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# **Identification and characterization of CD300H, a new member of the human CD300 immunoreceptor family**

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Running title: Identification and characterization of CD300H

**Background:** CD300-family molecules regulate innate immune responses.

**Results:** We identified and characterized a novel immunoreceptor, CD300H.

**Conclusion:** CD300H mediates neutrophil chemoattractants production.

**Significance:** CD300H may play an important role in innate immunity.

## Abstract

Recruitment of circulating monocytes and neutrophils to infection sites is essential for host defense against infections. Here, we identified a previously unannotated gene that encodes an immunoglobulin-like receptor, designated CD300H, and is located in the CD300 gene cluster. CD300H has a short cytoplasmic tail and associates with the signaling adaptor proteins, DAP12 and DAP10. CD300H is expressed on CD16<sup>+</sup> monocytes and myeloid dendritic cells. Ligation of CD300H on CD16<sup>+</sup> monocytes and myeloid dendritic cells with anti-CD300H monoclonal antibody induced the production of neutrophil chemoattractants. Interestingly, CD300H expression varied among healthy subjects, who could be classified into two groups according to “positive” and “negative” expression. Genomic sequence analysis revealed a single-nucleotide substitution [rs905709 (G>A)] at a splice donor site on intron 1 on either one or both alleles. The international HapMap project database has demonstrated that homozygosity for the A allele of SNP rs905709 (“negative” expression) is highly frequent in Han Chinese in Beijing, Japanese in Tokyo, and Europeans (A/A genotype frequencies 0.349, 0.167, and 0.138 respectively) but extremely rare in Sub-Saharan African populations. Together, these results suggest that CD300H may play an important role in innate immunity, at least in populations that carry the G/G or G/A genotype of *CD300H*.

## Introduction

Recruitment of blood leukocytes to sites of infection is essential for host defense against infection. Circulating monocytes (Mo) and neutrophils are especially important effectors in the initiation of inflammatory responses to microbes (1). Mo in the human peripheral blood are divided into subsets on the basis of their cell-surface expression of CD14 and CD16 (2). A major subset with a phenotype of CD14<sup>+</sup>CD16<sup>-</sup> is referred to as classical or inflammatory Mo (iMo); these are similar to Ly6C<sup>hi</sup>CCR2<sup>hi</sup> Mo in mice (3). The CD16<sup>+</sup> Mo subset can further be divided into two subsets, namely CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>dim</sup>CD16<sup>+</sup>, on the basis of CD14 expression (4). CD14<sup>dim</sup>CD16<sup>+</sup> Mo resemble Ly6C<sup>low</sup>Cx3CR1<sup>hi</sup> Mo in mice and are referred to as patrolling Mo (pMo) (5-7). pMo crawl along the luminal side of the vascular endothelium in the steady state. CD14<sup>+</sup>CD16<sup>+</sup> Mo are referred to as intermediate monocytes (intMo) and are found at low frequency (approximately 5% of blood monocytes) (8) in the peripheral blood; they represent a continuous transition from iMo (CD14<sup>+</sup>CD16<sup>-</sup>) to pMo (CD14<sup>dim</sup>CD16<sup>+</sup>) (7,9). iMo produce TNF- $\alpha$ , IL-6, and IL-1 in response to bacteria-associated signals. In contrast, pMo respond poorly to bacterial components but potently to viruses and nucleic acids; they thus selectively detect viral infection and injury and produce inflammatory cytokines (4). In mice, Ly6C<sup>low</sup> pMo provide immune surveillance and rapidly extravasate before neutrophils in response to *Listeria monocytogenes* infection (5). In addition,

activation of intravascular Ly6C<sup>low</sup> pMo is responsible for neutrophil recruitment via TLR7-dependent CXCL1 production (6). However, the precise mechanism by which pMo produce chemokines—particularly in humans—remains unclear.

CD300-family molecules are type 1 immunoreceptors belonging to the immunoglobulin superfamily and are encoded by seven genes on human chromosome 17 and nine genes on mouse chromosome 11 (10,11). They are expressed on myeloid lineage cells, including monocytes–macrophages, granulocytes, dendritic cells, and mast cells, suggesting that they play an important role in innate immunity. CD300A (also named MAIR-I (12), LMIR1 (13), and CLM-8 (14) in mice) and CD300LF (MAIR-V (15,16), LMIR3 (17-20), and CLM-1 (21) in mice) mediate inhibitory signals via the immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic regions. By contrast, CD300LB (LMIR5 (22)), CD300C, CD300LD (MAIR-IV (23), LMIR4 (17,24), and CLM-5 (25)), and CD300E have short cytoplasmic tails with no signaling motifs. However, CD300LB and CD300E each contain a positively charged lysine (K) residue, whereas CD300C and CD300LD contain a negatively charged glutamic acid (E) in their transmembrane domains (10) (11). These receptors noncovalently associated with membrane-bound signaling adaptor proteins, including DNAX adaptor protein 12 (DAP12), DAP10, and the  $\gamma$  chain of the Fc receptor for IgE (Fc $\epsilon$ RI $\gamma$ ) through interaction with a

negatively charged amino acid [aspartic acid (D)] in the transmembrane domain of the adaptors and thus transmit activating signals (10,11). DAP12 and Fc $\epsilon$ RI $\gamma$  contains ITAM in its cytoplasmic region, whereas DAP10 contains a YxxM motif, a potential src homology 2 (SH2) domain-binding site for the p85 regulatory subunit of the phosphatidylinositol (PI)3-kinase. Among the seven genes encoding human CD300 molecules, those encoding CD300A, CD300LB, CD300C, CD300E, CD300LF, and CD300LG each have mouse homologs, as determined from their functional, molecular, and genetic characteristics (10,11). However, the mouse counterpart of human CD300LD and the human counterpart of mouse MAIR-II (CLM-4/LMIR2) and MAIR-VI (CLM-3/LMIR7) are unclear.

Here, we identified a previously unannotated gene encoding a novel molecule, designated CD300H, in the CD300 family gene cluster on human chromosome 17. We demonstrate that CD300H is expressed on CD14<sup>+</sup>CD16<sup>+</sup> intMo and CD14<sup>dim</sup>CD16<sup>+</sup> pMo and associates with DAP12 or DAP10. Upon crosslinking of CD300H on CD16<sup>+</sup>Mo and CD11c<sup>+</sup> dendritic cell (DC), it mediates an activating signal for the production of neutrophil chemoattractants.

## **Experimental procedures**

### **Cloning of human *CD300H* cDNA**

Full-length cDNA of human *CD300H* was isolated from human CD14<sup>+</sup> monocyte-derived cDNA by reverse transcription–polymerase chain reaction (RT-PCR) using *CD300H*-specific primers (5'-ATGACCCAGAGGGCTGGGGC-3' and 5'-TCATGACTCTGTCCAAGGAG-3').

### **Generation of Fc fusion protein**

Fusion proteins of the entire extracellular domains of CD300H, CD300A, and CD300C with the Fc portion of human immunoglobulin (Ig) G1 (CD300H-Fc, CD300A-Fc, and CD300C-Fc, respectively) were generated as described previously (26).

### **Antibodies and flow cytometry analyses**

Monoclonal antibody (mAb) against CD300H (TX93; mouse IgG2a) was generated in our laboratory by immunizing mice with CD300H-Fc (12); mAb against CD300C (TX47; mouse IgG1) was generated by immunizing mice with BW5147 transfectant expressing CD300C, as described previously (12). MAb against CD300A and CD300C (TX49; mouse IgG1) was generated in our laboratory, as described previously (27). MAbs specific to human CD3 (clone UCHT1), CD14 (clone M5E2), CD56 (clone B159), CD11c (clone B-ly6), CD16 (clone 3G8), HLA-DR (clone G46-6), CD40 (clone 5C3), CD80 (clone BB1), and CD86 (clone 2331) were purchased from BD Biosciences (San Jose, CA). MAbs specific to human CD123 (clone

AC145), CD203c (clone FR3-16A11), and CD304 (clone AD5-17F6) were purchased from Miltenyi Biotec (Auburn, CA). Flow cytometry analyses were performed with a FACS Fortessa flow cytometer (BD Biosciences). FlowJo software (Tree Star, Ashland, OR) was used for data analyses.

### **Phylogenetic analysis**

The protein sequences of human CD300-family molecules were aligned by using the ClustalW program (28). NJplot was used to construct tree diagrams (29).

### **Cells and transfectants**

Murine T cell lines 2B4 and BW5147, human monocytic cell lines THP-1 and U937 and human embryonic kidney cell line 293T were used; they were cultured in RPMI1640 medium containing 5% fetal calf serum (FCS) except for 293T. 293T were cultured in DMEM containing 10% FBS. Blood samples were collected from healthy donors at the University of Tsukuba under an institutional review board–approved protocol. Written informed consent was obtained from the donors. Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood with LymphoPrep (Axis-Shield, Oslo, Norway) in accordance with the manufacturer's protocol. CD16<sup>+</sup> Mo were isolated from PBMCs by using the MACS cell separation system (Miltenyi Biotec); NK cells were depleted from the PBMCs by using anti-CD56 MicroBeads, and then CD16<sup>+</sup> Mo were purified from the NK cell-depleted PBMCs by using anti-CD16

MicroBeads. The purity of the CD16<sup>+</sup> Mo was more than 85%, as determined by flow cytometry. CD16<sup>-</sup> Mo were isolated from PBMCs by using the MACS cell separation system (Miltenyi Biotec): CD16<sup>+</sup> cells were depleted from the PBMCs by using anti-CD16 MicroBeads, and then CD16<sup>-</sup> Mo were purified from the CD16<sup>+</sup> cell-depleted PBMCs by using anti-CD14 MicroBeads. The purity of the CD16<sup>-</sup> Mo was more than 90%, as determined by flow cytometry. CD11c<sup>+</sup> dendritic cells (DCs) were isolated from PBMCs by using the Myeloid dendritic cell isolation kit (Miltenyi Biotec). The purity of the CD11c<sup>+</sup> DC was more than 95%, as determined by flow cytometry. 2B4, U937, and BW5147 transfectants stably expressing CD300H, CD300A, or CD300C tagged with Flag or HA at the N-terminus were established, as described previously (12). 293T transfectant transiently expressing Flag-tagged CD300Hs was established by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

### **SNP genotyping**

Genomic DNA was extracted and purified from peripheral blood leukocyte using the QuickGene 610 L system (Fujifilm, Tokyo, Japan). Polymorphism was analyzed by genomic PCR with primer pairs 5'-AAACCCAGAAGAGGCCAGAG-3' and 5'-GGTTTGGACCTTGACTGTGC-3' and subsequent direct sequencing with the Big Dye Termination Kit (Applied Biosystems, Foster City, CA, USA).

### **Biochemical analyses**

Cells were lysed in lysis buffer containing 1% NP-40 (Sigma-Aldrich, St. Louis, MO) or digitonin (Calbiochem, Darmstadt, Germany) supplemented with protease inhibitors, as described previously (26). Cell lysates were immunoprecipitated with control Ig, anti-Flag mAb (M2; Sigma-Aldrich), anti-HA (3F10; Roche Applied Science, Mannheim, Germany), anti-DAP12 polyclonal antibody (FL-113; Santa Cruz Biotechnology, Santa Cruz, CA), anti-DAP10 (H-2; Santa Cruz Biotechnology) or anti-FcεRIγ-chain polyclonal antibody (06-727; Millipore, Billerica, MA). Immunoprecipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes by electroblotting, and then immunoblotted with anti-Flag polyclonal antibody (F7425; Sigma-Aldrich), anti-HA (3F10; Roche Applied Science), anti-DAP12 (FL-113; Santa Cruz Biotechnology), anti-DAP10 (H-2; Santa Cruz Biotechnology), or anti-FcεRIγ chain (T2040; US Biological) followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (eB182; eBioscience, San Diego, CA), anti-mouse IgG (eB144; eBioscience), or anti-rat IgG (NA935; GE Healthcare UK Limited, Buckinghamshire, UK).

### **Stimulation of CD300H for cytokine and chemokine production**

CD16<sup>+</sup> Mo ( $5 \times 10^4$  or  $1 \times 10^5$ /well), CD16<sup>-</sup> Mo, and CD11c<sup>+</sup> DC ( $5 \times 10^4$  / well) were plated into 96-well flat-bottom plates precoated

with 10 µg/mL of F(ab')<sub>2</sub> fragments of either anti-CD300H (TX93) or control mouse IgG and cultured for 4 or 24 h at 37 °C in 5% CO<sub>2</sub>. TNF-α concentration in the culture supernatants were measured with ELISA kits purchased from BD Biosciences, concentrations of IL-12p70, TNF-α, IL-10, IL-6, IL-1β and IL-8 in the culture supernatants were measured with BD<sup>TM</sup> cytometric bead array (BD Biosciences), and the mRNAs of *IL6*, *CXCL1*, *CXCL2*, *CXCL5*, and *CXCL8* were quantified by real-time quantitative PCR. The sequences of the specific primers were:

*IL-6* forward,  
5'-GATGAGTACAAAAGTCCTGATCCA-3',  
*IL-6* reverse,  
5'-CTGCAGCCACTGGTTCTGT-3';  
*CXCL1* forward,  
5'-TCCTGCATCCCCATAGTTA-3',  
*CXCL-1* reverse,  
5'-CTTCAGGAACAGCCACCAGT-3';  
*CXCL2* forward,  
5'-CTTGTCTCAACCCCGCATCG-3',  
*CXCL2* reverse,  
5'-TCCTTCAGGAACAGCCACCA-3';  
*CXCL5* forward,  
5'-TTCGCCATAGGCCACAGT-3',  
*CXCL5* reverse,  
5'-TTTCCATGCGTGCTCATTTCTC-3'; and  
*CXCL8* forward,  
5'-AGACAGCAGAGCACACAAGC-3',  
*CXCL8* reverse,  
5'-CACAGTGAGATGGTTCCTTCC-3'.

#### cDNA synthesis and RT-PCR

Total RNA was extracted with Isogen reagent

(Nippon Gene, Tokyo, Japan), and cDNA was synthesized by using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed with SYBR Green master mix (Applied Biosystems). Expression of each target gene was normalized against that of *GAPDH* (primer sequences 5'-CTTCACCACCATGGAGAAGGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3').

#### Neutrophil migration assay

Neutrophils were isolated from whole blood with Polymorphprep (Axis-Shield) in accordance with the manufacturer's protocol. Supernatant obtained from TX93-stimulated CD16<sup>+</sup> Mo was added to the lower compartment of 96-well Transwell plates (pore size 3.0 µm; Corning, New York, USA) at a total volume of 235 µL per well. Neutrophils (1 × 10<sup>5</sup>) were placed in the upper compartment at a total volume of 75 µL, and then the plates were incubated for 30 min at 37 °C in 5% CO<sub>2</sub>. Cells in the lower compartment were collected and counted by using a Guava easyCyte Mini flow cytometer (Millipore).

#### Statistical analyses

The unpaired Student's *t*-test was used for statistical analyses. *P* values of less than 0.05 were considered statistically significant. All statistical analyses were carried out using GraphPad Prism 5.0c software (GraphPad Software, San Diego, CA, USA).



## Results

### *Cloning of the gene encoding human CD300H*

In our search for a human homolog of MAIR-II in the National Center for Biotechnology Information database, we found a previously unannotated gene located in the human CD300 gene cluster on chromosome 17 (Figure 1A). We isolated full-length cDNA from human CD14<sup>+</sup> Mo-derived cDNA by RT-PCR. Sequence analysis revealed that an open reading frame of 603 bp encoded a type I transmembrane receptor, which contained a 20-amino-acid (aa) leader sequence, a 148-aa extracellular region containing an Ig-like domain, a 23-aa transmembrane region, and a 10-aa cytoplasmic domain with no signaling motif (Figure 1B). The Ig-like domain contained a potential N-glycosylation site. The transmembrane domain contained a positively charged lysine residue (Figure 1C). Because this homolog was the eighth member of the human CD300 family of receptors, we termed it CD300H. We also identified a splicing variant that lacked a transmembrane domain; we called this variant CD300Hs (Figure 1B, C). A database search demonstrated that CD300H had 44% amino acid identity with mouse MAIR-II/CLM-4/LMIR2 and CD300c/CLM-6, but it had lower rates of identity with CD300e/CLM-2/LMIR6 (42%), CLM-3/LMIR7 (35%), CD300ld/CLM-5/LMIR4 (40%), CD300lb/CLM-7/LMIR5 (35%), and CD300lg/CLM-9 (35%) among the non-ITIM-bearing receptors. The gene

encoding CD300H was located between *CD300C* and *CD300LD* on human chromosome 17q25.1 (Figure 1A). We analyzed the degree of identity of the extracellular Ig domain of CD300H with the sequences of CD300 family members and constructed a molecular phylogenetic tree by using ClustalW2 phylogeny. The Ig-like domains of CD300A and CD300C had the greatest identity (53%) with that of CD300H; other members of the family had lower identity levels (Figure 1D).

### *Expression of CD300H*

To investigate the cellular distribution of CD300H, we generated a mAb against CD300H (clone TX93) and analyzed its specificity. TX47 and TX49 were previously generated mAbs against CD300C, and CD300A and CD300C, respectively (Figure 2B and C) (27). Although CD300H had a high level of identity with CD300A and CD300C, TX93 mAb bound specifically to CD300H-expressing THP-1 and 2B4 transfectants but not to CD300A- or CD300C-expressing BW5147 transfectants (Figure 2A and B). In addition, TX93 mAb bound to CD300H-Fc fusion protein but not to CD300A-Fc or CD300C-Fc protein (Figure 2C). These results indicated that TX93 mAb specifically recognized CD300H.

Flow cytometry analyses of human peripheral blood cells demonstrated that CD300H was expressed on myeloid lineage cells, including CD16<sup>+</sup> Mo (Figure 3E and H) and myeloid DCs (n=9, Figure 3C and H) but

not on plasmacytoid DCs (n=3, Figure 3D). By contrast, CD300H was not expressed on lymphocytes (T cells, B cells, and NK cells) (n=3 in each, Figure 3A and B) or granulocytes (eosinophils, neutrophils, and basophils) (n=3 in each, Figure 3F and G). CD16<sup>+</sup> Mo can be divided into two subsets (namely CD14<sup>dim</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>+</sup> Mo) (4) on the basis of their CD14 expression; mean fluorescence intensity (MFI) of CD300H expression was comparable between these subsets ( $p=0.5816$ ,  $n=13$  in each, Figure 3E and H). Compared to CD14<sup>dim</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>+</sup> Mo, CD16<sup>-</sup> Mo (n=13) exhibited significantly low-level of CD300H expression ( $p=0.0234$  and  $p=0.0270$ , respectively, Figure 3E and H).

#### ***Individual differences in CD300H expression***

Genomic DNA database analysis demonstrated that *CD300H* consisted of four exons (Figure 4A), but the gene encoding CD300Hs lacked exon 3, which encoded the transmembrane region. Interestingly, we observed that 4 out of the 13 individuals examined in Figure 3 showed no detectable expression of CD300H on CD16<sup>+</sup> Mo (open circle, Figure 3H). Genomic DNA analyses of these individuals demonstrated a single-nucleotide substitution (rs905709; G>A) of *CD300H*, that abolished the intron 1 donor splice site (Figure 4A). This can lead to loss of CD300H transcript expression through the action of nonsense-mediated mRNA decay machinery (30). Indeed, persons carrying the rs905709 A/A genotype showed a loss of CD300H transcripts (Figure

4B) and protein (Figure 3H and 4C). In contrast, CD16<sup>+</sup> Mo derived from subjects with G/A or G/G genotype expressed the CD300H transcript (Figure 4B) and protein on their cell surfaces (Figure 3H and 4C). The A/A genotype frequency of rs905709 differed significantly in the different ethnic populations and is observed at high frequency in Han Chinese in Beijing, China (HCB), Japanese in Tokyo, Japan (JPT) and CEPH (Utah Residents with Northern and Western European Ancestry, CEU) (A/A genotype frequencies 0.349, 0.167, and 0.138, respectively). However, it is extremely rare in Yoruba in Ibadan, Nigeria (YRI) (Figure 4D) (31).

#### ***CD300H associates with DAP12 and DAP10 in U937 cells***

To analyze the biochemical characteristics of CD300H, we generated a BW5147 transfectant stably expressing Flag-tagged CD300H at the N-terminus. Immunoprecipitation of CD300H protein from lysates of BW5147 transfectant and subsequent immunoblotting with anti-Flag antibody revealed that human CD300H had a molecular weight of ~25 kDa under reducing conditions and ~50 kDa under non-reducing conditions (Figure 5A), suggesting that CD300H forms dimer formation. To determine whether CD300H forms homodimeric or heterodimeric structure, we generated BW5147 transfectant simultaneously expressing Flag-tagged and hemagglutinin (HA)-tagged CD300H and performed co-immunoprecipitation assay. We found that HA-tagged CD300H was not

co-immunoprecipitated with Flag-tagged CD300H and vice versa (Figure 5B), suggesting that CD300H forms heterodimeric structure with an undetermined molecule with a molecular weight of ~25 kDa expressed in BW5147 cells.

Next, we analyzed to determine whether CD300Hs is a secreted form of CD300H. We performed immunoblot analysis of culture supernatant of 293T transfectant transiently expressing CD300Hs tagged with flag at the N-terminus and found that CD300Hs was a secreted form of CD300H (Figure 5C).

CD300H possesses a lysine residue in the transmembrane region, suggesting that CD300H associates with DAP12, DAP10 and/or FcεRIγ chain. We found that flag-tagged CD300H was co-immunoprecipitated with endogenous DAP12 and DAP10, and vice versa, but not with FcεRIγ chain, in U937 cells (Figure 5D). This result indicates that, unlike mouse MAIR-II, CD300H associates with DAP12 but not with the FcεRIγ chain in U937 cells.

### ***CD300H transduces an activating signal in CD16<sup>+</sup> Mo***

Our findings that CD300H was expressed on CD16<sup>+</sup> Mo and was associated with DAP12 and DAP10 in U937 cell line suggested that CD300H was an activating receptor on CD16<sup>+</sup> Mo. Human CD16<sup>+</sup> Mo consists of two distinct subsets. CD14<sup>dim</sup>CD16<sup>+</sup> Mo resemble murine Ly6C<sup>low</sup> Mo (5), which are called as pMo and produce chemokines. On the other hand, CD14<sup>+</sup>CD16<sup>+</sup> Mo are described as intMo (32),

main producers of inflammatory cytokines such as TNF-α and IL-1β (33). Therefore, we investigated whether CD300H mediated signaling for inflammatory cytokine and chemokines production in CD16<sup>+</sup> Mo. We sorted CD16<sup>+</sup> Mo from the PBMCs of healthy donors with the G/G (CD300H<sup>+</sup>) or A/A genotype (CD300H<sup>-</sup>) and then stimulated them with plate-coated F(ab')<sub>2</sub> fragments of TX93 mAb or control Ig (Figure 6A and B). Stimulation of CD300H<sup>+</sup> CD16<sup>+</sup> Mo with TX93 mAb induced significantly more TNF-α and IL-6 production than did stimulation with control Ig. In contrast, stimulation with TX93 mAb did not increase cytokine production by CD300H<sup>-</sup> CD16<sup>+</sup> Mo compared with stimulation with control Ig (Figure 6B and C). Thus CD300H transduces an activating signal for cytokine production in CD16<sup>+</sup> Mo. A recent study has demonstrated that murine Ly6C<sup>low</sup> Mo recruit neutrophils in a TLR7-dependent manner to mediate focal necrosis of endothelial cells (6). CD16<sup>+</sup> Mo produce chemoattractants for neutrophils. To examine whether CD300H is involved in this process, CD16<sup>+</sup> Mo were stimulated with plate-coated F(ab')<sub>2</sub> fragments of TX93 mAb or control Ig for 24 h; the culture supernatant was then plated in the lower compartment of a Transwell, and neutrophils derived from the peripheral blood were added to the upper compartment. In the case of CD300H<sup>+</sup>CD16<sup>+</sup> Mo but not CD300H<sup>-</sup>CD16<sup>+</sup> Mo, the average number of neutrophils that migrated from the upper to the lower compartment upon TX93 mAb stimulation was significantly greater than that with control

Ig-stimulated culture supernatant (approximately 1.3-fold increase,  $p < 0.05$ ) (Figure 6D). These results suggested that CD300H mediated a signal for the production of a neutrophil chemoattractant. Indeed, the expression of *CXCL8*, *CXCL1*, *CXCL2*, and *CXCL5*, which encode chemokines for neutrophil attraction, was significantly greater in CD300H<sup>+</sup> Mo, but not CD300H<sup>-</sup> Mo, than in the controls after stimulation with plate-coated F(ab')<sub>2</sub> fragments of TX93 mAb (Figure 6E).

#### ***CD300H had no effect on IL-8 production in CD16<sup>-</sup> Mo***

Since CD16<sup>-</sup> Mo also expresses CD300H (Figure 3E), we analyzed the CD300H activity on CD16<sup>-</sup>CD14<sup>+</sup> classical Mo sorted from the peripheral blood from donors with G/G- (CD300H<sup>+</sup>) or A/A-genotype (CD300H<sup>-</sup>). We analyzed the production of cytokines, including CXCL8 (IL-8), IL-12p70, TNF- $\alpha$ , IL-10, IL-6, and IL-1 $\beta$  after stimulation with anti-CD300H mAb by using cytometric bead array (CBA). However, we could not detect a significant amount of cytokines except IL-8 and observed no significant differences in IL-8 production between stimulation with control antibody and anti-CD300 mAb (Figure 6F and G). This might be resulted from the low level of CD300H expression on CD16<sup>-</sup> Mo compared with CD16<sup>+</sup> Mo.

#### ***CD300H transduces an activating signal in CD11c<sup>+</sup> DC***

CD11c<sup>+</sup> DC also express CD300H at comparable level to CD16<sup>+</sup> Mo. We addressed

whether CD300H transduces activating signal in CD11c<sup>+</sup> DC. We purified CD11c<sup>+</sup> cells by MACS sorting system (Fig. 7A), stimulated them with plate-coated F(ab')<sub>2</sub> fragment of TX93 mAb and analyzed surface expression of co-stimulatory molecules and cytokines production by flow cytometry. We found that TX93 stimulation did not enhance the expression level of co-stimulatory molecules, such as CD40, CD86, CD80, and HLA-DR on G/G-genotype (CD300H<sup>+</sup>)-derived CD11c<sup>+</sup> DC (Figure 7B). However, CD11c<sup>+</sup> DC from G/G-genotype, but not A/A-genotype (CD300H<sup>-</sup>), produced IL-8 upon CD300H stimulation (Fig. 7C). These results indicate that CD300H induced IL-8 production by CD16<sup>+</sup> Mo and CD11c<sup>+</sup> DC and may contribute to neutrophil attraction.

## Discussion

We identified and characterized a new member of the human CD300 family of immunoreceptors, which we designated CD300H. It is expressed on CD14<sup>+</sup>CD16<sup>+</sup> intMo and CD14<sup>dim</sup>CD16<sup>+</sup> pMo in the peripheral blood and transduces an activating signal for the production of inflammatory cytokines (TNF- $\alpha$  and IL-6) and chemokines (CXCL1, CXCL2, CXCL5, and IL-8) by CD16<sup>+</sup> Mo. In addition, CD300H induces IL-8 production by CD11c<sup>+</sup> DC. Importantly, we demonstrated that CD300H mediates neutrophil recruitment through the production of chemoattractants for those cells, suggesting that CD300H plays an important role in host immunity against bacterial and viral infections.

Despite recent progress in human Mo studies, the physiological roles of CD16<sup>+</sup> Mo have not been fully elucidated. One recent report described a healthy family with CD16<sup>+</sup> Mo deficiency in the blood (34), suggesting that the function of CD16<sup>+</sup> Mo is redundant. In accordance with this idea, we also found a subpopulation of healthy people whose CD16<sup>+</sup> Mo did not express CD300H, suggesting that CD300H on CD16<sup>+</sup> Mo is functionally redundant. However, because CD16<sup>+</sup> pMo are implicated in inflammatory diseases such as arthritis and atherosclerosis (35-37) or autoimmune diseases such as SLE (4), deficiency of CD16<sup>+</sup> pMo or CD300H on CD16<sup>+</sup> pMo might be potentially advantageous, rather than disadvantageous, for

maintaining homeostasis. The genetic mutation known as CCR5-delta 32 is a well-studied example of an advantageous allele with a well-characterized geographic distribution. This mutation plays an important role in human immunodeficiency virus (HIV)-1 resistance because it prevents functional expression of the CCR5 chemokine receptor, the entry receptor for HIV-1 (38). The mutation is found principally in Europe and Western Asia, where average frequencies are approximately 10%, but not in Western and Central Africa (38). The mutant allele, which can be regarded as a natural knockout in humans, is not accompanied by an obvious phenotype in homozygous individuals, although CCR5 has a protective role against *Listeria monocytogenes* infection in mice (39). Additional studies are required to determine the functional role of CD300H in immunity and inflammation.

CD300H has a splicing variant, CD300Hs, which lack the transmembrane domain. We found that CD300Hs is a secreted form of CD300H. Recent findings demonstrated that activating immunoglobulin-like receptors CD300b and Triggering receptor expressed on myeloid cells (TREM-1) also produce soluble forms sCD300b and sTREM-1, respectively, by proteolytic cleavage (40,41). While sCD300b amplifies LPS-induced inflammation (40,42), sTREM-1 attenuates excessive inflammatory response by counterregulating TREM-1 (41)

probably by acting as a decoy receptor, as observed in the TNF- $\alpha$  system (43). Therefore, expression of CD300Hs may modulate CD300H-mediated inflammatory responses in vivo.

Our studies suggested that CD300H may form a heterodimeric structure in mouse T cell lymphoma, BW5147. Recent evidences demonstrated that CD300C forms either homodimer or heterodimer with all CD300 family members. Although these dimer formation requires immunoglobulin (Ig)-like domains, CD300C did not bind other Ig-like receptors, such as TREM-1 and CD28 (44) (45). The Ig-like domain of CD300H has 53% homology to that of CD300C, suggesting that CD300H may also form heterodimer with mouse CD300 family members expressed on BW5147 cell line. Since we did not detect surface expression of mouse CD300a, MAIR-II and CD300lf on BW5147 cells by flow cytometry using TX41(12), TX52 (46), and TX70 (15) mAbs, respectively (data not shown), CD300H may interact with mouse CD300 family members other than these Ig-like receptors. Unlike CD300C, CD300H has a positively-charged lysine residue in its transmembrane region and interact with DAP12 and DAP10. Thus, CD300H may be able to mediate an activating signal upon ligand binding. However, the heterodimer of CD300H with other CD300 receptors may positively or negatively modulate the signaling.

To elucidate the physiological roles of CD300H on CD16<sup>+</sup> pMo in vivo, the counterpart

of CD300H in mice needs to be identified. Although we identified CD300H in our search for a human homolog of MAIR-II, there are several differences between MAIR-II and CD300H. First, MAIR-II inhibits B-cell receptor-mediated signaling in B cells (47), whereas CD300H expression on human peripheral blood B cells was not detected by flow cytometry using TX93 mAb (Figure 3). Second, MAIR-II is a unique receptor that binds to either DAP12 or Fc $\epsilon$ RI $\gamma$ ; however, CD300H did not show an association with Fc $\epsilon$ RI $\gamma$  in U937 cells. Third, *CD300H* is located adjacent to *CD300C* on human chromosome 17, whereas *Cd300ld/MAIR-IV/LMIR4/Clm-5*, but not *MAIR-II*, is located adjacent to *Cd300c* on mouse chromosome 11. Recent studies have demonstrated that CD300 family receptors recognize lipids such as phosphatidylserine (PS), ceramide, and lipoprotein as ligands that mediate signals in mast cells and macrophages via these receptors (48-50). We and others reported that human CD300A binds to PS and phosphatidylethanolamine (PE) (11,27,50). Because CD300H has high amino acid identity with CD300A, we assessed whether CD300H also bind to PS and PE by dot blot analyses using a lipid-bound membrane. However, CD300H bound to neither PS nor PE (data not shown). Future studies are required for identification of a ligand for CD300H to understand the function of CD300H in vitro and in vivo.

**Accession codes**

GenBank Accession Number: human CD300H, LC013475; human CD300Hs, LC013476.

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**Competing financial interests**

The authors declare no competing financial interests.

**Author contributions**

K.N. conducted the experiments and analyzed the data. S.T-H. designed the experiments, analyzed the data, and wrote the paper. E.N. analyzed the data. A.S. supervised the overall project and wrote the paper.

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## Figure Legends

**Figure 1. Molecular characteristics of CD300H.** (A) Localization on mouse and human CD300 family gene maps. Mouse and human family genes are mapped on chromosomes 11 and 17, respectively. (B) Amino acid sequences of CD300H and CD300Hs. Numbers in parentheses (left margin) indicate amino acid (aa) positions; underlines indicate putative leader and transmembrane sequences; circles denote potential N-linked glycosylation sites in the extracellular domain and the charged aa residue in the transmembrane region; \* indicates cysteine residues potentially involved in disulfide bonding of the immunoglobulin-like domains. (C) Schematic diagram of CD300H (left) and CD300Hs (right) proteins. The pair of cysteine residues in the extracellular portion is potentially able to participate in intra-chain disulfide bonding for the formation of Ig-like domains. (D) Phylogenetic tree showing the relationships among the Ig-like-domain amino acid sequences of human CD300 molecules. Each neighbor-joining phylogenetic tree was generated by aligning the translated sequences of the open reading frames for CD300 proteins by using the ClustalW algorithm.

**Figure 2. Characterization of the anti-CD300H monoclonal antibody, TX93.** (A) Flow cytometry of THP-1 cells or THP-1 transfectants expressing CD300H ( $5 \times 10^5$  cells per experiment) were stained with biotinylated control mouse immunoglobulin (shaded) or anti-CD300H (TX93) (thick lines) followed by allophycocyanin-conjugated streptavidin. (B) Flow cytometry of BW5147 transfectants expressing CD300A or CD300C or 2B4 transfectants expressing CD300H ( $5 \times 10^5$  cells per experiment) were stained with control mouse immunoglobulin (shaded), TX93, anti-CD300C (TX47), or anti-CD300A and CD300C (TX49) (thick lines), followed by phycoerythrin-conjugated goat F(ab')<sub>2</sub> fragment anti-mouse IgG. (C) Microtiter plates coated with control human IgG, CD300H-Fc, CD300A-Fc, or CD300C-Fc were incubated with control mouse IgG, TX93, TX47, or TX49, and binding was analyzed by ELISA. Data are representative of two independent experiments.

**Figure 3. Expression of human CD300H protein.** (A-G) Flow cytometry of peripheral blood mononuclear cells ( $5 \times 10^5$  cells per experiment) from healthy donors. Cells were stained with specific antibodies to distinguish different cell subsets and simultaneously stained with biotinylated control mouse immunoglobulin (shaded) or anti-CD300H (TX93) (thick lines), followed by PE/Cy-7 streptavidin. PI positive dead cells were excluded. Cells in outlined areas were gated and analyzed for CD300H expression. Cells in (C) are Lineage (CD3, CD19, CD56, CD14)<sup>-</sup>. Cells in (E) are Lineage (CD3, CD19, CD56)<sup>-</sup> HLA-DR<sup>+</sup>. HLA-DR, human leucocyte antigen-DR; FSC-A, forward scatter-area; B, B cell; T, T cell; NK, natural killer cell; mDC,

myeloid dendritic cell; pDC, plasmacytoid dendritic cell; Eos, eosinophil; Neu, neutrophil; Baso, basophil. Data are representative of at least three independent experiments. (H) The mean fluorescence intensity (MFI) of CD300H expression on each cell type from each individual carrying A/A (circle), G/A (triangle), or G/G (square) genotype is shown.  $\Delta$ MFI: MFI of stained with isotype control was subtracted from MFI of CD300H stained with anti-CD300H (TX93). \* $P < 0.05$ ; NS, not significant.

**Figure 4. A single-nucleotide polymorphism, rs905709 (allele A), at the splice donor site on intron 1 of *CD300H* is responsible for the loss of CD300H expression.** (A) Schematic structure of the exon-intron of *CD300H*. F and R are the sites of primers. Peripheral blood mononuclear cells derived from healthy donors carrying the A/A, G/A, or G/G genotype were subjected to RT-PCR analysis for the expression of CD300H and GAPDH transcripts (B) or were analyzed for surface expression of CD300H on CD14<sup>dim</sup>CD16<sup>+</sup> Mo by flow cytometry analysis (C), as described in the caption to Figure 3. Data are representative of two independent experiments. (D) Table from the National Center for Biotechnology Information single nucleotide polymorphism (SNP) web site showing the population diversity of SNP rs905709 in Utah residents with ancestry from Northern and Western Europe (CEU), Han Chinese in Beijing (HCB), Japanese in Tokyo (JPT), and Yoruba in Ibadan, Nigeria (YRI) populations. HWP, Hardy-Weinberg probability; Chrom. Sample Cnt, chromosome sample count; IG, individual genotype.

**Figure 5. Biochemical analyses of CD300H.** (A) BW5147 cells and transfectants expressing Flag-tagged CD300H ( $5 \times 10^6$  cells per experiment) were lysed in 1% NP-40 buffer, immunoprecipitated with anti-Flag, and immunoblotted with anti-Flag. (B) BW5147 transfectants simultaneously expressing Flag-CD300H and HA-CD300H were lysed in digitonin buffer, immunoprecipitated with anti-HA or anti-Flag, and immunoblotted with anti-HA or anti-Flag in reducing condition. (C) Culture supernatant from 293T cells transiently expressing Flag-tagged CD300Hs or mock were immunoprecipitated with anti-Flag and immunoblotted with anti-Flag. (D) U937 cells and transfectants stably expressing Flag-tagged CD300H were lysed in digitonin buffer, immunoprecipitated with anti-Flag, anti-DAP12, anti-DAP10, or anti-Fc $\epsilon$ RI $\gamma$ , and immunoblotted with anti-DAP12, anti-DAP10, or anti-Fc $\epsilon$ RI $\gamma$ . Data are representative of two independent experiments.

**Figure 6. CD300H ligation in CD16<sup>+</sup> monocytes (Mo) induces cytokine and chemokine secretion.** CD16<sup>+</sup> Mo (A-E) and CD16<sup>-</sup> Mo (F, G) were isolated from peripheral blood

mononuclear cells by using the MACS system. Their purity was then analyzed by using flow cytometry (A, F). MACS-isolated Mo from G/G (CD300H<sup>+</sup>) or A/A (CD300H<sup>-</sup>) genotype donors were stimulated with plate-coated F(ab')<sub>2</sub> fragment of TX93 or control Ig for 24 h (B, D, G) or 4 h (C, E). Culture supernatant was analyzed by ELISA (B), subjected to neutrophil migration assay (D), or cytometric bead array (G). Cells were analyzed for IL-6 (C) and chemokines (E) expression by using quantitative polymerase chain reaction. In (D), culture supernatant was transferred to the lower compartment of Transwells, and neutrophils were placed in the upper compartment. This was followed by a 30-min incubation. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; NS, not significant. All data are representative of three independent experiments. Data in (C) and (E) are shown as mean ± SD of four (CD300H<sup>+</sup>) and three (CD300H<sup>-</sup>) different donors.

**Figure 7. CD300H ligation in CD11c<sup>+</sup> dendritic cell (DC) induces IL-8 production.**

CD11c<sup>+</sup> DC were isolated from peripheral blood mononuclear cells by using the MACS system. (A) The purity was analyzed by using flow cytometry. MACS-isolated DC from G/G (CD300H<sup>+</sup>) or A/A (CD300H<sup>-</sup>) genotype donors were stimulated with plate-coated F(ab')<sub>2</sub> fragment of TX93 or control Ig for 24 h. (B) Cells were analyzed for expression of co-stimulatory molecules by flow cytometry. (C) Culture supernatant was analyzed by cytometric bead array. Lineage: CD3, CD19, CD56, CD14. \*\**P* < 0.01; NS, not significant. Data are representative of three independent experiments.

Fig1

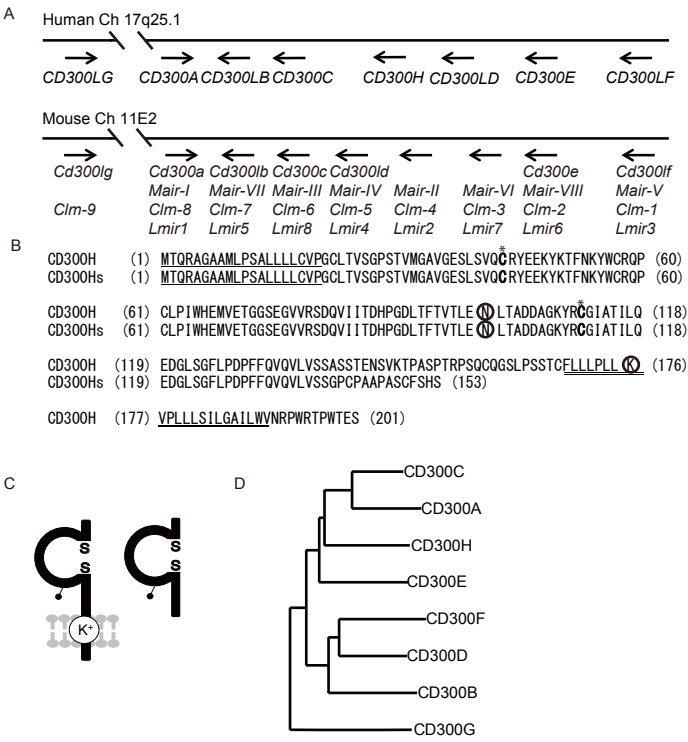




Fig2

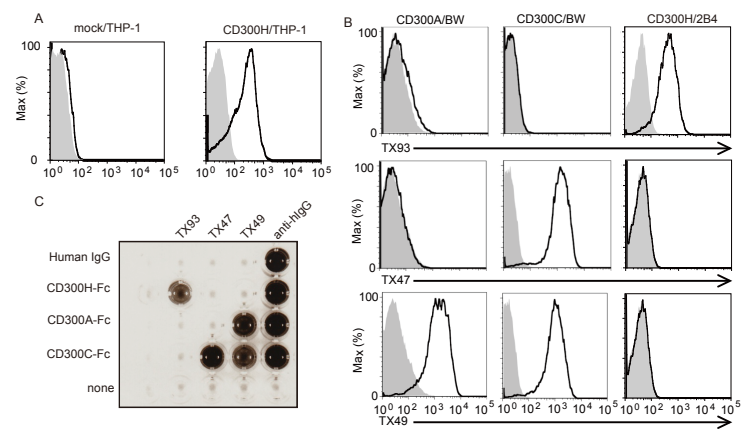
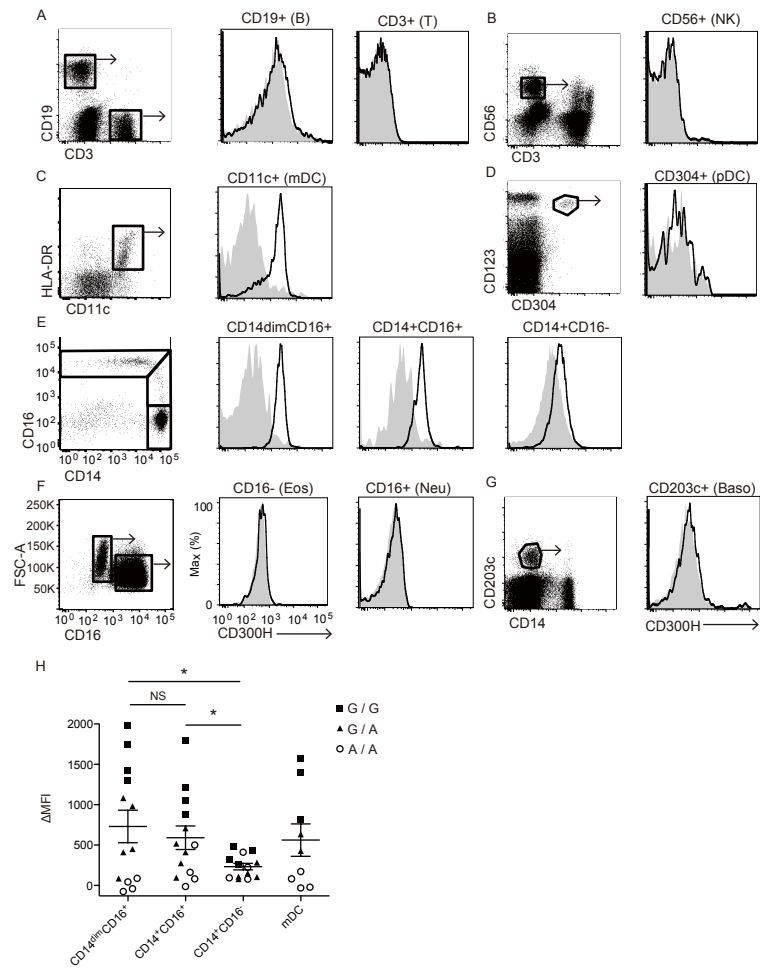
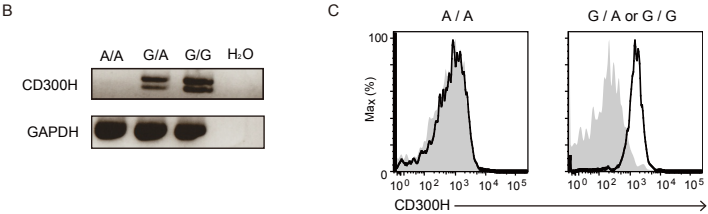
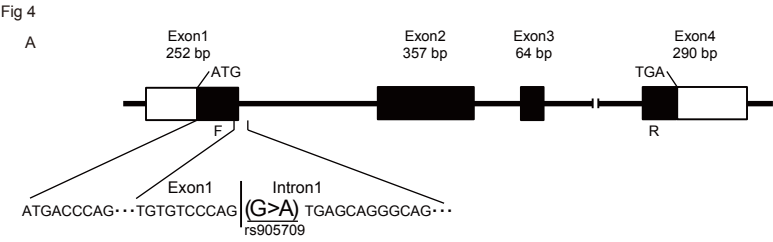


Fig3





**D**

| Sample Ascertainment |                     |                    |        | Genotype Detail |       |       |       | Alleles |       |
|----------------------|---------------------|--------------------|--------|-----------------|-------|-------|-------|---------|-------|
| Population           | Individual group    | Chrom. Sample Cnt. | Source | G / G           | G / A | A / A | HWP   | G       | A     |
| HapMap -CEU          | European            | 116                | IG     | 0.379           | 0.483 | 0.138 | 1.000 | 0.621   | 0.379 |
| HapMap -HCB          | Asian               | 86                 | IG     | 0.163           | 0.488 | 0.349 | 1.000 | 0.407   | 0.619 |
| HapMap -JPT          | Asian               | 84                 | IG     | 0.405           | 0.429 | 0.167 | 0.584 | 0.619   | 0.381 |
| HapMap -YRI          | Sub-Saharan African | 120                | IG     | 0.783           | 0.217 |       | 0.752 | 0.892   | 0.108 |

Fig5

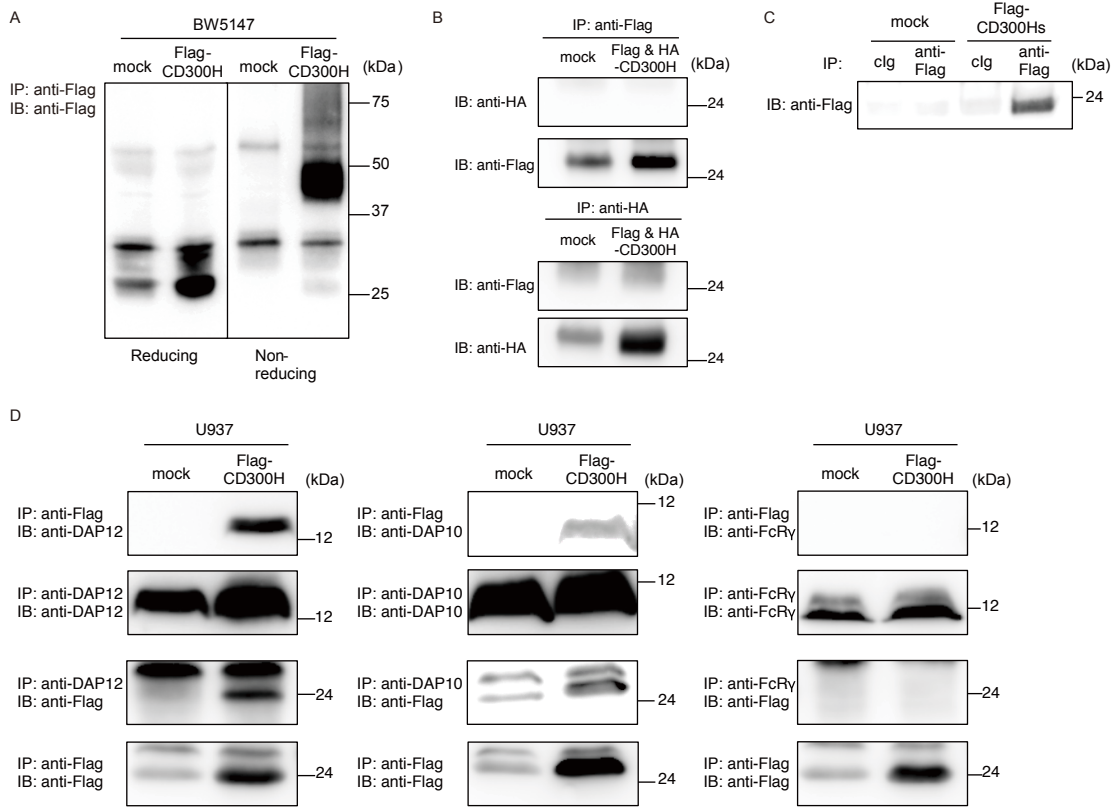


Fig 6

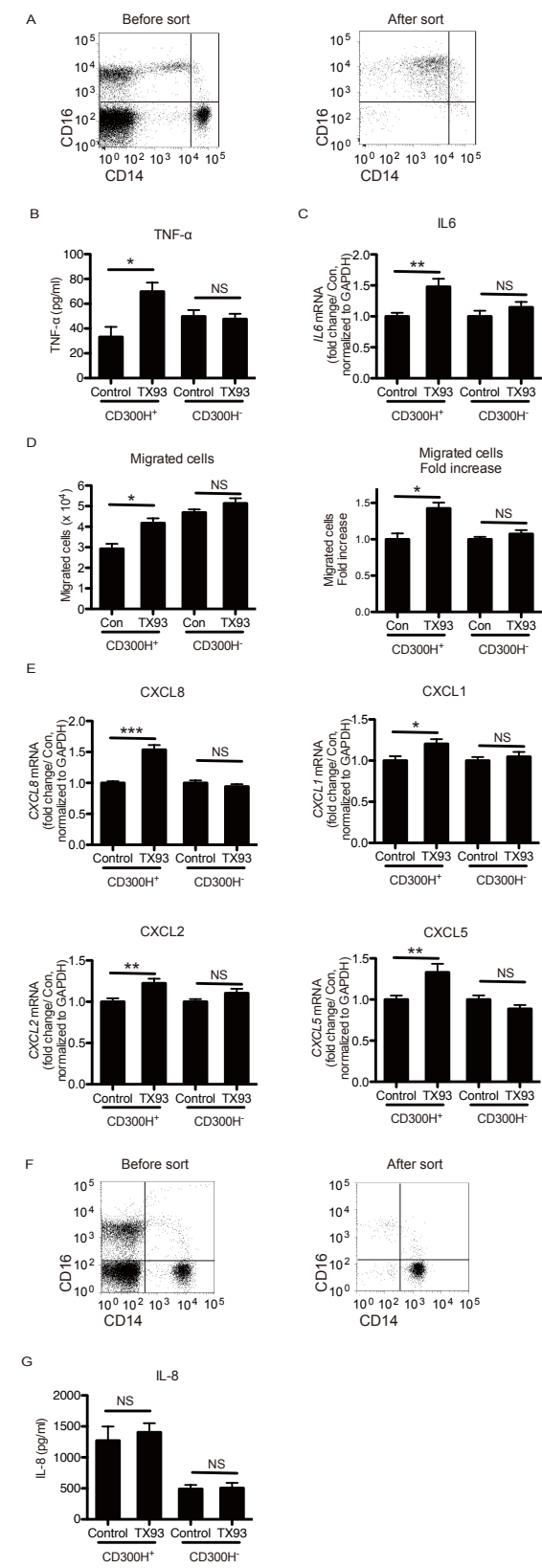


Fig 7

