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CD300a and CD300f differentially regulate the MyD88 and TRIF-mediated TLR signalling pathways through activation of SHP-1 and/or SHP-2 in human monocytic cell lines

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Summary

CD300a, a membrane protein expressed on myeloid lineages and specific subsets of CD4$^+$ T cells, has been reported to have inhibitory activities in cellular activation. However, the role of CD300a in Toll-like receptor (TLR) -mediated macrophage activation has not been investigated. The human monocytic cell lines THP-1 and U937 were stimulated with various TLR ligands after triggering of CD300a with specific monoclonal antibody. Interestingly, CD300a blocked TLR4-mediated and TLR9-mediated expression of pro-inflammatory mediators without affecting TLR3-mediated events. In contrast, CD300f, another member of the CD300 family, blocked the activation of cells induced by all TLR ligands. A transient transfection assay using luciferase reporter gene under the regulation of nuclear factor-κB binding sites indicated that co-transfection of CD300f blocked reporter expression induced by over-expression of both myeloid differentiation factor 88 (MyD88) and toll-interleukin 1 receptor-domain-containing adapter-inducing interferon-β (TRIF), whereas CD300a blocked only MyD88-induced events. Synthetic peptides representing immunoreceptor tyrosine-based inhibitory motifs of CD300a or CD300f mimicked the differential inhibition patterns of their original molecules. The use of various signalling inhibitors and Western blotting analysis revealed that TLR9/MyD88-mediated signalling was regulated mainly by SH2-containing tyrosine phosphatase 1 (SHP-1), which could be activated by CD300a or CD300f. In contrast, regulation of the TLR3/TRIF-mediated pathway required the combined action of SHP-1 and SHP-2, which could be accomplished by CD300f but not CD300a. These data indicate that CD300a and CD300f regulate the MyD88 and TRIF-mediated TLR signalling pathways through differential activation of SHP-1 and SHP-2.

Keywords: CD300a; inflammation; macrophage; Toll-like receptor signalling

Introduction

CD300a (IRp60/CMRF-35H), as a member of the CD300 family of leucocyte surface receptors that share a single immunoglobulin variable-like extracellular domain, modulates a diverse array of cellular processes in leucocytes including natural killer cells,[1,2] CD4$^+$ T cells,[3,4] neutrophils,[5] eosinophils,[6] dendritic cells[7] and mast cells.[8] CD300a was initially found in natural killer cells as an inhibitory receptor that suppressed cytotoxic activity.[1] Later, the expression of CD300a was found in other cells of myeloid lineage. In neutrophils, co-ligation of CD300a with FcγRIIa (an activating receptor) was found to result in the inhibition of FcγRIIa-mediated activation signalling.[5] In mast cells, triggering of CD300a by cross-linking antibodies was shown to inhibit IgE-induced degranulation and stem cell factor-mediated survival.[8] In eosinophils, CD300a cross-linking inhibited the eotaxin-dependent transmigration of eosinophils as well as the anti-apoptotic effect of interleukin-5 (IL-5)/granulocyte–macrophage colony-stimulating factor. In human plasmacytoid dendritic cells, cross-linking of CD300a (or CD300c) was found to reduce tumour necrosis factor-α and increase interferon-α secretion.[7] The inhibitory activity of CD300a has been

further demonstrated using bi-specific antibodies that link CD300a with cell surface receptors expressed on mast cells.[9–12] In a murine model of allergic peritonitis, administration of neutralizing antibodies against LMIR1 (the murine homologue of CD300a) was found to significantly augment the release of inflammatory mediators and eosinophilic infiltration.[8] Furthermore, CD300a-based therapy has been shown to be useful for the treatment in animal models of cutaneous anaphylaxis, allergic airway inflammation and chronic experimental asthma.[9,10,12]

The inhibitory effects of CD300a are believed to be mediated by three putative immunoreceptor tyrosine-based inhibitory motifs (ITIMs) present in the intracellular region that can be tyrosine phosphorylated and therefore interact with SH2-containing tyrosine phosphatase 1 (SHP-1), SHP-2[1] and/or SH2-containing inositol-5-phosphatase-1 (SHIP-1).[6,9] Although CD300a expression has been detected in macrophages,[1] the role of CD300a in macrophage function has not been investigated. To investigate the role of CD300a in processes associated with macrophage function, CD300a was triggered by a specific monoclonal antibody (mAb) in human monocytic cell lines. CD300a triggering exhibited inhibitory effects against cellular signalling mediated by Toll-like receptor 4 (TLR4) and TLR9, but not against TLR3-mediated events. The molecular mechanism of this inhibitory effect was investigated in comparison with CD300f, another member of the CD300 family that has been shown to have an inhibitory action toward both the myeloid differentiation factor 88 (MyD88) -mediated and toll-interleukin 1 receptor-domain-containing adapter-inducing interferon-β (TRIF) -mediated TLR signalling pathways.[13]

Materials and methods

Antibodies, cell lines and reagents

CD300a-specific mAb (clone MEM-260) was purchased from AbD Serotec (Oxford, UK) and mouse IgG2a was obtained from BD Biosciences (San Jose, CA). CD300f-specific mAbs were generated in our laboratory (clones 2-3.6D and 2-1.7B) as described previously.[14] Polyclonal antibodies against SHP-1 and phospho-SHP-1 were purchased from Abcam (Cambridge, MA) and polyclonal antibodies against SHP-2 and phospho-SHP-2 were purchased from Cell Signaling (Danvers, MA). Human monocytic leukaemia cell lines THP-1 and U937 and the human embryonic kidney cell line 293T were obtained from the American Type Culture Collection (Rockville, MD). Protein-tyrosine phosphatase (PTP) inhibitor III, PTP inhibitor IV and PP2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Bacterial lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was purchased from Sigma (St Louis, MO); polyinosine-polycytidylic acid (PolyI:C) double-stranded RNA was from GE Healthcare (Little Chalfont, Bucks, UK), and cytosine phosphodiester guanine oligodeoxynucleotides (CpG ODN) 1826 (TLR9 ligand) was from Invivo-Gen (San Diego, CA). The CD300a expression construct (a full-length CD300a gene cloned into the mammalian expression vector pCMV-SPOR6) was purchased from 21C Frontier Human Gene Bank, KRIBB (Daejeon, Korea). For construction of CD300f-expressing constructs, full-length CD300f or its cytoplasmic domain deletion mutant was PCR-amplified and cloned into pGEM-T Easy vector, which was further digested by *Eco*RI for cloning into the corresponding site of the pCI-neo mammalian expression vector. Expression constructs for CD4-TLR4, death domain of MyD88, wild-type TRIF and tumour necrosis factor receptor-associated factor 6 (TRAF6), luciferase reporter gene under the control of nuclear factor-κB (NF-κB) binding sites, and the Renilla-luciferase construct for transfection control were described previously.[15–18] Fusion peptides containing ITIM of CD300a (or CD300f) and HIV-$TAT_{48–57}$ (TAT-YADL) were custom-designed and synthesized by Peptron Inc. (Daejeon, Korea).

Luciferase reporter assay

293T cells were seeded (1×10^4 cells/well, triplicate/sample) in 96-well plates and incubated overnight before transfection with 200 ng/well of total DNA that had been mixed with 2·5 μl SuperFect transfection reagent (Qiagen, Valencia, CA), and the reactions were performed as suggested by the manufacturer. Cell lysates were obtained 24 hr after transfection in lysis buffer and the luciferase activities were determined using the Dual-Luciferase reporter Assay System (Promega, Madison, WI) according to the manufacturer's recommended protocol. Relative firefly luciferase activity was determined by normalization with Renilla-luciferase activity.

Flow cytometry

Flow cytometry was performed on the FACScalibur system (Becton-Dickinson, Mountain View, CA). Cells (5×10^5) were pelleted and incubated with 0·3 μg antibodies in 30 μl FACS solution (a PBS containing 0·5% BSA and 0·1% sodium azide) for 20 min on ice. Cells were washed twice and incubated with 0·3 μg FITC-labelled goat anti-mouse IgG in 30 μl FACS solution. For background fluorescence, cells were stained with an isotype-matching control antibody. The fluorescence profiles of 1×10^4 cells were collected and analysed.

Gelatin zymogram and ELISA

The cells were activated by adding 1 μg/ml TLR ligands to medium containing 1×10^6/ml THP-1 cells in

RPMI-1640 supplemented with 0·1% fetal bovine serum. A sandwich ELISA (R&D systems Inc., Minneapolis, MN) measured the levels of IL-8 in the supernatants. The detection limit was < 10 pg/ml. The matrix metalloproteinase (MMP) activity in the culture supernatant was determined using substrate gel electrophoresis, as previously described.[19]

Statistical analysis

All data are presented as the mean values ± SD, with the number of independent experiments indicated in the figure legends. Statistical analyses were performed using SPSS software with one-way analysis of variance or paired or unpaired Student's *t*-test, as appropriate. Differences were considered significant at $P < 0{\cdot}05$.

Results and discussion

CD300a displays a differential effect on MyD88-mediated and TRIF-mediated TLR signalling

The expression pattern of CD300a was tested in THP-1 cells using a specific mAb. As shown in Fig. 1(a), THP-1 cells expressed moderate levels of CD300a. As CD300a has demonstrated inhibitory effects in various cell types of myeloid lineage as well as certain subsets of $CD4^+$ T cells, THP-1 cells were stimulated with a TLR4 ligand in the presence or absence of an anti-CD300a mAb capable of cross-linking cell surface CD300a molecules. Stimulation of cells with LPS, a TLR4 ligand, resulted in the induction of MMP-9 and IL-8. Pre-incubation of the cells with anti-CD300a mAb but not isotype-matching mouse IgG resulted in dose-dependent inhibition of the expression of both MMP-9 and IL-8 after stimulation with LPS (Fig. 1b). Similarly, treatment with CD300a resulted in the inhibition of CpG ODN-induced expression of MMP-9 and IL-8 (data not shown).

Two main pathways mediate signalling induced by TLR4. A complex containing MyD88 and toll-IL-1 receptor domain containing adaptor protein mediates one pathway whereas the other is mediated by a TRIF and TRIF-related adaptor molecule complex (reviewed in ref. 20). CD300a cross-linkage blocked TLR4-mediated cellular activation so it is necessary to determine through which of the two pathways CD300a manifests its effect or if it is through both.

To determine whether CD300a affects the pathway mediated by MyD88, THP-1 cells were stimulated with CpG ODN, a ligand for TLR9. Ligation of TLR9 can activate macrophages through the MyD88-mediated cellular signalling pathway. As shown in Fig. 2, treatment of THP-1 with anti-CD300a mAb but not mouse IgG resulted in the inhibition of TLR9-mediated induction of MMP-9 and IL-8 expression. THP-1 cells were then stimulated with PolyI:C, which is a well-known ligand of TLR3. Ligation of TLR3 can activate macrophages through the TRIF-mediated cellular signalling pathway. Interestingly, cross-linkage of CD300a with a specific mAb tended to reduce PolyI:C-

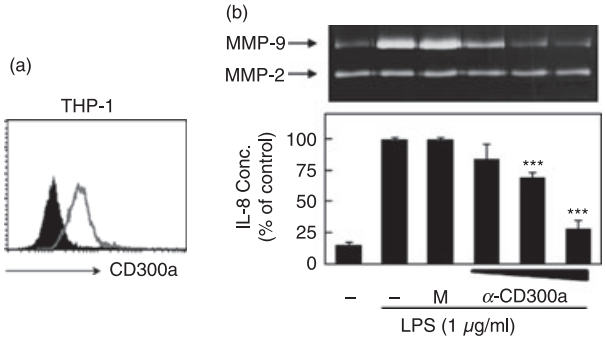

Figure 1. Triggering of CD300a results in the suppression of lipopolysaccharide (LPS) -induced matrix metalloproteinase 9 (MMP-9) and interleukin-8 (IL-8) expression. (a) Cells were stained with anti-CD300a monoclonal antibody (mAb; empty area) for flow cytometry. Background fluorescence levels (black area) were obtained by staining the cells with isotype-matching mouse IgG. (b) Cells were pre-treated with 0·1, 0·3 or 1 μg/ml anti-CD300a mAb or 1 μg/ml mouse IgG (M) for 30 min and then stimulated with 1 μg/ml of LPS. Culture supernatants were collected 24 hr after activation for the analysis of MMP-9/MMP-2 activity using gelatin zymography and measurement of IL-8 concentrations using double sandwich ELISA. Data points are represented as a percentage of positive control (LPS-treated samples) ($n = 3$, $^{***}P < 0{\cdot}001$ when compared with positive control samples).

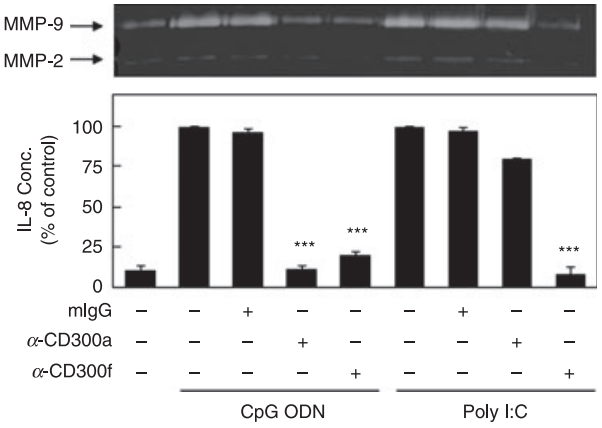

Figure 2. Treatment with anti-CD300a monoclonal antibody (mAb) blocks the expression of interleukin-8 (IL-8) and matrix metalloproteinase 9 (MMP-9) in cells stimulated with ligands for Toll-like receptor 9 (TLR9) but not TLR3. THP-1 cells were pre-treated with 1 μg/ml anti-CD300a, anti-CD300f mAb or mouse IgG (M) for 30 min and then stimulated with 1 μg/ml CpG ODN or Poly I:C. Culture supernatants were collected 24 hr after activation for the measurement of MMP-9 activity and IL-8 concentrations ($n = 3$, $^{***}P < 0{\cdot}001$ when compared with corresponding positive control samples).

induced expression of MMP-9 and IL-8 but failed to leach a statistically significant level (Fig. 2). These data indicate that triggering of CD300a exerts its inhibitory activity by blocking MyD88-mediated cell signalling but not TRIF-mediated cell signalling.

Macrophages, along with other cells of myeloid lineage, are under the regulation of another inhibitory receptor called CD300f (IREM-1/IgSF13).[21–24] The inhibitory activity of CD300f affects various biological processes such as mast cell activation,[21,25] osteoclastogenesis[23] and experimental autoimmune encephalomyelitis.[26] Recently, CD300f was shown to have an inhibitory effect on macrophage activation by blocking the B-cell-activating factor-mediated activation of THP-1 cells.[14] CD300f also blocks both MyD88-mediated and TRIF-mediated activation of THP-1 cells.[13] To compare the inhibitory effects of CD300f with those of CD300a, THP-1 cells were stimulated with TLR3 or TLR9 ligands in the presence of either CD300a or CD300f-specific mAb. As shown in Fig. 2, triggering of CD300f blocked cellular activation induced by both TLR3 and TLR9 ligands. These data demonstrate that CD300a and CD300f have different patterns of regulation.

Although THP-1 cells have macrophage-like properties, they may not fully represent the monocyte/macrophage lineage with respect to CD300a-mediated inhibition of TLR signalling. Consequently, another human monocytic cell line U937 was subjected CD300a-mediated inhibition of TLR signalling. As shown in Fig. 3(a), U937 cells expressed high levels of CD300a on the cell surface. These cells were pre-treated with anti-CD300a mAb and then stimulated with various TLR ligands. Triggering of CD300a exhibited inhibitory effects on inflammatory activation of U937 cells, which was mediated by TLR4 and TLR9 but not TLR3 (Fig. 3b). These data indicate that it is highly likely that CD300a has an inhibitory effect on TLR4-mediated and TLR9-mediated activation of monocytes/macrophages.

Luciferase reporter assay confirms that CD300a affects MyD88-mediated cell signalling but not TRIF-mediated cell signalling

Activation of the MyD88-mediated signalling pathway leads to activation of TRAF6, which then activates the IκB kinase (IKK) complex.[27–29] The activated IKK complex then phosphorylates IκB, which undergoes ubiquitin-mediated degradation. Degradation of IκB liberates bound NF-κB, which then translocates into the cell nucleus and activates the expression of various pro-inflammatory mediators such as cytokines, adhesion molecules and matrix degrading enzymes. In contrast, activation of the TRIF-mediated signalling pathway leads to two downstream signalling pathways; one passes through receptor-interacting protein 1 (RIP1) and pellino homolog 1 (PELI1), which then induces IKK activation, degradation of IκB, and subsequent activation of NF-κB,[30,31] whereas the other is mediated by serine/threonine-protein kinase 1 and leads to the activation of interferon regulatory factor 3, which is responsible for the expression of type I interferon.[32]

To confirm the differential effects of CD300a on MyD88-mediated and TRIF-mediated cell signalling, transient transfection assay was performed using the luciferase reporter gene under the control of NF-κB binding sites. Stimulation of NF-κB activity was achieved by transfection of the cells with DNA constructs for CD4-TLR4 fusion protein containing the extracellular domain of CD4 and the transmembrane and intracellular domains of TLR4. CD4-TLR4 generates TLR4 signalling constitutively in the absence of LPS.[33] Over-expression of signalling adaptors tends to induce downstream signalling pathways even in the absence of appropriate activation from upstream signalling molecules. This over-expression-related signal generation has been observed with TLR signalling molecules such as TRIF, TRAF2 and TRAF6.[34–38] Similarly, over-expression of CD300f results in its own phosphorylation followed by interaction with downstream mediators

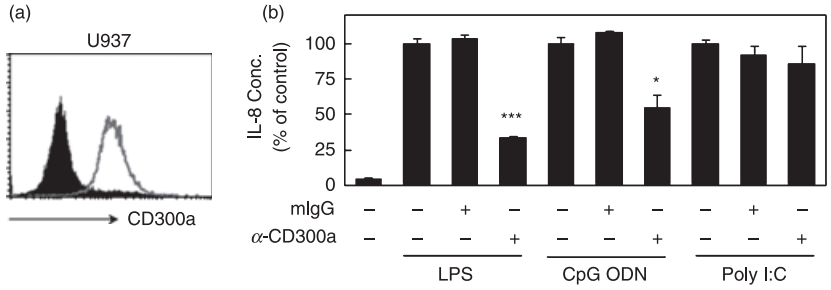

Figure 3. Treatment with anti-CD300a monoclonal antibody (mAb) blocks the expression of interleukin-8 (IL-8) in U937 cells stimulated with lipopolysaccharide (LPS) and CpG PDN, but not PolyI:C. (a) U937 cells were analysed for their expression levels of CD300a using flow cytometry. Background fluorescence levels (black area) were obtained by staining the cells with isotype-matching mouse IgG. (b) Cells were pre-treated with 1 μg/ml of anti-CD300a mAb or mouse IgG for 30 min and then stimulated with 1 μg/ml of LPS, CpG ODN or Poly I:C. Culture supernatants were collected 24 hr after activation for the measurement of IL-8 concentrations ($n = 3$, $^{*}P < 0{\cdot}05$ and $^{***}P < 0{\cdot}001$ when compared with corresponding positive control samples).

without the need for triggering by mAb.[13] Under the assumption that over-expression of CD300a generates inhibitory signals from it without the addition of agonistic mAb, 293T cells were transfected with CD300a expression construct (Fig. 4a). As shown in Fig. 4(b), co-transfection of HEK293T cells with the CD300a expression construct resulted in significant inhibition of reporter activity that was induced by CD4-TLR4.

Activity of NF-κB was then induced by transfection of cells with the death domain of MyD88, which is constitutively active.[37] Co-expression of CD300a resulted in a significant reduction in reporter activity induced by the constitutively active form of MyD88. In contrast, reporter activity induced by over-expression of wild-type TRIF was not blocked by CD300a (Fig. 4b). This result is in agreement with the analysis using THP-1 cells, which showed that CD300a cross-linking blocked CpG ODN-induced events that require MyD88 without affecting PolyI:C-induced events that require TRIF (Fig. 2b). The blocking effect of CD300a on TRAF6 activity was next tested in HEK293T cells over-expressing wild-type TRAF6, which is the downstream mediator of MyD88. Co-transfection of the CD300a expression construct blocked reporter gene expression that was induced by TRAF6 (Fig. 4b). These data confirm that CD300a can affect MyD88-TRAF6-mediated cellular signalling without affecting TRIF-mediated events.

The inhibitory activity of CD300a was then compared with that of CD300f in HEK293T cells. Transfection of THP-1 cells with differing amounts of CD300a or CD300f expression constructs resulted in dose-dependent inhibition of the reporter activity induced by the constitutively active forms of TLR4, MyD88 and TRAF6 (Fig. 5). However, TRIF-induced expression of the reporter gene was inhibited only by CD300f expression. Although transfection of large amounts of CD300a tended to reduce reporter activity, the reduction was not statistically significant (Fig. 5). The intracellular domain of CD300f contains five putative ITIMs, and deletion of the intracellular domain abolished the inhibitory effect (Fig. 5). This confirms the essential role of the intracellular domain in the inhibitory activity. These data further demonstrate that CD300f and CD300a differentially regulate TLR-mediated signalling pathways: CD300f blocks both the MyD88 and TRIF pathways while CD300a blocks only the MyD88 pathway.

ITIM-containing peptides derived from CD300a block TLR signalling mediated by MYD88 but not TRIF

The cytoplasmic tail of CD300a contains three ITIM-like sequences, each of which has one tyrosine residue (Y231, Y255 and Y267). The ITIM encompassing Y267 is known to have the strongest inhibitory function.[39] In an effort to generate immunosuppressive peptides that mimic the inhibitory action of CD300a, decapeptides representing the ITIM sequences encompassing Y231 or Y267 of CD300a were synthesized in linkage with HIV-TAT$_{48–57}$ (TAT-YANL or TAT-YASV, respectively) (Table 1). A fragment (AA48-57) from HIV-TAT was added to facilitate the internalization of peptide into cells. Internalization of the peptides was detected as early as 10 min after treatment (data not shown), in agreement with previous

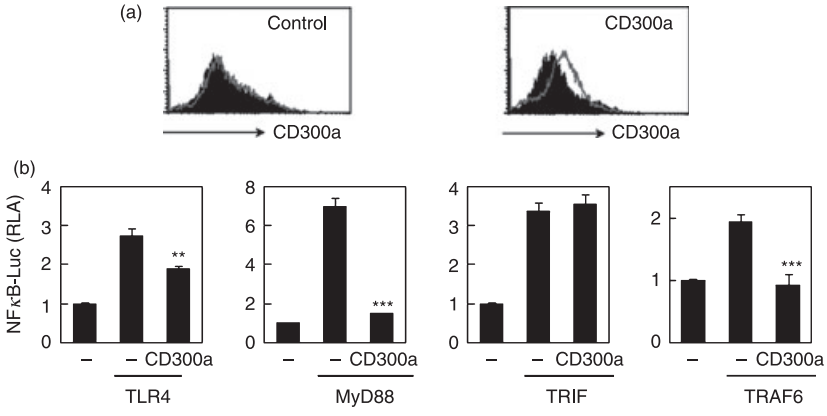

Figure 4. CD300a blocks Toll-like receptor (TLR) -mediated nuclear factor-κB (NF-κB) activation through inhibition of a MyD88-mediated but not a toll-interleukin 1 receptor-domain-containing adapter-inducing interferon-β (TRIF) -mediated pathway. (a) 293T cells were transfected with empty vector (control) or CD300a expression vector. The expression levels of CD300a were tested after 48 hr using flow cytometry. Background fluorescence levels (black area) were obtained by staining the cells with isotype-matching mouse IgG. (b) 293T cells were transfected with a luciferase reporter plasmid containing NF-κB binding sites (4 ×) and an expression plasmid containing Renilla-luciferase as an internal control. For the induction of NF-κB activity, cells were transfected with the constitutively active forms of TLR4, MyD88, TRIF or tumour necrosis factor receptor-associated factor 6 (TRAF6); 0·2 μg/well). For inhibition, CD300a expression construct (0·2 μg/well) was co-transfected. Relative luciferase activity (RLA) was determined at 24 hr after transfection after normalization with Renilla-luciferase activity ($n = 3$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$ when compared with corresponding positive control).

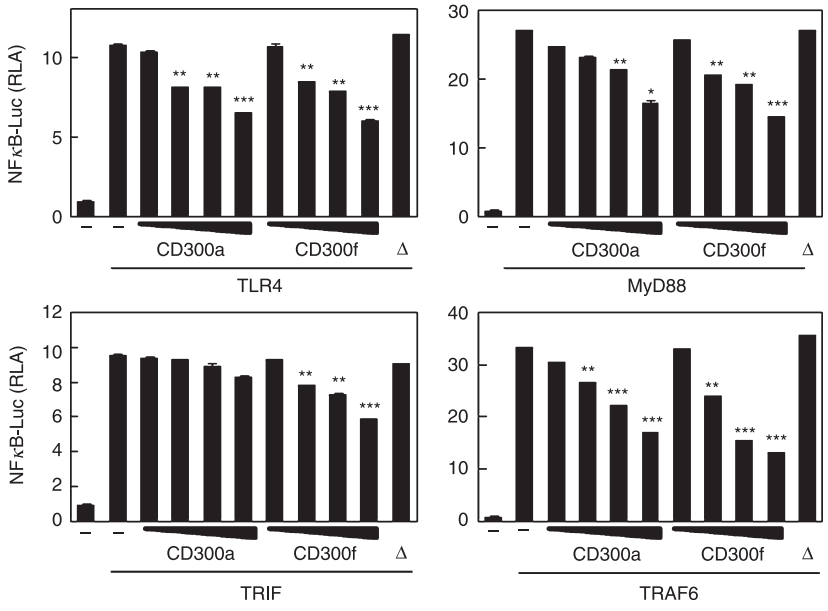

Figure 5. CD300a and CD300f differentially affects Toll-like receptor (TLR) -mediated activation of toll-interleukin 1 receptor-domain-containing adapter-inducing interferon-β (TRIF). 293T cells were transfected with a luciferase reporter plasmid containing nuclear factor-κB (NF-κB) binding sites (4 ×) and an expression plasmid containing Renilla-luciferase as an internal control. For the induction of NF-κB activity, cells were co-transfected with the constitutively active forms of TLR4, MyD88, TRIF or tumour necrosis factor receptor-associated factor 6 (TRAF6) (0·2 μg/well). For inhibition, cells were co-transfected with 0·1, 0·2, 0·3 and 0·4 μg/well of expression plasmids for CD300a, full-length CD300f, or 0·4 μg/well of cytoplasmic deletion mutant of CD300f (Δ). Relative luciferase activity (RLA) was determined at 24 hr after transfection ($n = 3$, $^{*}P < 0{\cdot}05$ when compared with corresponding positive control, $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$).

Table 1. Synthetic peptides used in the experiments

Name	Sequence	Source
TAT-YADL	GRKKRRQRRR-GDLCYADLTL	CD300f (AA 201–210)
TAT-YANL	GRKKRRQRRR-SELHYANLEL	CD300a (AA 227–236)
TAT-YASV	GRKKRRQRRR-EELHYASVVF	CD300a (AA 263–272)
TAT-FASV	GRKKRRQRRR-EELHFASVVF	CD300a (AA 263–272, Y267F)
TAT	GRKKRRQRRR	–

experiments using TAT-containing peptides.[14,40] As controls, a peptide containing only HIV-TAT$_{48–57}$ (named TAT) and a tyrosine-to-phenylalanine substitution mutant of TAT-YASV (named TAT-FASV) were used. When these peptides were pre-treated to THP-1 cells, LPS-induced and CpG ODN-induced expression of IL-8 was blocked by TAT-YASV and TAT-YANL but not by TAT-FASV (Fig. 6a). As expected, PolyI:C-induced expression of IL-8 was not blocked by the synthetic peptides. These data indicate that ITIM-containing synthetic peptides derived from CD300a can mimic the inhibitory action of CD300a and that tyrosine plays an important role.

The inhibitory action of ITIM-containing peptides derived from CD300a was then compared with another ITIM-containing peptide (named TAT-YADL) derived from the intracellular domain of CD300f. TAT-YADL has already been shown to mimic the inhibitory action of CD300f in such a way that the peptide inhibits both MyD88 and TRIF-mediated signalling events.[13] As shown in Fig. 6(b), TAT-YADL exhibited inhibitory action against the constitutively active forms of TLR4, MyD88 and TRIF, whereas synthetic peptides representing ITIMs of CD300a (TAT-YASV and TAT-YANL) exhibited inhibitory action against the constitutively active forms of TLR4 and MyD88 but not TRIF. These data indicate that the synthetic peptides derived from the ITIM-like sequences of CD300a and CD300f mimic the inhibitory activities of their original molecules and differentially inhibit MyD88-mediated and TRIF-mediated cell signalling.

The inhibitory action of CD300a is mediated by SHP-1 but that of CD300f is mediated by both SHP-1 and SHP-2

Tyrosine phosphorylation of ITIMs is mediated by src-related kinases. When THP-1 cells were treated with a

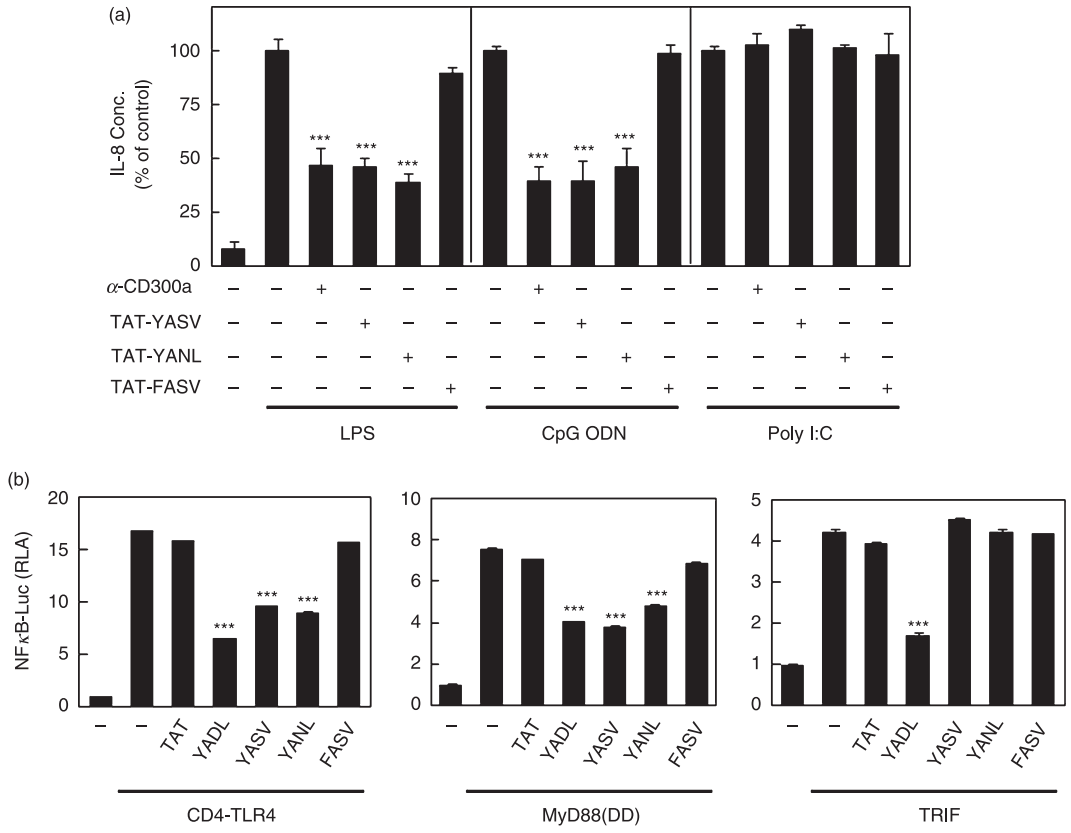

Figure 6. Synthetic peptides containing immunoreceptor tyrosine-based inhibitory motif-like sequences of CD300a mimics the inhibitory activities of CD300a. (a) THP-1 cells were pre-treated with 1 μg/ml anti-CD300a mAb or 5 μM synthetic peptides as indicated for 30 min. Cells were then stimulated with 1 μg/ml lipopolysaccharide (LPS), CpG ODN or PolyI:C for 24 hr. Culture supernatants were then collected for the measurement of IL-8 concentrations using ELISA. (b) 293T cells were transfected with a luciferase reporter plasmid containing nuclear factor-κB (NF-κB) binding sites (4 ×) and an expression plasmid containing Renilla-luciferase as an internal control. For the induction of NF-κB activity, cells were co-transfected with the constitutively active forms of Toll-like receptor 4 (TLR4), MyD88 or toll-interleukin 1 receptor-domain-containing adapter-inducing interferon-β (TRIF). Three hours after transfection, cells were treated with 5 μM synthetic peptides for additional 20 hr before measurement of relative luciferase activity (RLA; $n = 3$, ***$P<0{\cdot}001$ when compared with corresponding positive control samples).

well-known src kinase inhibitor PP2, the inhibitory actions of both CD300a and CD300f were blocked (Fig. 7a). PP2 treatment alone, however, did not affect LPS-induced expression of IL-8 (data not shown).

Phosphorylated ITIMs are known to interact with SH2-domain-containing signalling adapters. SHP-1 and SHP-2 have been shown to mediate the inhibitory effects in NK cells through their interactions with ITIM-like sequences in the cytoplasmic domain of CD300a.[1] In contrast, the inhibitory effects of CD300a are mediated by SHP-1 but not by SHP-2 in peripheral blood eosinophils.[6] To test whether or not both members of the SHP family are involved in CD300a-mediated inhibition of TLR signalling in monocytic cells, THP-1 cells were treated with LPS and anti-CD300a mAb in the presence of inhibitors specific for SHP-1 (PTP inhibitor III) or SHP-2 (PTP inhibitor IV). PTP inhibitor III has been shown to inactivate SHP-1 through binding to its catalytic domain.[41] PTP inhibitor IV has been shown to specifically inhibit SHP-2.[42] Interestingly, the inhibitory action of CD300a was blocked by PTP inhibitor III but not by PTP inhibitor IV whereas the inhibitory action of CD300f was blocked by both inhibitors (Fig. 7b). Stimulation of cells with LPS in the presence of PTP inhibitor III or PTP inhibitor IV did not affect the expression of IL-8 (data not shown).

Experiments in Fig. 7 suggest that the inhibitory action of CD300f was mediated by both SHP-1 and SHP-2, whereas the inhibitory action of CD300a was mediated by only SHP-1. Western blot analysis was then performed to confirm the differential effects of CD300a and CD300f on SHP-1 and SHP-2. As shown in Fig. 8, treatment of THP-1 cells with anti-CD300f resulted in the phosphorylation of both SHP-1 and SHP-2. In contrast, stimulation of CD300a induced phosphorylation of only SHP-1. These data indicate that SHP-1 is the downstream adaptor molecule of CD300a whereas CD300f mediates its effect through both SHP-1 and SHP-2.

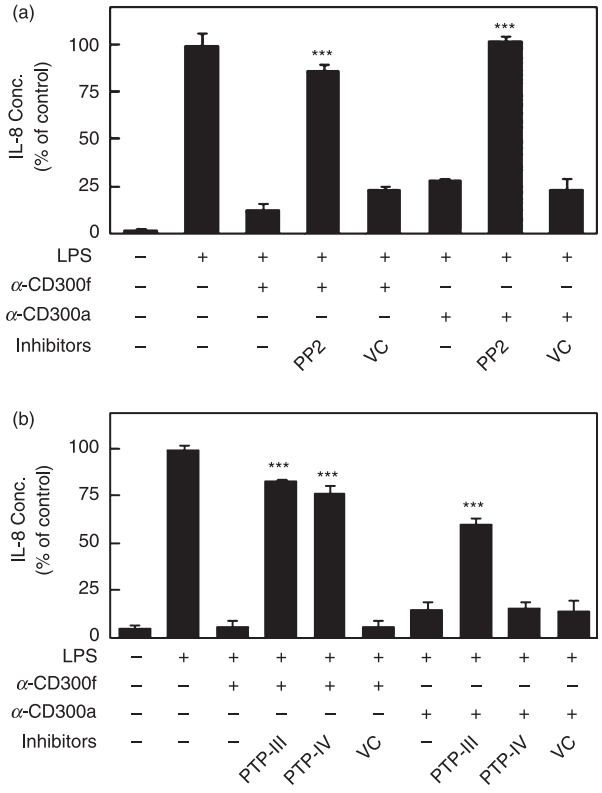

Figure 7. Inhibitory function of CD300a and CD300f is differentially regulated by specific inhibitors for SHP-1 and SHP-2. (a) THP-1 cells were sequentially pre-treated with 100 nM of PP2 or 0·2% DMSO (vehicle control, VC) for 30 min, followed by 1 μg/ml of monoclonal antibodies (mAbs) against CD300a or CD300f. Finally, cells were stimulated with 1 μg/ml lipopolysaccharide (LPS) for 24 hr. Culture supernatants were collected in 24 hr for the measurement of interleukin-8 (IL-8) concentrations. (b) THP-1 cells were sequentially pre-treated with 1 mM of PTP inhibitor III (PTP-III), 1 mM of PTP inhibitor IV (PTP-IV) or 0·2% DMSO for 30 min, followed by 1 μg/ml of mAb against CD300a or CD300f. Finally, cells were stimulated with 1 μg/ml of LPS for 24 hr. Culture supernatants were collected for the measurement of IL-8 concentrations ($n = 3$, ***$P<0·001$ when compared with corresponding control samples treated with LPS and anti-CD300a or anti-CD300f mAb).

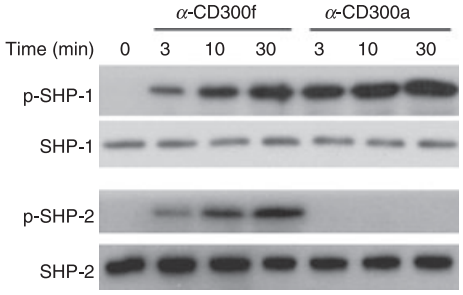

Figure 8. Triggering CD300f stimulates both SHP-1 and SHP-2 while CD300a stimulates only SHP-1. THP-1 cells were treated with 1 μg/ml monoclonal antibodies (mAbs) against CD300f or CD300a for the indicated times. Cell lysates were then collected for Western blot analysis for SHP-1/SHP-2 and their phosphorylated forms. The experiment was performed twice with essentially the same results.

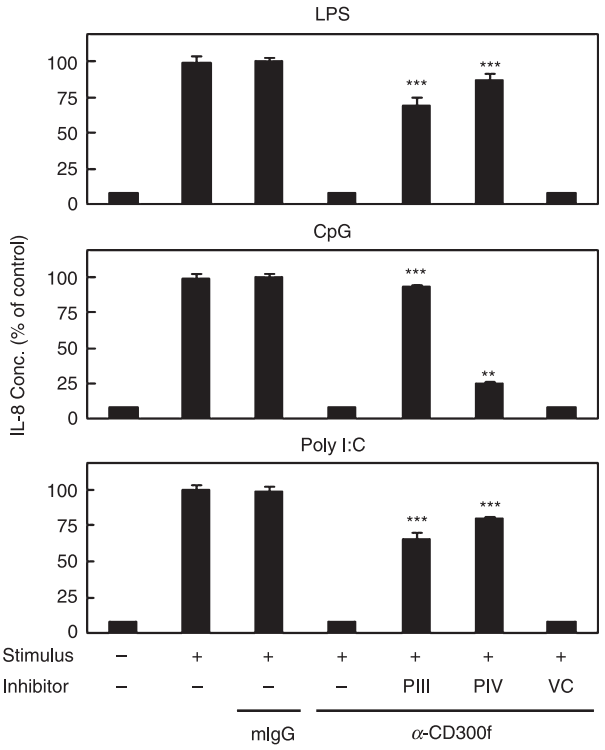

Figure 9. Inhibitors of SHP-1 and SHP-2 differentially block the inhibitory activities of CD300f under various Toll-like receptor (TLR) ligands. THP-1 cells were sequentially pre-treated with 1 mM of PTP inhibitor III (PIII), 1 mM of PTP inhibitor IV (PIV) or 0·2% DMSO (vehicle control, VC) for 30 min, followed by 1 μg/ml mIgG or monoclonal antibody (mAb) against CD300f for another 30 min. Finally, cells were stimulated with 1 μg/ml lipopolysaccharide (LPS), CpG ODN or PolyI:C for 24 hr. Culture supernatants were collected for the measurement of IL-8 concentrations ($n = 3$, **$P < 0·01$ and ***$P<0·001$ when compared with control samples treated with corresponding TLR ligand and anti-CD300f mAb).

Inhibition of the MyD88 pathway requires SHP-1 activity but inhibition of the TRIF pathway requires activities of both SHP-1 and SHP-2

Previous experiments described in Figs 7 and 8 suggest that CD300a and CD300f differentially affect the activation of SHP-1 and SHP-2. To determine whether or not SHP-1 and SHP-2 have differential effects on the MyD88 and TRIF pathways, a CD300f-mediated inhibition assay was performed in THP-1 cells in the presence or absence of PTP inhibitor III or PTP inhibitor IV. As shown in Fig. 9, PTP inhibitor III blocked the inhibitory effects of CD300f in cells activated with either CpG or PolyI:C by more than 90% or 60%, respectively. In contrast, PTP inhibitor IV blocked the inhibitory effect of CD300f in cells activated with CpG by only about 20% but PolyI:C-induced activation by about 75%. These data

suggest that SHP-1 activity has a major effect on CpG-induced (MyD88-mediated) events, whereas SHP-2 activity has a minimal effect. In contrast, PolyI:C-induced (TRIF-mediated) events are affected by SHP-1 and SHP-2 to a similar extent.

These differential effects of SHP-1 and SHP-2 on the MyD88-mediated and TRIF-mediated pathways are in agreement with the differential inhibitory effects of CD300a and CD300f on the MyD88-mediated and TRIF-mediated pathways. Triggering of CD300a induced phosphorylation of only SHP-1 and exhibited an inhibitory effect on CpG-induced (MyD88-mediated) cellular activation, which was mainly affected by SHP-1. CD300a did not show inhibitory activity against PolyI:C-induced (TRIF-mediated) activation, which was affected equally by SHP-1 and SHP-2. In contrast, triggering of CD300f induced phosphorylation of both SHP-1 and SHP-2 and had an inhibitory effect on both CpG-induced and PolyI:C-induced cellular activation. The fact that CD300a could not significantly inhibit PolyI:C-induced IL-8 expression suggests that inhibition of PolyI:C-induced (TRIF-mediated) activation requires the combined action of SHP-1 and SHP-2.

As SHIP-1 has been reported to be another downstream mediator of CD300a,[6,9] phosphorylation of SHIP-1 was tested by Western blot analysis using antibodies specific for phospho-SHIP-1 and SHIP-1 after triggering CD300a. Phosphorylation of SHIP-1, however, was not detected after treatment with anti-CD300a mAb (data not shown), suggesting that SHIP-1 may not be the downstream adapter for CD300a in monocytic cells.

Current data provide the first demonstration of the inhibitory effects of CD300a and its ITIM-containing peptides on the TLR-mediated signalling pathway. Triggering of CD300a induces phosphorylation (and subsequent activation) of SHP-1 but not SHP-2. As a result, CD300a is able to inhibit the MyD88 signalling pathway because it is regulated mainly by SHP-1. In contrast, CD300a is not able to regulate the TRIF pathway because inhibition requires the combined action of SHP-1 and SHP-2. It is required to confirm the differential effect of CD300a and CD300f against TLR-mediated induction of pro-inflammatory mediator expression using human primary macrophages in the future. The synthetic peptides representing the ITIM-like sequences of CD300a mimic these activities of CD300a, therefore they could be used for the regulation of immune responses where inflammation plays an important role.

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