards of University of Tsukuba.

## 3. Results and discussion

### 3.1. DNAM-1 expression on leukocytes in mouse peripheral blood

Although DNAM-1 expression in mouse splenocytes was reported (Tahara-Hanaoka et al., 2005), DNAM-1 expression profiles on leukocyte subsets in mouse peripheral blood remains unclear. In addition, although the function of DNAM-1 on T cells and NK cells are well known (Bottino et al., 2003; Iguchi-Manaka et al., 2008; Martinet and Smyth, 2015; Nabekura et al., 2010; Tahara-Hanaoka, 2004; Verhoeven et al., 2008), the functional role of DNAM-1 in myeloid cells is incompletely understood. Therefore we aimed to investigate expression profile of DNAM-1 on mouse peripheral blood cells, especially on circulating myeloid cell populations. Peripheral bloods and splenocytes from wild type (WT) and DNAM-1-deficient (*Cd226*$^{-/-}$) mice were collected and DNAM-1 expression on myeloid cell subsets and lymphocytes subsets were analyzed by flowcytometry. After CD11c$^+$ DCs and Ly6G$^+$ neutrophils in the peripheral blood were gated out, CD11b$^+$ monocytes were divided into two populations on the basis of Ly6C expression (Fig. 1A and B). Eosinophils were gated by Siglec-F (Fig. 1C). Among myeloid cell subsets, we found that Ly6C$^{hi}$ iMos obtained from WT mice strongly expressed DNAM-1. In contrast, Ly6C$^{lo}$ pMos did not express DNAM-1, showing a striking difference of DNAM-1 expression on these distinct monocyte subsets (Fig. 1A and B).

Surprisingly, DNAM-1 was expressed on most circulating neutrophils at an intermediate level (Fig. 1A). This result was contrary to splenic neutrophils of which only a small subset expressed low levels of DNAM-1 (Supplementary figure), indicating that expression of DNAM-1 on neutrophils is different between the peripheral blood and the spleen. DNAM-1 was also expressed on most eosinophils and on a small population of dendritic cells (Fig. 1A and C). DNAM-1 expression on CD4$^+$ and CD8$^+$ T cells and NK cells in peripheral blood of mice (Fig. 1D) were similar to that in spleen cells (Supplementary figure).