

Expressions and inhibitory functions of CD300a receptors on purified human basophils

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Abstract: The inhibitory myeloid immunoglobulin receptor CD300a (IRp60) has been shown to downregulate mast cell and eosinophil activities, thereby serving as a potential target for inhibiting allergic effector cell input in allergy. Our aims were to study the expression and functional properties of this receptor in purified human basophils, cells that crucially contribute to Th2-type immunity and allergy. Basophils homogeneously expressed CD300a as well as the inhibitory receptor CD200R on their cell surface, and these expressions increased after anti-IgE stimulation. IgE-mediated basophil degranulation was also significantly inhibited by crosslinking of either CD200R or CD300a (by 90% and 50%,

respectively). Inhibitory SHIP-1 phosphorylations were also induced by CD200R and CD300a, although they were not noticeably increased by IgE-dependent activation. We conclude that both CD200R and CD300a play a role in reducing IgE-mediated basophil function and may crucially govern the known differential activities of these cells *in vivo*.

Key words: allergy – basophils – CD200R – CD300a – SHIP-1

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Background

The inhibitory immunoglobulin-like receptor CD300a has been shown to downregulate immune cell function including those of mast cells (1) and eosinophils (2), thereby serving as a potential target for inhibiting allergic effector cell input in allergy. Similar actions have also been shown in human basophils following binding of either CD200 or viral CD200 analogues (e.g. human herpesvirus-6, -7 and -8) to the inhibitory CD200R causing a reduction in IgE-mediated histamine release from these cells (3).

These inhibitory receptors (IRs) are thought to downregulate high-affinity IgE-receptor (FcεRI) triggering because of their immunoreceptor tyrosine-based inhibition motifs (ITIMs) (4–7). Upon activation, ITIMs recruit phosphotyrosine phosphatases (e.g. SHP-1, SHP-2) or inositol-phosphatases (e.g. SHIP-1), which serve to terminate cellular activation. CD300a receptors belong to a multigene family of membrane receptors that contain 4 immunoreceptor tyrosine-based inhibitory motifs and are constitutively expressed on a variety of immune cells. CD300a has been shown to block IgE-mediated mast cell degranulation and cytokine production (1), thus indicating potential anti-allergic properties that may be exploited therapeutically. We recently showed that CD300a is upregulated in basophils in mixed leucocyte cultures (8), and our current aims were to assess its effects on mediator release and signalling using purified basophils. As basophils contribute to the pathophysiology of several skin diseases, particularly allergies (9,10), as well as potentially regulating key immunological (9,10) and even neuroendocrine functions (11), there is a pressing need to investigate inhibitory mechanisms that can be used as therapeutic strategies.

Questions addressed

We investigated the expressions of CD300a receptors on human basophils in comparison with a previously reported inhibitory

receptor, CD200R (3). We also asked whether the activation of these receptors could prevent IgE-dependent and IgE-independent histamine release from these cells and investigated possible modes of action of these receptors.

Experimental design

Human basophils were isolated from buffy coats obtained from healthy donors, following NHS-REC ethical approval, and purified to >90% by negative cell sorting (12). IR expressions were assessed using either Western blotting, in-cell-assays, immunofluorescence microscopy or flow cytometry. Histamine releases were measured spectrofluorometrically and SHIP-1 phosphorylations by Western blotting and assessed using an Odyssey imaging system (Supporting Information).

Results

We first assessed the extent of CD300a and CD200R expression using Western blotting (Fig. 1a). CD300a was homogeneously expressed between different donors than the more variable CD200R, and no correlations between the expressions of these two receptors were observed (not shown). Using non-permeabilizing conditions, we next asked whether these receptors are expressed on the cell surface of basophils. This was confirmed by in-cell assays (Fig. 1b), fluorescence microscopy (Fig. S1) and flow cytometry (Fig. S2A–C). Interestingly, we also observed that these expressions are rapidly increased following brief stimulation with anti-IgE (Fig. S2D–F).

Treatment of basophils with anti-CD200R or anti-CD300a significantly reduced spontaneous histamine release as well as that caused by anti-IgE stimulation (Fig. 2). However, CD300a-mediated inhibitory effects were highly variable among different donors compared with CD200R, even though expressions of the latter varied more than CD300a. Overall, CD300a triggering inhibited histamine release by 50% (range, 16–100%) compared with nearly

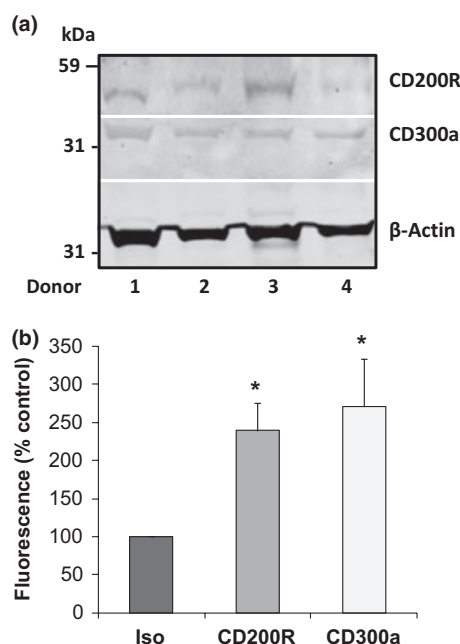


Figure 1. CD200R and CD300a expression in purified human basophils as judged by Western blotting (a) and in-cell assays (b), using fluorescent labelling ($n = 4-6$).

90% for CD200R in basophils from 15 separate donors. Increasing the antibody concentrations or varying the incubation periods did not increase inhibitory responses, and no further inhibitory effects were seen using anti-mouse F(ab)2 to increase receptor aggregation. However, a weak positive correlation ($r = 0.4$) was observed between the level of inhibitory action with CD200R and that caused by CD300a (not shown).

The precise mechanisms governing the inhibitory actions of CD300a have not been resolved but are thought to arise from an ITIM contained within the intracellular domain of the receptor forming a docking site for the inositol 5-phosphatase SHIP (13, reviewed in 14). CD200R is tyrosine phosphorylated upon activation and associates with adaptor proteins, such as Dok1 that also recruits SHIP (15). In a previous study, we showed that SHIP-1 is involved in reducing basophil responses following FcεRI triggering (16), and, in mast cells, Bachelet *et al.* showed that it is involved in the inhibitory actions of CD300a (1). However, our data in human basophils show only weak CD200R or CD300a-mediated SHIP-1 phosphorylations (Fig. S3A–B). Furthermore, although SHIP-1 phosphorylations were slightly enhanced by IR triggering alone, we failed to observe any marked further increases following IgE-dependent activation (not shown). This suggests either that SHIP-1 is not centrally involved in IR signalling or that its early, albeit weak, recruitment is sufficient to terminate signalling events that control mediator release. We have previously observed that poor basophil responders to IgE-induced stimulation have elevated levels of constitutive SHIP-1 phosphorylation (16), which may therefore suffice in controlling subsequent stimulation.

In support for a possible non-essential role for SHIP-1 in IR action were observations showing that both anti-CD200R and anti-CD300a reduced calcium ionophore (A23187)-induced basophil activation (Fig. S3C), although fMLP-induced histamine release was only moderately affected by anti-CD200R. A 2-h incu-

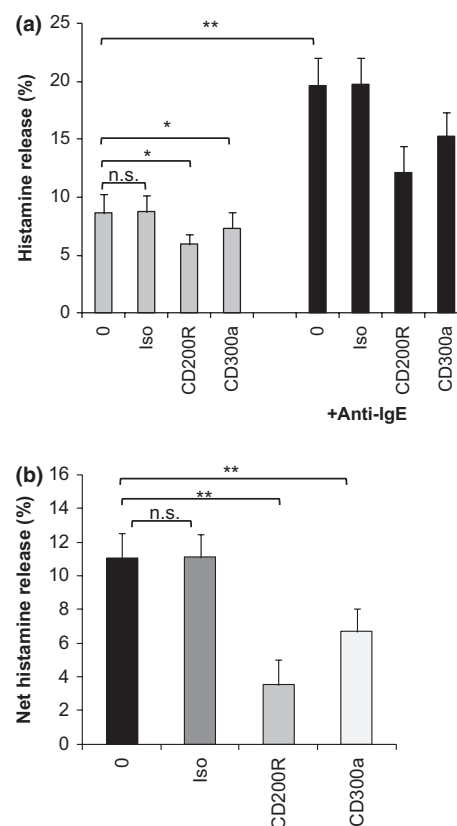


Figure 2. (a) Effects of anti-CD200R and anti-CD300a on IgE-dependent basophil histamine release (stimulated with 0.1 $\mu\text{g/ml}$ anti-IgE). (b) Results from panel A expressed as mean relative percentage histamine releases, corrected for spontaneous secretions, $\pm$ SEM for 15 separate experiments. * and ** represent significant ($P < 0.05$ and $P < 0.01$, respectively) differences using a paired Student's *t*-test.

bation of basophils with anti-CD200R/CD300a also slightly, but significantly, affected cell viability (Fig. S3D), suggesting involvement of cell death mechanisms too. In mast cells, CD300a triggering blocks CD117-mediated cell survival (1,13), although it remains to be clarified whether similar mechanisms apply to basophils regarding the anti-apoptotic effect of IL-3 (an autocrine agent (17)) and IR.

Our data generally support those obtained using human mast cells (1,13), although the inhibitory effects of CD300a are less pronounced in basophils compared with CD200R. This may be due to differences inhibitory CD300a versus non-inhibitory CD300c receptor expressions, which are highly homologous and indistinguishable on the cell surface using currently available antibodies (13). However, as the net effect of CD300 stimulation in basophils was inhibitory, we assume that CD300a functional effects predominate.

Conclusions

CD300a is expressed and functional on human basophils and, along with CD200R, can reduce basophil mediator secretion. The mode of action involves, at least in part, signalling downstream of calcium mobilization as well as SHIP-1 recruitment and possibly also cell death mechanisms. Overall, our data suggest that these IRs may serve as targets for anti-allergic therapy, although their mode of action appears to differ from those in mast cells.

Acknowledgements

BFG conceived the original study together with FLS, who, together with MBZ, contributed to the data analysis, presentation and correction of the manuscript. BFG conducted the experiments and generated the first draft of the manuscript. VS, CHB and BDE contributed to the design of the experiments regarding CD300a function in basophils and provided FACS

data. All authors assisted in preparing the final draft of the manuscript. The authors would like to acknowledge the support of the BMBS COST Action BM1007 COST action 'Mast Cells and Basophils - Targets for Innovative Therapies' which facilitated their collaboration on this study.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary methods.

Figure S1. Purified human basophil express CD200R and CD300a as determined by immunofluorescence ($n = 3$).

Figure S2. Flow-cytometric evaluation of CD300a and CD200R on resting (A–C) and anti-IgE stimulated basophils (D–F).

Figure S3. (A) Effect of CD200R and CD300a receptor triggering on SHIP-1 phosphorylation in purified human basophils.

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Letter to the Editor

In vitro formation of organized structure between keratinocytes and dorsal-root-ganglion cells

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Abstract: We recently found that the morphology in a co-culture system of keratinocytes and dorsal-root ganglion-derived cells depended on the timing of seeding of the two cell types. In skin, epidermis is formed first, followed by construction of peripheral nerve structure. Therefore, we hypothesized that formation of peripheral nerve structure in the epidermis might be driven by interaction between keratinocytes and nerve cells. In the present study, we tested this idea by incubating keratinocytes and dorsal-root ganglion cells in a spatially separated manner and observing the morphological changes in the co-culture system. Extension of

nerve fibre-like structures from the ganglion cells was observed, and within 3 days after seeding, many nerve fibre-like extensions penetrated into the keratinocyte cluster, subsequently forming a network that appeared to resemble the cutaneous peripheral nervous system. Our present model may be useful for studying the formation of peripheral nerve structure in the skin.

Key words: co-culture – epidermis – nerve fiber – peripheral nerve structure – Schwann cell

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Background

We recently demonstrated that co-culture systems of epidermal keratinocytes and dorsal-root ganglion-derived cells exhibited different morphological features depending upon the timing of seeding of the two cell types (1). Further, anatomical studies of skin have shown that unmyelinated nerve fibres exist in the epidermis, and there is a

horizontal net-like structure at the upper layer of the dermis (2). Martin has suggested that during skin development, the epidermis is formed first and then the peripheral nerve system develops (3).

Question addressed

How do primary afferent neurons interact with keratinocytes to innervate the epidermis?

LETTER TO THE EDITORS

Supplementary Material

Expressions and Inhibitory Functions of CD300a Receptors on Purified Human Peripheral Blood Basophils.

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Supplementary Methods

Cell stimulation and mediator assay

Following 15 min blocking at 4°C with 10% goat serum, purified basophils were washed and incubated with 10 µg/ml of either mouse-anti-human CD300a (clone 232612), mouse-anti-human CD200R (clone OX-108) or isotype control (and buffer control) for 30 min at 4°C. Following 2 washes, basophils ($1-2 \times 10^5$ cells per vial) were resuspended in HEPES-buffered-Tyrodé's solution, supplemented with 1 mM CaCl_2 , and then incubated for 15 min at 37°C before stimulation with either anti-human-IgE (0.1 µg/ml; polyclonal goat anti-human IgE; Sigma, Gillingham, UK), fMLP or A23187 (or buffer control) for 10-30 min. Histamine releases (shown as a percentage of total content) were determined following 30 min stimulation with anti-IgE and histamine contents in the supernatants and lysed cell pellets were measured spectrofluorometrically. In some experiments, cells were incubated for 2h for Trypan blue exclusion analysis for cell viability. Statistical significances were assessed using a paired Student's t-test.

Immunocytochemistry

Purified basophils, in Ca^{2+} -free media, were centrifuged onto glass microscope slides ($50-100 \times 10^5$ cells per slide). After drying, slides were incubated with primary antibodies for 4 h at 4°C. In the case of slides treated with PE-conjugated anti-CD300a (clone E59.126; Beckman Coulter, High Wycombe, UK), after 4 washes over a period of 15 min, slides were analyzed by immunofluorescence microscopy. Slides treated with CD200R (clone OX-108; Abcam, Cambridge, UK) or isotype control (Dako, Glostrup, Denmark) primary antibodies for 4 h, were then washed and

incubated with FITC-labeled secondary antibodies for 2h at RT (in the dark) before washing and microscopy.

In-cell assays and FACS analysis

50-100 x 10⁵ purified basophils were incubated with primary antibodies for 4h at 4°C. After 3 washes, in calcium-free buffer, cells were then incubated with fluorochrome-labeled secondary antibodies for 2h at 4°C. After washing, 100 µl cell suspensions were pipetted onto a 96-well plate and analysed using the Li-Cor Odyssey fluoroimaging system (Cambridge, UK). Flow cytometric analysis was performed on basophils using CD203c and IgE as selection markers and PE-labeled CD300a and CD200R antibodies, as we previously described [8].

Western blot analysis

Purified basophils (2 x 10⁵/sample) were treated as described above for 10 min in order to assess SHIP-1 phosphorylations. Reactions were terminated by placing tubes in ice and centrifugation. Cell pellets were prepared for SDS-PAGE and Western blotting as previously reported [8]. Blotted nitrocellulose membranes were blocked for 3 hours in 5 % skimmed milk in TBST buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1 % Tween 20) and then incubated overnight (at 4 °C) with specific antibodies to either phosphorylated SHIP-1 or beta actin (to gauge cell loading). After 5 x 3 min washes in TBST, membranes were incubated with fluorochrome-labeled secondary antibodies and, after a final washing cycle, analysed using the Odyssey imaging system. Mouse anti-human CD200R/CD300a primary antibodies were employed in a further series of Western blots using unstimulated purified basophils from various donors in order to assess receptor expressions.

Legends to supplementary figures

Figure 1

Purified human basophil express CD200R and CD300a as determined by immunofluorescence (n=3). (A) PE isotype control vs. (B) PE-conjugated anti-CD300a. (C) Isotype control vs. (D) anti-CD200R (in both cases using FITC labelling). (E) The same slide as shown in panel D counterstained with May Grünwald (MG) stain (in order to verify basophil morphology). Immunofluorescence images were acquired with a monochrome CCD camera and converted to false colour.

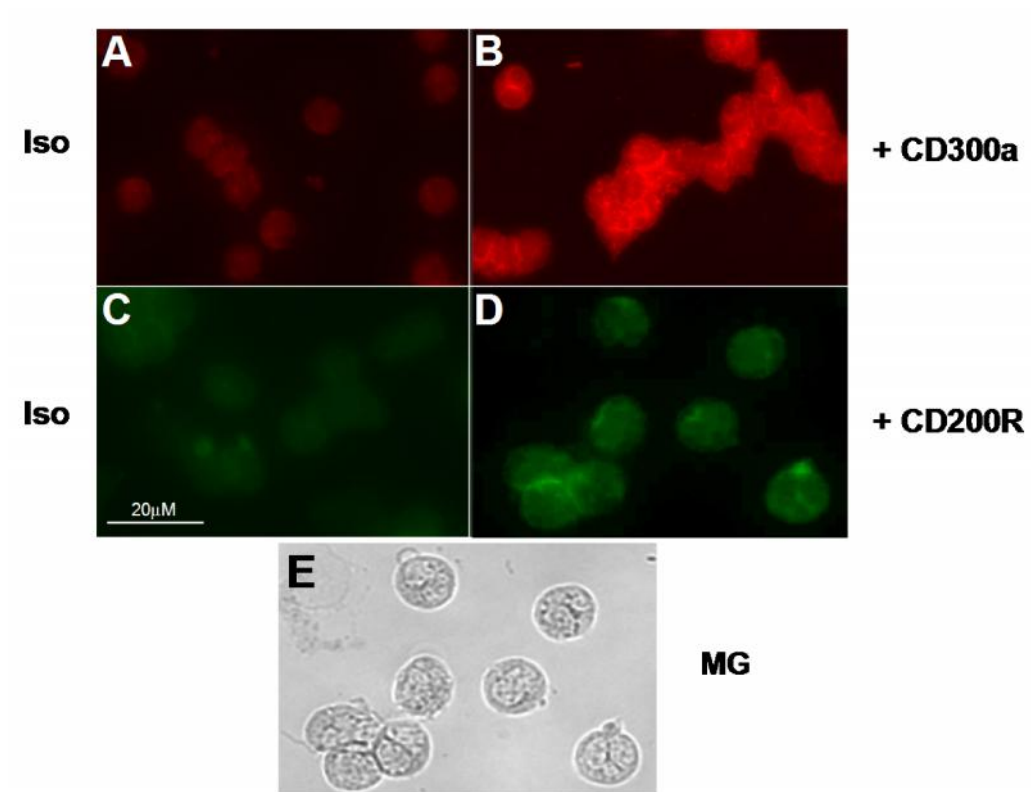
Figure 2

Flow-cytometric evaluation of CD300a and CD200R on resting (A-C) and anti-IgE stimulated basophils (D-F). CD63 and CD203c expressions in unstimulated (A) basophils and those stimulated with anti-IgE for 20 min (D). CD300a (B) and CD200R (C) are homogeneously expressed on resting basophils. Anti-IgE-activated basophils show a unimodal (expression in almost all cells) upregulation of both CD300a (E) and CD200R (F). P1 denotes the basal expression.

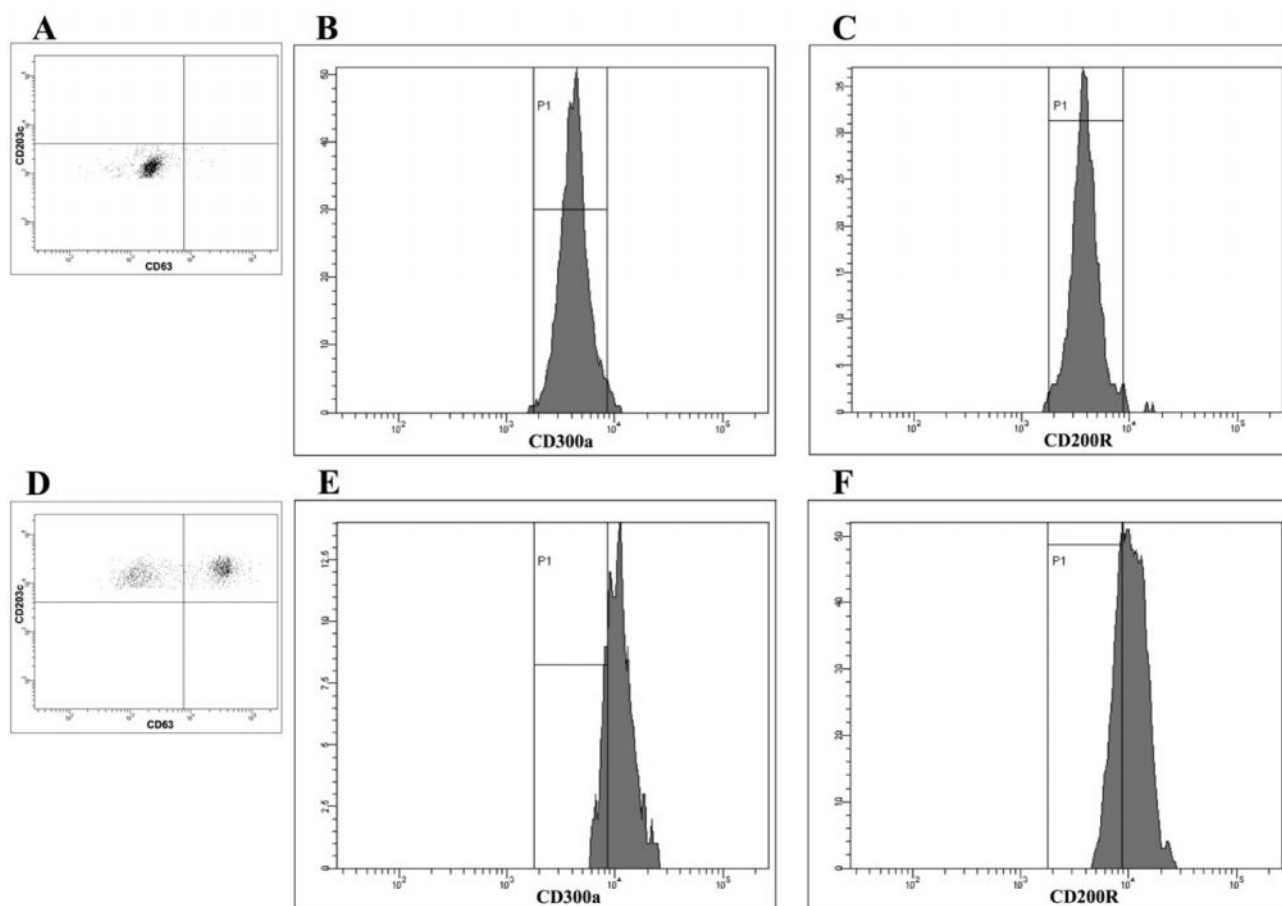
Figure 3

(A) Effect of CD200R and CD300a receptor triggering on SHIP-1 phosphorylation in purified human basophils. Results are shown from one representative experiment from a total of 6 separate basophil donors. (B) Densitometric analyses of SHIP-1

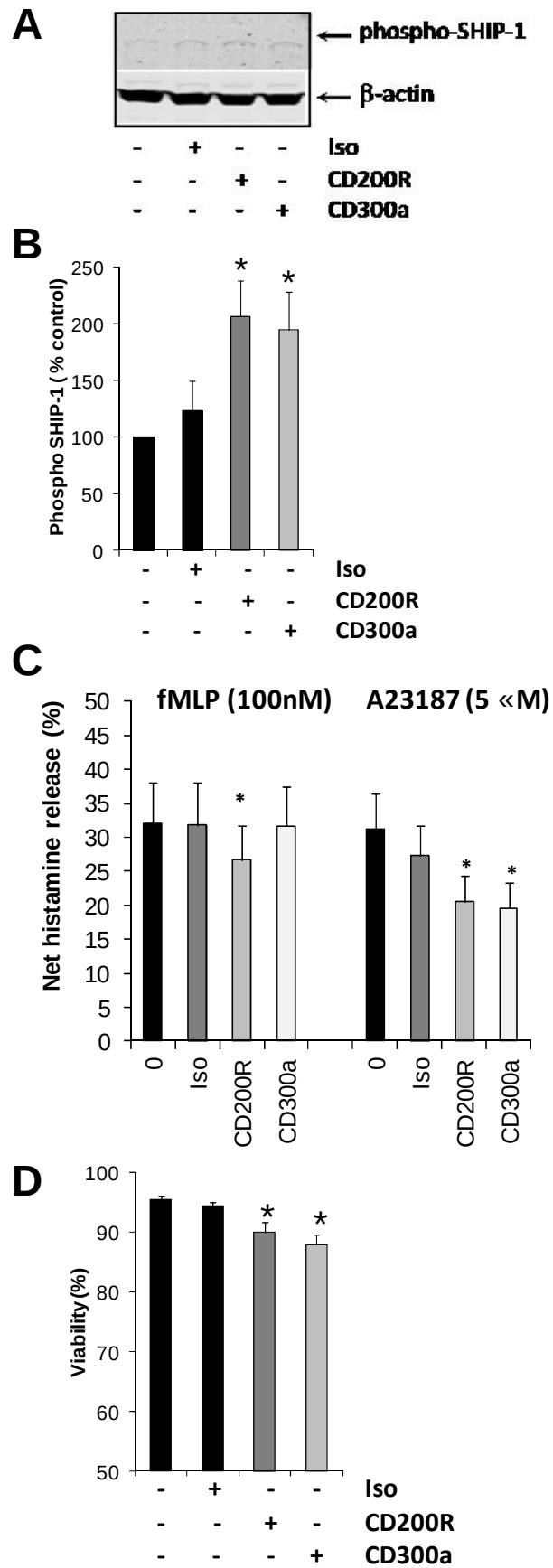
phosphorylations compared to actin expressions (n=6). (C) Effects of anti-CD200R and anti-CD300a on calcium ionophore (A23187) and fMLP-induced basophil histamine release. Results are shown as net percentage histamine release $\pm$ SEM (corrected for 9.2 ± 1.5 % spontaneous control). (D) Effect of CD200R and CD300a receptor triggering on basophil viability following 2h incubation at 37°C (n=6). * represents significant ($p < 0.05$) differences compared to isotype controls using a paired Student's t-test.



Supplementary figure 1



Supplementary figure 2



Supplementary figure 3