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.

Key words
DNAM-1 (CD226); inflammatory monocytes; patrolling monocytes; adhesion

Abbreviations
DNAM-1, 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

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 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 2×10^4 cells/well, and then incubated for 1 hour 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 minutes 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, 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, 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, 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).

3.2. DNAM-1 expression on human monocytes.

We next investigated DNAM-1 expression on human counterparts of monocyte subsets. PBMCs were isolated from healthy donors and analyzed by flowcytometry. After excluding $CD14^-CD16^-$ cells (T cells, B cells, and DCs) and $HLA\text{-}DR^-CD16^+$ cells (contaminated neutrophils and NK cells) (Abeles et al., 2012), $CD14^+CD16^-$ iMos and $CD14^{lo}CD16^+$ pMos were analyzed. Similar to mouse iMos, $CD14^+CD16^-$ human iMos strongly expressed DNAM-1 (Fig. 2A). In contrast, pMos, defined as $CD14^{lo}CD16^+$ cells, scarcely expressed DNAM-1 (Fig. 2A). Five independent donors were studied and the mean

fluorescent intensity of DNAM-1 on iMos was significantly higher than that of pMos (Fig. 2B). Thus, selective expression of DNAM-1 on iMos is conserved between mice and humans, suggesting that DNAM-1 is evolutionally conserved and plays an important role in the function of iMos. It is known that heterogeneity of monocytes is conserved among mammalian species including human, mouse, rat, and pig (Ancuta et al., 2009; Gordon and Taylor, 2005). Expression of some chemokine receptors and adhesion molecules is conserved between species. Among these, stronger expression of surface molecules that contribute to the major function of each subsets, such as CCR2 and CD62L on iMos and CX_3CR1 on pMos, appears to be well conserved (Gordon and Taylor, 2005). In this context, DNAM-1 can be newly recognized as surface molecule that defines two subsets of monocytes. DNAM-1 expression on other mammalian species is of interest.

3.3. DNAM-1 is involved in cell adhesion of mouse iMos.

Because DNAM-1 is an adhesion molecule (Shibuya et al., 1996), we next addressed the involvement of DNAM-1 in adhesion ability of iMos. Although CD155 and CD112 are ligands for DNAM-1 and both ligands are expressed on human endotherial cells (Lopez et al., 1998; Reymond et al., 2004), a previous report suggested that CD155 is solely

an important ligand on human endothelial cells for DNAM-1 (Reymond et al., 2004). Therefore we examined the role of DNAM-1 on iMos in adhesion to CD155. Ba/F3 or Ba/F3 transfectant expressing CD155 (Fig.3A) were seeded on a 96 well cell culture plate, and then CFSE-labeled iMos from peripheral blood of WT or *Cd226*$^{-/-}$ mice were added over the plate. After washing, remaining of iMos was counted under fluolescent microscope. iMos from *Cd226*$^{-/-}$ mice showed lower ability of adhesion to CD155-expressing Ba/F3 transfectants compared with those from WT mice; in contrast, this difference in adhesion ability was not observed in Ba/F3 parental cells (Fig. 3B, C). Furthermore, adhesion of iMos was downregulated when iMos were pre-incubated with anti-DNAM-1 neutralizing antibody (Fig. 3D). Taken together, these results indicate that DNAM-1 is involved in iMos adhesion to CD155-expressing cells. Given that CD155 is expressed on mouse endothelial cells (Maier et al., 2007), our results suggest that DNAM-1 may be involved in transendotherial migration of mouse iMos. Although previous reports showed that interaction of DNAM-1 on human monocytes with CD155 on endothelial cells was involved in transmigration *in vitro* (Manes and Pober, 2011; Reymond et al., 2004; Sullivan et al., 2013), physiological role of DNAM-1 −CD155 interaction in monocyte transmigration has not been addressed *in vivo*. Since iMos highly expressed DNAM-1 in mice as well, contribution of DNAM-1−CD155 interaction

could be observed *in vivo* model in mice.

pMos crawl along the endothelial cells of blood vessel in steady state and rapidly migrate out of the circulation into inflamed tissue within 1 hour after inflammation occurs (Auffray et al., 2009, 2007; Geissmann et al., 2003; Soehnlein and Lindbom, 2010). In contrast, iMos are selectively recruited into inflamed tissues and lymph nodes after several hours from the initiation of infection (Auffray et al., 2009, 2007; Shi and Pamer, 2011). The difference of the migratory characteristics of these monocyte subsets has been explained by expression profile of chemokine receptors such as CCR2 and CX_3CR1 (Ancuta et al., 2009; Gordon and Taylor, 2005). Here we revealed that DNAM-1 is expressed on iMos, but not on pMos, in humans and mice and that DNAM-1 on mouse iMos is involved in iMos adhesion to CD155-expressing cells, suggesting that DNAM-1 is involved in transmigration of iMos through endothelial cells, which express CD155, into inflamed tissues. Although iMos in the bloodstream are derived from the bone marrow following bacterial infection (Ginhoux and Jung, 2014; Serbina and Pamer, 2006; Shi and Pamer, 2011), the dynamics of DNAM-1 expression on iMos in the bone marrow, blood and inflamed tissue remain unclear. However, since DNAM-1 expression is upregulated on T cells, NK cells, and platelets during their proliferation and/or activation (Alici et al., 2008; Caruso et al., 2015; Nabekura et al., 2010),

1 it might be possible that activation of iMos after infection upregulates DNAM-1 expression
2 on iMos. Nonetheless, since CD155 expression is upregulated in inflamed liver (Erickson et
3 al., 2006), transmigration of iMos trough endothelial cells may be promoted at inflamed sites
4 as a result of increased interaction between DNAM-1 and CD155.
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Figure Legends

Figure 1. DNAM-1 expression on leukocytes in mouse peripheral blood.

DNAM-1 expression on leukocytes populations in peripheral blood from naïve C57BL/6 wild-type (WT) or DNAM-1-deficient (*Cd226*$^{-/-}$) mice was detected by flow cytometry. After gating PI^{-} viable cells, CD11c^{+} dendritic cells (DC), Ly6G^{+} Neutrophils, Ly6C^{-} patrolling monocytes (pMo), and Ly6C^{hi} inflammatory monocytes (iMo) (A, B), Siglec-F^{+} eosinophils (C), B220^{+} B cells, CD4^{+} T cells, CD8^{+} T cells, and NK cells (D) were analyzed.

(A, C, D) Representative plots of staining of surface markers and DNAM-1. Open and shaded histograms indicate staining with anti-mouse DNAM-1 and isotype control mAb, respectively. Numbers indicate percentage of the population in each region and the mean fluorescence intensity (MFI). Data are representative of three independent experiments. (B) Scatter plot of MFI value of DNAM-1 expression and percentage of DNAM-1-positive cells in peripheral blood monocytes from WT (n=5) or *Cd226*$^{-/-}$ (n=3) mice. Error bars indicate SEM. **P* <0.05. The MFI was determined by subtracting the MFI by staining with isotype control mAb from that by staining with anti-mouse DNAM-1 mAb.

Figure 2. DNAM-1 expression on human monocytes.

DNAM-1 expression on monocyte subsets in peripheral blood mononuclear cells from healthy donor was detected by flowcytometry. After gating monocytes based on FSC/SSC plot and gating PI^- viable cells, HLA-DR^-CD16^+ cells (contaminated neutrophils and NK cells) were excluded, and then $CD14^{lo}CD16^+$ patrolling monocytes (pMo) and $CD14^+CD16^-$ inflammatory monocytes (iMo) were analyzed.

(A) Representative plots of staining of surface markers and DNAM-1. Numbers indicate percentages of the population in each region. Open histograms indicate staining with anti-human DNAM-1 mAb and shaded histograms indicate staining with isotype control.

(B) Scatter plot of MFI value of DNAM-1 expression on monocytes from 5 different donors. The value was obtained by subtracting the MFI of the anti-human DNAM-1 mAb stained cells from the MFI of isotype control. Error bars indicate SEM. $*P < 0.05$.

Figure 3. DNAM-1 is involved in cell adhesion of mouse inflammatory monocytes.

(A) Expression of CD155 on Ba/F3 transfectant expressing mouse CD155 was detected by using anti-mCD155 (open histogram). Shaded histograms indicate staining with isotype control.

(B, C) Ba/F3 and its transfectants expressing CD155 were seeded on a 96 well cell culture plate and cultured overnight. $CCR2^{hi}$ inflammatory monocytes (iMos) were MACS-isolated from peripheral blood of wild type (WT) and DNAM-1 deficient ($Cd226^{-/-}$) mice, labeled with CFSE and plated over the pre-coated transfectants, following incubation for 1 hour. The cell-culture medium was aspirated and the cells were washed with PBS. The adherent cells were determined by counting CFSE-positive cells under fluorescence microscope. All cells in wells were counted. Representative images of WT and $Cd226^{-/-}$ iMos on CD155-expressing transfectants (B); Scale bar = 100 μm. Bar graph shows the average of percentages of adherent cells in triplicate wells (C).

(D) Transfectants expressing CD155 were prepared as in B. iMos were isolated from wild type mice, labeled with CFSE as in B and pre-incubated with blocking anti-DNAM-1 antibody (anti-DNAM-1) or its isotype control rat IgG2a (control Ig) before being plated over the transfectants. After incubation for 1 hour, the cells were PBS-washed and observed as in B. Bar graph shows the average of percentages of adherent cells in triplicate wells.

Percentages of adherent cells were calculated as (%) = (# adherent cells) / (# cells plated). Error bars indicate SEM. $*P < 0.05$. *NS*, not significant. Data are representative of two independent experiments.

1

2

Supplementary Figure Legends

Supplementary Figure. DNAM-1 expression on leukocytes in mouse spleen.

Spleen cells from wild-type (WT) or DNAM-1-deficient (*Cd226*$^{-/-}$) C57BL/6 mice was stained with anti-mouse DNAM-1 mAb (open histogram) or isotype control mAb. (shaded histograms) together with mAbs indicated against each lineage marker. After gating PI$^-$ viable cells, CD11c$^+$ dendritic cells (DC) (A), CD11b$^+$Ly6G$^+$ neutrophils (B), CD11c$^-$Ly6G$^-$CD11b$^+$ macrophages (C), Siglec-F$^+$ eosinophils (D), B220$^+$ B cells, CD4$^+$ T cells, CD8$^+$ T cells, and NK cells (E) were analyzed for DNAM-1 expression. Numbers indicate percentage of the population in each region and MFI.

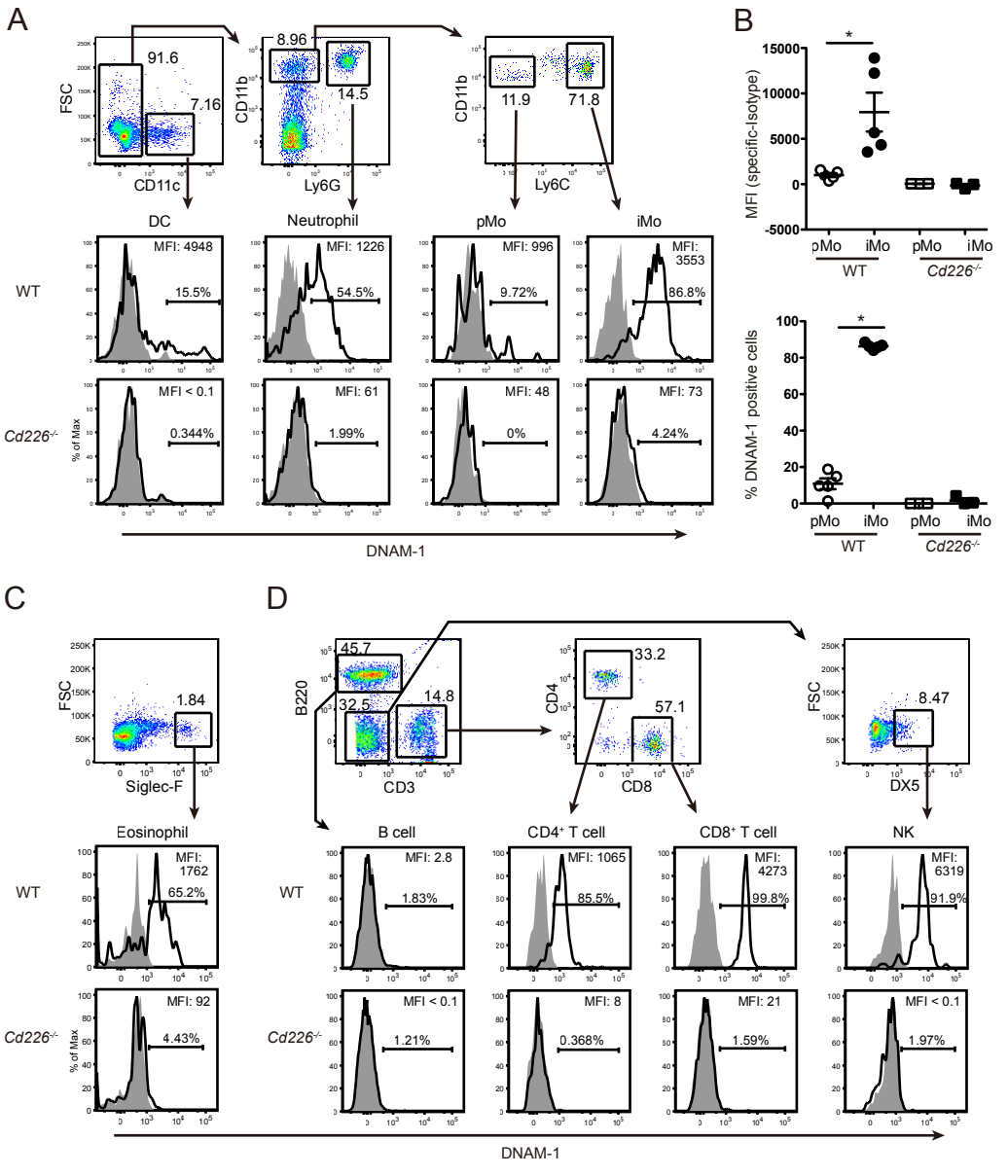

A
91.6
7.16
FSC
CD11c
8.96
14.5
CD11b
Ly6G
11.9
71.8
CD11b
Ly6C
DC
Neutrophil
pMo
iMo
WT
MFI: 4948
15.5%
MFI: 1226
54.5%
MFI: 996
9.72%
MFI: 3553
86.8%
Cd226-/-
MFI < 0.1
0.344%
MFI: 61
1.99%
MFI: 48
0%
MFI: 73
4.24%
% of Max
DNAM-1
B
MFI (specific-Isotype)
15000
10000
5000
0
-5000
*
pMo
iMo
pMo
iMo
WT
Cd226-/-
% DNAM-1 positive cells
100
80
60
40
20
0
*
pMo
iMo
pMo
iMo
WT
Cd226-/-
C
FSC
1.84
Siglec-F
Eosinophil
WT
MFI: 1762
65.2%
Cd226-/-
MFI: 92
4.43%
% of Max
D
45.7
32.5
14.8
B220
CD3
33.2
57.1
CD4
CD8
FSC
8.47
DX5
B cell
CD4+ T cell
CD8+ T cell
NK
WT
MFI: 2.8
1.83%
MFI: 1065
85.5%
MFI: 4273
99.8%
MFI: 6319
91.9%
Cd226-/-
MFI < 0.1
1.21%
MFI: 8
0.368%
MFI: 21
1.59%
MFI < 0.1
1.97%
DNAM-1

Figure 1.

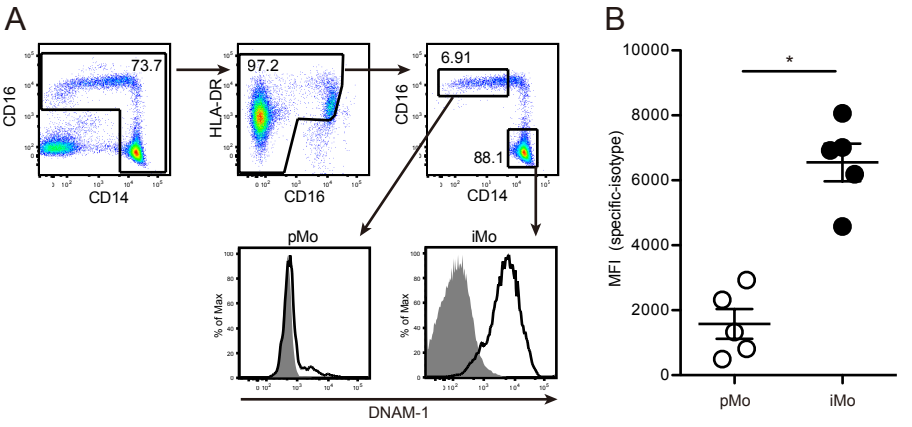

A
73.7
CD16
CD14
97.2
HLA-DR
CD16
6.91
88.1
CD16
CD14
pMo
iMo
% of Max
% of Max
DNAM-1
B
10000
8000
6000
4000
2000
0
MFI (specific-isotype)
*
pMo
iMo

Figure 2.

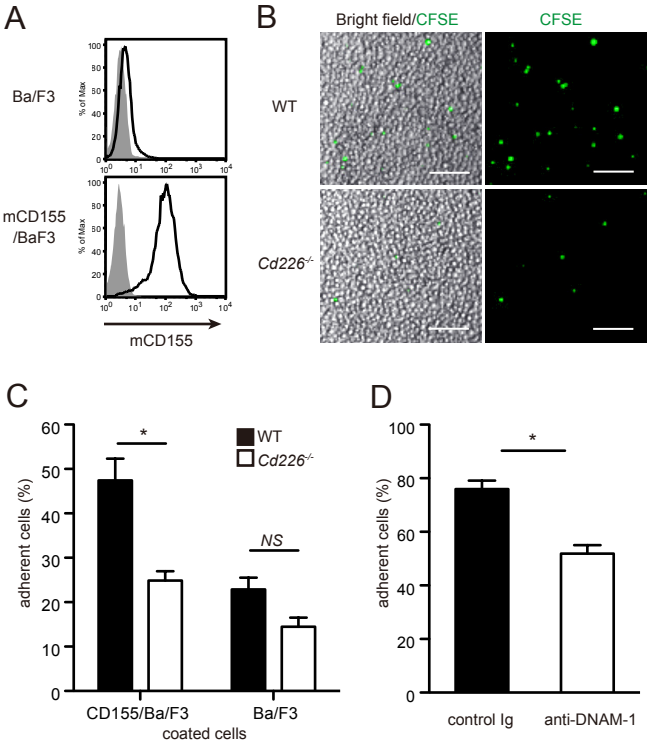
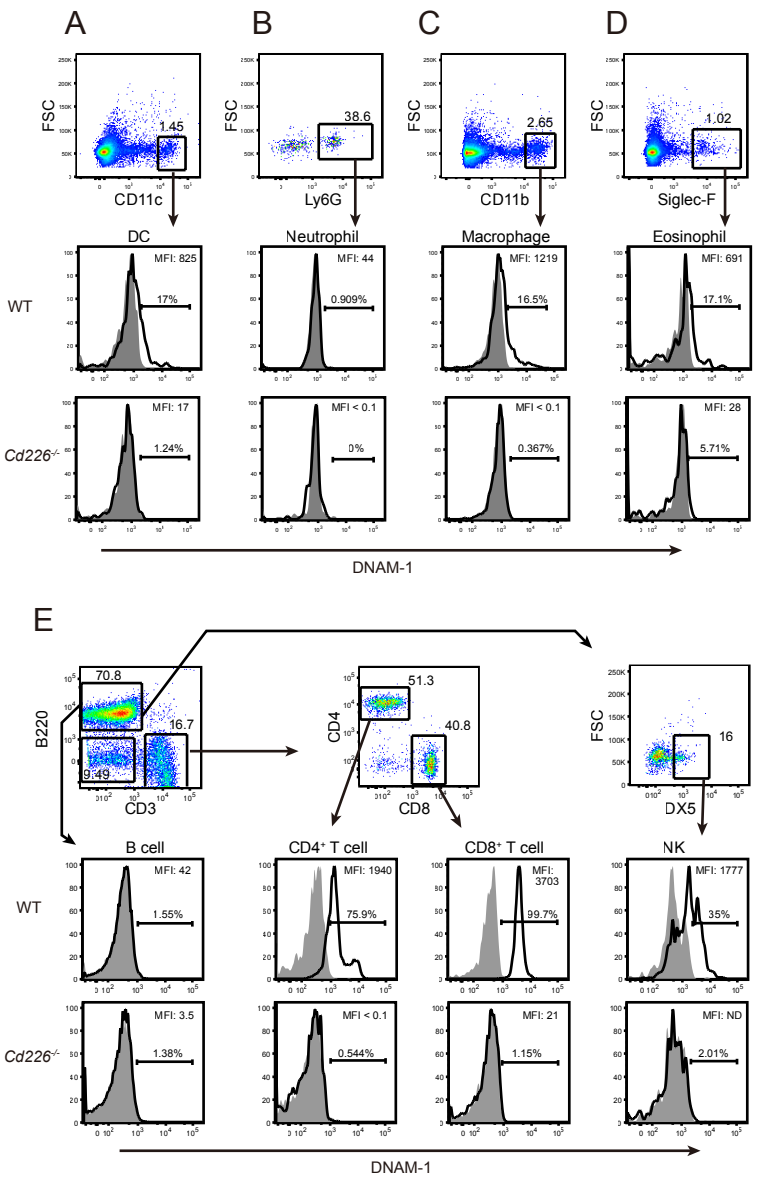

A
Ba/F3
mCD155
/BaF3
% of Max
mCD155
B
Bright field/CFSE
CFSE
WT
Cd226-/-
C
adherent cells (%)
WT
Cd226-/-
*
NS
CD155/Ba/F3
Ba/F3
coated cells
D
adherent cells (%)
*
control Ig
anti-DNAM-1

Figure 3.



Supplementary Figure