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Suppression of Normal and Malignant Kit Signaling by a Bispecific Antibody Linking Kit with CD300a[1]

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Through its receptor Kit (CD117), stem cell factor (SCF) critically regulates human mast cell (MC) differentiation, survival, priming, and activation. The dominance of SCF in setting these parameters compels stringent contra-regulation to maintain a balanced MC phenotype. We have synthesized a library of bispecific Ab fragments to examine the effect of linking Kit with CD300a. In this study, we report that CD300a exerts a strong inhibitory effect on Kit-mediated SCF-induced signaling, consequently impairing MC differentiation, survival, and activation in vitro. This effect derives from Kit-mediated tyrosine phosphorylation of CD300a and recruitment of the SHIP-1 but not of SH2-containing protein phosphatase 1. CD300a inhibits the constitutive activation of the human leukemic HMC-1 cells but not their survival. Finally, CD300a abrogates the allergic reaction induced by SCF in a murine model of cutaneous anaphylaxis. Our findings highlight CD300a as a novel regulator of Kit in human MC and suggest roles for this receptor as a suppressor of Kit signaling in MC-related disorders. ***The Journal of Immunology,*** **2008, 180: 6064–6069.**

Since their emergence as important effector cells in a variety of conditions other than allergy, mast cells (MC)[3] have been the focus of extensive research (1). Despite this, many factors regulating their differentiation, homing, survival, death, and activity are still not completely defined.

Among these, the cytokine known as stem cell factor (SCF) or steel factor is a uniquely critical determinant in MC biology (2). The presence of SCF alone is sufficient to direct human hematopoietic progenitors to become MC in vitro (3–6). SCF is the most potent promoter of MC survival and priming, and also induces their activation (7, 8). Finally, it functions as a MC chemoattractant (9) and a costimulator of activation by eosinophil major basic protein (10). Murine MC are subject to integrated regulation by SCF and IL-3 (11, 12).

SCF functions are mediated through a specific receptor termed Kit (CD117), which is abundantly expressed on MC. Binding of SCF to Kit triggers the latter's tyrosine kinase activity. This subsequently initiates a pathway involving PI3K, Btk, Non-T cell activation linker, and Syk, and results in promoting survival, mediator synthesis, and release (13–16). Mutations leading to aberrant Kit signaling induce mastocytosis (17, 18), gastrointestinal stromal tumors (19), and other malignant disorders, and its intact negative control is therefore vital.

Despite their importance, the mechanisms responsible for negative regulation of Kit in human MC are unclear. Malbec et al. (20) have elegantly shown that FcγRIIB (CD32) inhibits SCF-induced MC survival. However, other key factors such as differentiation and activation are also integrated in the phenotype of MC and the allergic response.

In this work, we demonstrate that SCF-induced survival, differentiation, and activation of MC are inhibited by the novel MC inhibitory receptor CD300a. Our findings show that CD300a regulates critical checkpoints in the "life-cycle" of MC and is also capable of suppressing constitutive activity of leukemic MC. CD300a may therefore be of value as a therapeutic target in MC-associated disorders.

Materials and Methods

Cell culture

MC were derived in vitro from human cord blood mononuclear cells as previously described (21). Cells were cultured in MEM Alpha (Biological Industries) enriched with 100 ng/ml SCF (a generous gift from Amgen, Thousand Oaks, CA), 10 ng/ml IL-6 (PeproTech), and prostaglandin E_2 (Sigma-Aldrich), with weekly refreshment of the medium. MC maturity was evaluated by toluidine blue and FACS analysis of tryptase (using anti-human tryptase clone AA1; DakoCytomation), and cells were used only when ≥95% were mature MC. HMC-1 cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and were cultured as previously described.

All experimental procedures involving cells from human cord blood were reviewed and approved by the Hadassah University Hospital Helsinki Committee, and its guidelines were strictly followed.

Bispecific Ab generation

Bispecific $F(ab')_2$ were generated as previously described (22, 23). Briefly, whole IgG(κ) Abs were digested using agarose-immobilized pepsin (Pierce) and reduced using cysteamine (Fluka) in the presence of sodium arsenite (Merck) to yield Fab′. Fab′ from different Ab species were activated using Ellman's reagent and then reconjugated to yield a bispecific $F(ab')_2$. The process was monitored by SDS-PAGE, spectrophotometry, and standard BCA (Bio-Rad) to assess yield and purity. FACS analysis was used to verify that the intermediate and final products were functional. Anti-human precursor Abs: anti-CD300a (clone P192) and CDw328 (clone

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[3] Abbreviations used in this paper: MC, mast cell; SCF, Stem cell factor; SHP-1/2, Src homology 2 containing protein tyrosine phosphatase 1 or 2; SHIP-1, Src homology 2 containing inositol 5′ phosphatase 1; LAT, linker for activation of T cells; PI, propidium iodide.

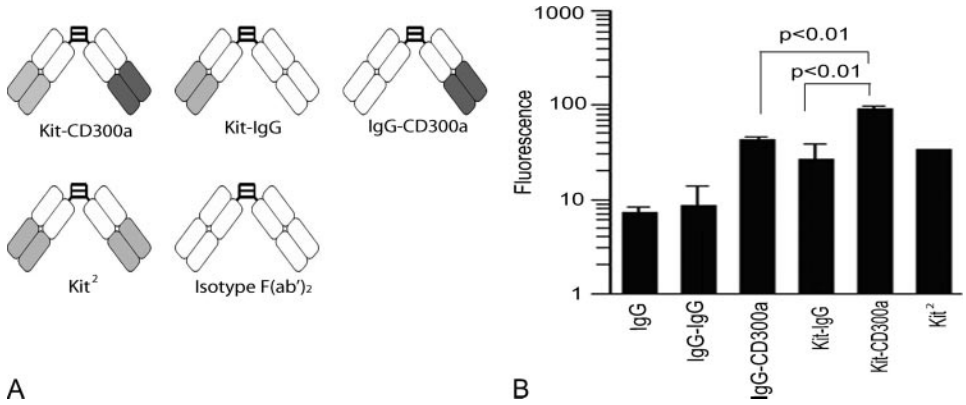

FIGURE 1. Ab fragments used in this study. *A*, The library of $F(ab')_2$ that were generated for this study. *B*, MC were incubated with the Ab fragments, followed by Cy^5-conjugated goat anti-mouse. The fluorescence intensity was analyzed by FACS. Iso = isotype match IgG ($n = 3$; $p < 0.001$).

QA79) (kindly provided by Drs. A. and L. Moretta, Genova, Italy), anti-Kit (clone YB5.B8; BD Biosciences), and negative control IgG (Dako-Cytomation). Anti-mouse precursor Abs: anti-CD300a (clone NKRL-1–172224.111; R&D Systems), and anti-Kit (clone 2B8; Biolegend).

Inhibition assays

For IgE-dependent activation, MC were cultured for 4 days in the presence of 5 μg/ml human myeloma IgE (Calbiochem). Cells were washed and placed in cold TG^{2+} buffer (137 m*M* NaCl, 12 m*M* $NaHCO_3$, 5.5 m*M* L-Glucose, 2 m*M* KCl, 0.3 m*M* Na_2HPO_4, 0.1% w/v gelatin, 1.8 m*M* $CaCl_2$, and 0.9 m*M* $MgCl_2$) and activated by addition of 5 μg/ml anti-human IgE (clone GE1; Sigma-Aldrich) for 30 min at 37°C. For SCF-dependent activation, MC were deprived from SCF for 24 h, washed and resuspended in TG^{2+}, and 0.25 μg/ml SCF were added for 30 min at 37°C.

Inhibition of activation was achieved by incubating the cells, before wash and activation, with the specified $F(ab')_2$ and concentrations for 15 min on ice. Inhibition of differentiation was achieved by adding the specified $F(ab')_2$ (0.01 μg/ml) to the culture medium once a week during cord blood MC growth. For this assay 2×10^6 cells were used as starting population to compensate for $F(ab')_2$-induced reduction in viable cells. Inhibition of survival was achieved by incubating the cells for 72 or 96 h (for HMC-1 and cord blood MC, respectively) in a 96-well plate coated with sheep anti-mouse (MP Biomedicals) followed by the specified $F(ab')_2$.

Inhibitory effects were measured as follows: for activation, tryptase and β-hexosaminidase release were measured using a chromogenic assay as previously described (21). For differentiation, cells were stained for tryptase followed by Cy (5)-conjugated goat anti-mouse, FITC-conjugated Annexin V (R&D Systems), and propidium iodide (PI) (Sigma). Only Annexin V^-/PI^- cells were analyzed for tryptase expression. For survival, cells were stained with PI, followed by FACS analysis.

Flow cytometry

For surface molecule detection, 10^5 cells/sample were incubated with primary Ab in cold HBA (0.1% w/v BSA and 0.05% w/v sodium azide in Hank's solution) for 30 min on ice, washed twice, and incubated with secondary Ab as above. For intracellular FACS, cells were first fixed in 2% formaldehyde in Hank's solution for 10 min on ice, then permeabilized and blocked using blocking buffer (10% w/v BSA, 1% v/v human serum, 0.1% w/v saponin, 10 m*M* HEPES, and 0.05% w/v sodium azide in Hank's solution) for 10 min on ice. Abs were added as in the surface FACS assay in HBA containing 0.1% w/v saponin and 10 m*M* HEPES. Anti-(phospho)Kit and anti-(phospho) linker for activation of T cells (LAT) were purchased from Cell Signaling. Anti-(phospho)SHIP-1 was purchased from Calbiochem.

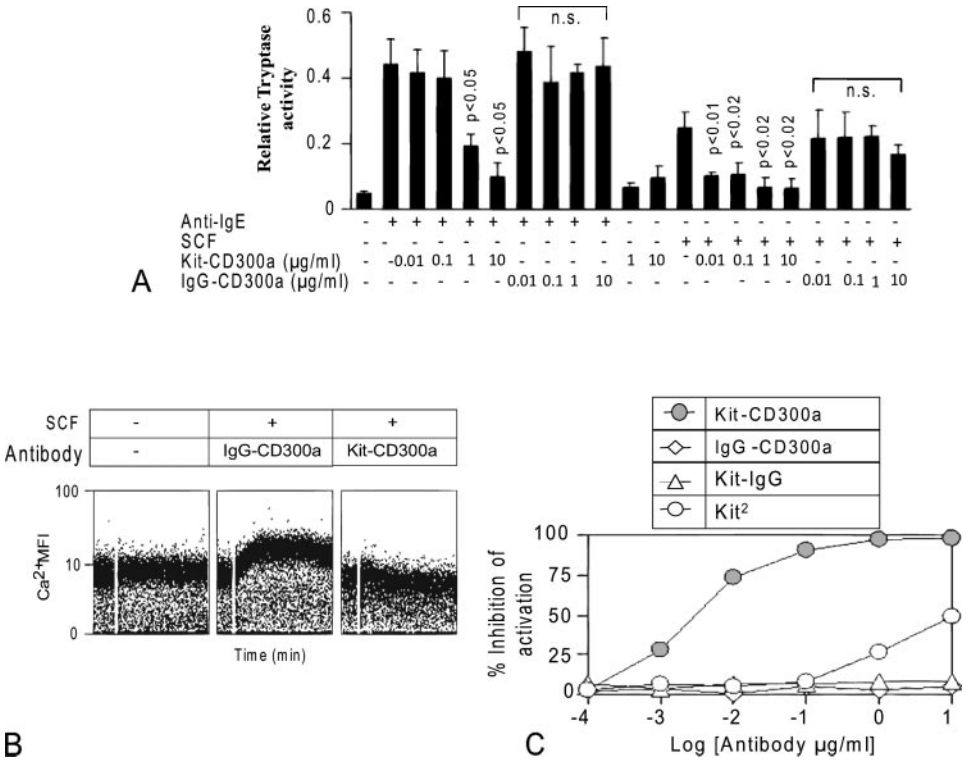

FIGURE 2. Kit-CD300a inhibits MC activation. *A*, IgE-sensitized MC were incubated with various Ab fragments and activated using anti-IgE or SCF. Tryptase activity in the supernatant was subsequently measured ($n = 4$, p see figure). *B*, IgE-sensitized cells loaded with Calcium Green-1AM were activated by SCF, and Ca^{2+} flux was measured by FACS ($n = 3$; $p < 0.001$). *C*, The dose-response curves (measured as dose: Ab concentration; μg/ml and response: Tryptase activity; OD) of the various fragments were plotted to quantify potency variation between Kit-CD300a and the potentially neutralizing Kit^2 ($n = 3$; $p < 0.001$).

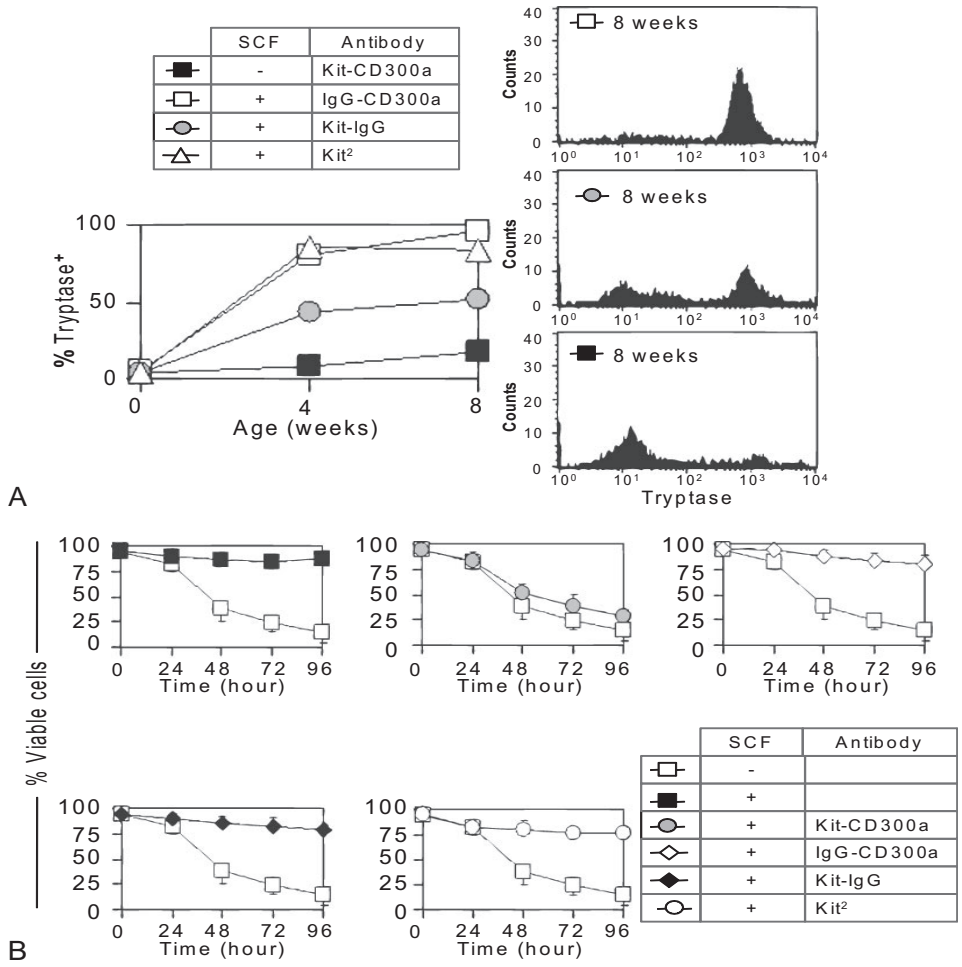

FIGURE 3. Kit-CD300a inhibits MC differentiation and survival. *A*, Cord blood mononuclear cells were cultured in the presence of SCF with weekly Kit-CD300a addition, and tryptase content was evaluated by FACS at wk 0, 4, and 8 ($n = 2$; $p < 0.01$). *B*, MC were cultured in the presence of SCF and Kit-CD300a (0.01 μg/ml), and viability was detected by PI staining analyzed by FACS ($n = 3$; $p < 0.001$).

Ca^{2+} mobilization

Calcium mobilization was performed as described previously (21). Briefly, cells deprived of SCF were loaded for 1 h with 5 μM of Calcium Green-1AM (Invitrogen), washed extensively with cold TG^{2+}, and activated by addition of 0.25 μg/ml SCF during flow. The buffer was prewarmed to 37°C before flow.

Immunoprecipitation and Western blot

Immunoprecipitation was performed using the Mammalian Seize-X Classic kit (Pierce) according to the manufacturer's instructions, using 3–6 $\times$ 10^6 cells/sample. Precipitating Ab was anti-CD300a clone E59 (kindly provided by Drs. A. and L. Moretta, Genova, Italy). Samples were run on standard SDS-PAGE, transferred to polyvinylidene difluoride membranes (Pierce) and blotted using the following Abs: anti-CD300a (clone P192), anti-phosphotyrosine, anti-SHP-1, and anti-SHIP-1 (Santa Cruz Biotechnology). Peroxidase-conjugated anti-mouse and anti-rabbit secondary Abs were purchased from Jackson ImmunoResearch Laboratories.

SCF-induced cutaneous anaphylaxis

SCF-induced cutaneous anaphylaxis was performed as previously described (7). Briefly, 8-wk-old female BALB/c mice were slightly anesthetized with inhaled isoflurane and gently shaved on the dorsal side. The following solutions were injected s.c., 25 μl on each of four dorsal points: saline, murine SCF (0.75 μg in saline), murine SCF (0.75 μg) mixed with Kit-CD300a $F(ab')_2$ termed IK1 (0.2 μg) in saline, and murine SCF (0.75 μg) mixed with an isotype control $F(ab')_2$ (0.2 μg) in saline. Immediately following these injections, the mice were injected i.v. with 1% Evan's Blue solution in saline and sacrificed 20 min later. Dye spots were assessed visually. Murine SCF was purchased from PeproTech.

Animal experimental procedures were reviewed and approved by the Faculty of Medicine Committee of Animal Experimentation, and its guidelines were strictly followed.

Statistical analysis

Data are presented as mean $\pm$ SD and analyzed by ANOVA followed by paired student's t test assuming equal variances. An $\alpha \leq 0.05$ is considered statistically significant throughout the study.

Results

Generation of a Kit-CD300a bispecific Ab fragment

Numerous observations have shown that inhibitory receptors exert an effect when linked with an activating receptor (24) or at least that this effect is more potent under this condition (21). Because the ligand of CD300a is unknown, we used a bispecific Ab approach to link Kit with CD300a. Using chemical conjugation, we generated a library of bispecific Ab fragments to test our hypothesis (Fig. 1*A*). To ensure that observed effects were specific to the action of Kit-CD300a and not a result of Kit neutralization or sterical interference with SCF binding, Kit^2 and Kit-IgG were generated. As an additional control for the construct IgG-CD300a bispecific Abs were synthesized. MC were brightly stained by Kit-CD300a. Kit^2 induced intermediate staining and IgG-CD300a and Kit-IgG induced weak to negligible staining (Fig. 1*B*).

Inhibition of SCF-induced MC activation

We first examined the effect of linking Kit to CD300a on the direct stimulatory effect of SCF. For this we used anti-IgE or SCF to activate MC previously incubated with Kit-CD300a or its control IgG-CD300a. Kit-CD300a inhibited both anti-IgE- and SCF-induced activation, the latter more potently (Fig. 2*A*). In addition, the

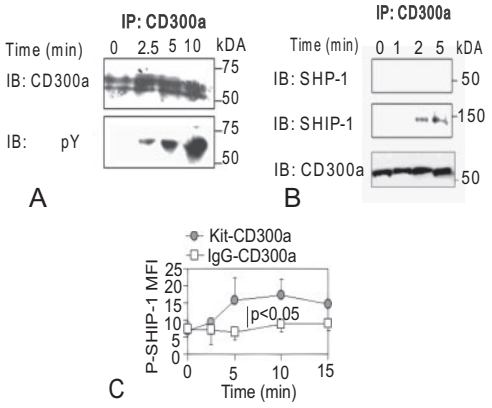

FIGURE 4. Kit-CD300a induces CD300a phosphorylation and SHIP-1 recruitment and activation. *A*, MC were incubated with SCF and Kit-CD300a for the indicated time points, CD300a was precipitated and blotted vs itself, phosphotyrosine, SHP-1, and SHIP-1 (*B*). *C*, MC were incubated with SCF and Kit-CD300a, and SHIP-1 activity was measured by intracellular FACS ($n = 3$; $p < 0.05$).

calcium influx induced by SCF was inhibited by Kit-CD300a as well (Fig. 2*B*).

A central question is whether the inhibitory effect exerted by Kit-CD300a derives partially from Kit neutralization by the Kit Fab′ in the construct. We have tested several anti-Kit clones and found that all had a certain degree of neutralizing capability in their native IgG form (data not shown). Therefore, we repeated the SCF activation experiment with increasing concentrations of Kit-CD300a, Kit-IgG, IgG-CD300a, and Kit^2 and measured the inhibitory effect of each construct. Although the isotype controls did not inhibit Kit signaling at any observed concentration, Kit^2 exerted 27 and 49% inhibition at 1 and 10 μg/ml, respectively (Fig. 2*C*). Kit-CD300a exerted 90 and 97.5% inhibition at 0.1 μg/ml, and 1 and 10 μg/ml, respectively. Because at 0.01 μg/ml the control constructs (Kit^2 and Kit-IgG) exhibit no significant inhibitory effect, we chose this concentration of kit-CD300a for our additional experiments. This concentration was also chosen to rule out the possibility that Kit^2 induces Kit dimerization and hence MC activation as previously reported (20).

Inhibition of MC differentiation and survival

The in vitro maturation of human MC from cord blood progenitors depends almost exclusively on SCF. We therefore hypothesized that inhibiting Kit would result in defective MC development. To test this, cord blood mononuclear cells were cultured in the presence of SCF and Kit-CD300a at 0.01 μg/ml and examined after 4 and 8 wk. Kit-CD300a inhibited the transition into fully mature MC by 50% as evaluated by tryptase expression (Fig. 3*A*).

Once mature, the survival of MC is maintained mainly by SCF both in vitro and in vivo. We used Kit-CD300a to examine the effect of CD300a on SCF-induced survival. In the absence of SCF, the number of viable cells decreased already within 24 h, declining dramatically starting from 48 h. When MC were cultured with SCF in a plate precoated with Kit-CD300a, culture viability displayed a similar pattern, in contrast to isotype constructs (i.e., Kit^2 and Kit-IgG) which did not exert this effect (Fig. 3*B*).

Kit induces CD300a phosphorylation and SHIP recruitment

To identify the mechanism by which CD300a regulates Kit signaling, we precipitated CD300a from Kit-CD300a treated MC and examined its activation state and interactions with phosphatases. Upon Kit linking, CD300a underwent rapid phosphorylation (Fig. 4*A*). This was not induced by isotype control constructs (data not shown). We had previously shown that CD300a operates via recruitment of SHP-1 and/or SHIP-1 (21, 25) and therefore examined whether these phosphatases are recruited under Kit linking. Surprisingly, we found that CD300a recruits only SHIP-1 but not SHP-1 (Fig. 4*B*). This result also correlated with the activation state of SHIP-1 that was induced by Kit linking (Fig. 4*C*) as shown by FACS analysis.

Inhibition of HMC-1 activity by CD300a

Various human malignancies are associated with mutations in Kit that impair its regulation. We have sought to define whether the constitutively active Kit in malignant cells is still prone to regulation by CD300a. For this, HMC-1 cells were treated with Kit-CD300a, and their activity and survival were measured.

We found that CD300a abrogated the constitutive activation of HMC-1 and their subsequent mediator release in a concentration-dependent fashion (Fig. 5*A*). However, the survival of HMC-1 was not inhibited (Fig. 5*B*). This could mean that the mutant Kit itself cannot be dephosphorylated by CD300a, but its second messenger

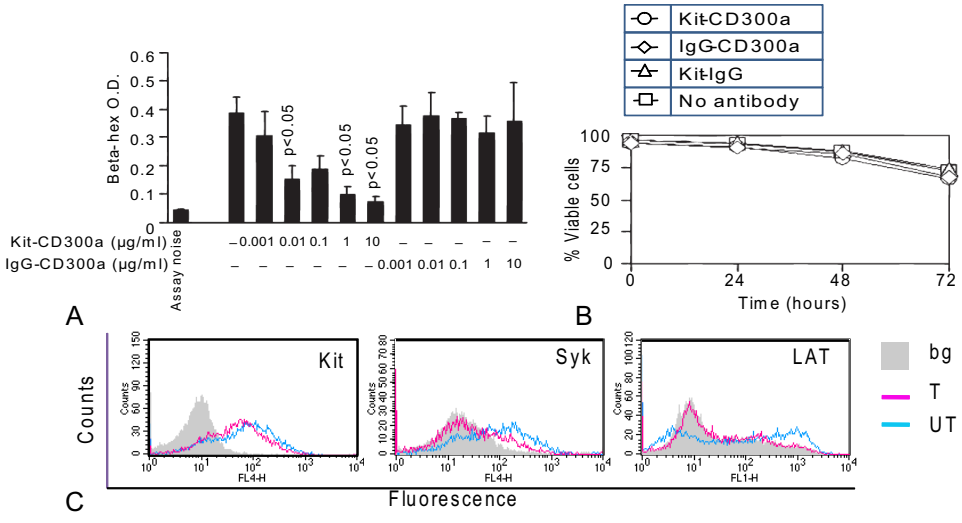

FIGURE 5. Kit-CD300a inhibits HMC-1 activity but not survival. *A*, HMC-1 cells were incubated with various concentrations of Kit-CD300a, and the levels of β-hexosaminidase release (OD) were measured by the chromogenic assay. Assay noise indicates nonspecific substrate cleavage by serum in the medium ($n = 4$; p see figure). *B*, HMC-1 cells were incubated with Kit-CD300a, and viability was measured by PI analyzed by FACS at the indicated time points ($n = 3$). *C*, After treatment with Kit-CD300a, the phosphorylation level of Kit, Syk, and LAT was measured using intracellular FACS. *UT* = Untreated; *T* = treated; and *bg* = background staining.

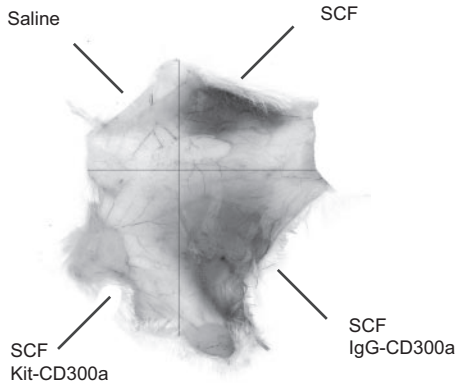

FIGURE 6. Kit-CD300a abrogates SCF-induced cutaneous anaphylaxis. SCF was subcutaneously injected to 3 of 4 dorsal locations, while the fourth was injected with saline alone. Murine-targeting Kit-CD300a IK1 or isotype control was injected simultaneously, immediately followed by i.v. Evans blue. Dye spots represent local MC degranulation events ($n = 3$; $p < 0.001$).

kinases, such as syk, can. To test this, we analyzed the phosphorylation state of Kit, syk, and the LAT, the latter being two important signaling molecules in the MC activation cascade. Although Kit was not significantly dephosphorylated, both syk and LAT were rapidly deactivated by Kit-CD300a following a 15-min incubation (Fig. 5*C*).

Inhibition of SCF-induced cutaneous anaphylaxis

Finally, we aimed to examine the effect of CD300a to regulate Kit signaling in vivo. For this, we tested a murine Kit-CD300a bispecific Ab in a mouse model of SCF-induced cutaneous anaphylaxis. Treatment with Kit-CD300a simultaneously with administration of SCF completely abrogated cutaneous MC degranulation as observed by lack of blue dye spot formation (Fig. 6).

Discussion

Immune functions at all levels are balanced in order for the effector response to be safe and efficient. In human MC, Kit provides a rare example for a signal with tremendous impact on numerous biological features. Hence, Kit is likely prone to regulation tighter than other signals with less influence.

Inhibitory receptors have been shown in recent years to be a critical source of regulatory signals and were subsequently targeted as means to treat immune disorders (22, 26–28). Despite extensive research, the full range of pathways subjected to inhibitory receptor-mediated regulation has not been defined as yet. In human MC, Kit was previously shown to be regulated by FcγRIIB (CD32) (20). Although very important, we hypothesized that this mechanism would not be the only one controlling this dominant signal.

We have shown recently that the inhibitory receptor CD300a is expressed on human MC, eosinophils, and basophils (data not shown), and that it serves as a critical regulator of multiple MC and eosinophil functions. Our goal in this study was therefore to define the regulatory cross-talk between Kit and CD300a.

Immune inhibitory receptors, including CD300a, recruit tyrosine phosphatases such as SHP-1 and inositol phosphatases such as SHIP-1. Both are responsible for deactivation of stimulatory pathway components and secondary messengers. In MC, two main axes mediate cell activation, one involving Syk, LAT/Non-T cell activation linker and phospholipase Cγ, and one involving Fyn and PI3K. Therefore, SHP-1 will eliminate tyrosine phosphorylation events (on which the former is based). Conversely, SHIP-1 will destroy the phosphoinositide messengers that eventually lead to calcium influx and degranulation. Based on this, we anticipated that CD300a, which was shown to recruit both phosphatases upon linking with FcεRI would be capable of shutting off Kit signaling. As we observed, linking CD300a with Kit induced neither SHP-1 nor SHP-2 recruitment (data not shown). This is in contrast to our observations in normal MC where CD300a recruits SHP-1 (21), but in agreement with the reports indicating SHP-1 degradation in leukemic MC (29), and also correlates with the observation that Kit dephosphorylation was not observed. However, the dephosphorylation of other kinases in the cascade raises the possibility that Kit-CD300a leads to recruitment of distinct phosphatases other than SHP-1 and 2.

In accordance with this, Kit-CD300a inhibited IgE-dependent activation of MC even though CD300a was not linked directly to FcεRI, as described in our previously published data. Importantly, this effect confirms previous and unpublished data from our laboratory in which CD300a triggering, regardless of its coupled receptor, is sufficient to inhibit multiple pathways, given that they depend on tyrosine phosphorylation, e.g., STAT-6 activation by IL-4 or innate functions mediated through TLRs (in the latter case, partial inhibitory effects were observed).

In the present work, we have shown that Kit-CD300a in addition to inhibiting IgE- and SCF-dependent activation, down-regulates cord blood mast cells survival in the presence of SCF. It is noteworthy that these inhibitory effects obtained with SCF as a stimulant were not due to sterical interference or neutralization by the kit Fab portion of the bispecific Ab. This was demonstrated by the lack of inhibition with Kit-IgG and Kit[2] as controls. Similarly, previous works that dealt with a bispecific Abs/fusion protein that inhibited MC activation by crosslinking FcεRI with FcγRII did not report any interference with the Ag binding (26, 28, 30). Moreover, it was reported that this specific fusion protein did not exhibit any inhibitory effect on MC derived from FcγRIIB-deficient mice clearly demonstrating that inhibitory ITIM-recruited machinery, rather than a sterical inhibition of the activating receptor, was responsible for the inhibitory effects (31).

Interestingly, CD300a was not able to inhibit HMC-1 survival, in accordance with previous reports showing similar outcome in other myeloid leukemias (32). We speculate that this is due to defective expression of signaling components critical for CD300a function. In addition to SHP-1 degradation mentioned above, the downstream of kinase signaling 1, an important adaptor protein for phosphatase recruitment (30), is very weakly expressed in HMC-1 cells compared with normal human MC (Bachelet and Levi-Schaffer, unpublished results). Conceivably, downstream of kinase signaling 1 enhances the phosphatase deployment effectiveness of CD300a, although in its absence CD300a still operates efficiently enough to abrogate activation signaling. Corollary to this, different signaling pathways may be thought of as having different "intensities." Thus, the mechanism interfering with them must be accordingly strong.

The ligand of CD300a is currently unknown, although it is probably not an HLA molecule (33). The fact that CD300a regulates MC differentiation suggests that its ligand(s) could be expressed within the bone marrow. We also speculate that varying levels of MC tumors might correlate with ligand expression levels. Further research is required to elucidate its localization and modulation during development and diseases.

Our findings indicate a novel role for CD300a in Kit signaling in MC. Along with our previously published data, we establish the value of inhibitory receptors as targets for immune disorders and other diseases. Identification of additional receptors will improve

our understanding of disease mechanisms and enhance the arsenal of available strategies to combat these conditions.

Disclosures

The Hebrew University of Jerusalem through its technology transfer company "Yissum" has a patent licensing with Teva Pharmaceuticals on the CD300a-cKit bispecific Abs platform which is based upon the work of Prof. Francesca Levi-Schaffer, Ido Bachelet, and Ariel Munitz.

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