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Mast Cell Costimulation by CD226/CD112 (DNAM-1/Nectin-2)

*A NOVEL INTERFACE IN THE ALLERGIC PROCESS**

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Mast cells have critical effector functions in various immune reactions. In allergic inflammation, mast cells interact with tissue-infiltrating eosinophils, forming a regulatory unit in the late and chronic phases of the allergic process. However, the pathways and molecules within this unit are still largely undefined. Here, we show that human mast cells and eosinophils express DNAX accessory molecule 1 (DNAM-1, CD226) and its ligand Nectin-2 (CD112). CD226 synergizes with FcεRI on mast cells, and its engagement augments degranulation through a pathway involving Fyn, linker of activation of T-cells, phospholipase C γ2, and CD18. This pathway is subject to negative interference by inhibitory receptors and is completely inhibited by linking IgE with IRp60 (CD300a) using a bispecific antibody. Moreover, blocking CD112 expressed on eosinophils using neutralizing antibodies normalized the hyperactivity resulting from IgE-dependent activation of mast cells co-cultured with eosinophils. Our findings demonstrate a novel interface between these two effector cells, implicating relevance for *in vivo* allergic states. Moreover, costimulatory responses might be a critical component in allergic reactions and may therefore become novel targets for anti-allergic therapy.

During the past decade, mast cells have been recognized as critical immune effector cells. Solid evidence indicates their involvement in various settings extending far beyond their traditionally associated context of type I hypersensitivity reactions, such as innate immunity (1), autoimmunity (2), atherosclerosis (3), and more. Yet many aspects of their effector functions, even in the classical context of allergic reactions, are still unknown.

Until recently, mast cell involvement in the allergic process was confined to its early/acute phase, of which FcεRI-dependent activation forms the central trigger (4). The late/chronic phase, characterized by tissue inflammation and remodeling, was attributed solely to infiltrating leukocytes, notably to the eosinophils. However, several lines of evidence indicate that mast cells participate in modulation of the late/chronic phase mainly by their interactions with eosinophils and T lymphocytes. For example, mast cells induce T lymphocyte proliferation and enhance their activity through OX40/OX40L engagement (5). Reciprocally, intracellular adhesion molecule 1 (ICAM-1)/LFA-1[2] interactions costimulate mast cells upon contact with T lymphocytes (6). FcεRI-dependent activation of mast cells was shown to be enhanced by several receptors, including c-Kit (7), 4-1BB (8), CD28 (9), and CCR1 (10). Mast cell activity is also enhanced by multiple soluble factors as well. Moreover, it is likely to assume that positive signals, especially if operating via separate routes, will stack and synergize with each other.

Although mast cell-eosinophil interactions were shown to enhance cytotoxic functions in helminthic infections, the data regarding mast cell-eosinophil cross-talk in allergic settings are very scarce. Early observations have demonstrated that eosinophil major basic protein and eosinophil cationic protein activate mast cells and basophils to degranulate (11, 12). Eosinophils produce stem cell factor, potentially contributing to mast cell survival and activity (13). On the other hand, mast cells produce IL-3, IL-5, and GM-CSF, important cytokines regulating eosinophil recruitment, survival, and activity (14). Mast cell tryptase evokes eosinophil degranulation through the protease-activated receptor PAR2 (15). However, it is likely that information transfer between cells is mediated more by direct cell-cell interactions, being more efficient, accurate, and liable than paracrine communication systems.

In this study, we demonstrate that human mast cells express DNAX accessory molecule 1 (DNAM-1, CD226) and its ligands Nectin-2 (CD112) and the poliovirus receptor PVR (CD155). We show that CD226 augments FcεRI-mediated activation of mast cells by enhancing a signaling pathway dependent in part on Fyn, LAT, and PLCγ2 but not on Syk. Moreover, this pathway involves association of CD226 with integrins, and preferentially to CD18. We also demonstrate that this pathway is prone to inhibition by inhibitory receptors. Finally, we show that in the presence of eosinophils, mast cell FcεRI-mediated activation is augmented and that this effect is mediated at least partially by a CD226/CD112 interaction. In conclusion, these findings demonstrate a novel interface between mast cells and eosinophils and suggest that CD226 and CD112 participate in modulation of the allergic response.

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[2] The abbreviations used are: LFA-1, leukocyte function-associated antigen 1; DNAM-1, DNAX accessory molecule 1; PVR, poliovirus receptor; ICAM-1, intracellular adhesion molecule 1; LAT, linker for activation of T cells; ERK, extracellular-signal regulated kinase; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; HMC-1, human mast cell leukemia 1; FACS, fluorescence-activated cell sorter; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—All the cell culture media, reagents, and buffers were purchased from Biological Industries, Beit Haemek, Israel. Stem cell factor is a kind gift from Amgen, Inc. (Thousand Oaks, CA). IL-6 was purchased from Peptrotech (Rocky Hill, NJ). The following mouse monoclonal antibodies were prepared as described (16, 17) and used: F22 (anti-DNAM-1/CD226, IgG1), F5 (anti-DNAM-1/CD226, IgM), L14 (anti-Nectin-2/CD112, IgG2a), L95 (anti-PVR/CD155, IgG1), E59 (anti-CD300a, IgG1), P192 (anti-CD300a, IgG2a). The following antibodies and reagents were purchased and used: rat anti-mouse CD300a (clones (NKRL1-172206.111, -172219.111, -172224.111, -172238.111)) from R&D Systems (Minneapolis, MN); mouse anti-human IgE (clone GE-1) from Sigma; anti-human tryptase (clone AA1) and isotype control (IgG1 and IgG2A) antibodies from Dako (Glostrup, Denmark); sheep anti-mouse $F(ab')_2$ from ICN Biomedicals (Aurora, OH); human myeloma IgE/κ from Calbiochem-Merck (Schwalbach, Germany); polyclonal anti-human phosphotyrosine, phosphoserine, Fyn, CD18 and CD29 from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-human phospho-syk, phospho-LAT, phospho-ERK, phospho-PLCγ1, and phospho-PLCγ2 from Cell Signaling (Beverly, MA); horseradish peroxidase-conjugated anti-rabbit and anti-mouse, fluorescein isothiocyanate-, Cy^5-, and phycoerythrin-conjugated secondary antibodies from Jackson Laboratories (West Grove, PA); chromogenic substrates from Sigma; Calcium Green-1AM from Molecular Probes (Eugene, OR); protein G from Pierce; and Ficoll-paque from Amersham Biosciences (Uppsala, Sweden). All other reagents were from Sigma, unless otherwise stated, and were of best chemical grade available. FcR blocking reagent 2.4G2 was purchased from BD Biosciences.

Cell Culture—Human cord blood-derived mast cells were obtained and cultured as described (16). Briefly, mononuclear progenitors were isolated from cord blood by Ficoll gradient and cultured in α-MEM/10% fetal calf serum in the presence of stem cell factor, IL-6, and prostaglandin E_2 for at least 7–8 weeks, with weekly refreshment of the culture medium. At weeks 7–8, mature mast cells were identified using toluidine blue and tryptase staining by FACS. Cells were used upon reaching >95% pure cultures.

Eosinophils were purified from peripheral blood of untreated mildly atopic individuals (blood eosinophil levels >5%) as described (18). Briefly, eosinophils were isolated by a serial procedure of dextran sedimentation, Ficoll gradient, and magnetic cell sorting using anti-CD3/anti-CD16 beads and brought to >98% purity in RPMI 1640/10% fetal calf serum, assessed by Kimura's staining.

HMC-1 cell line (a kind gift from Dr. J. Butterfield, Mayo Clinic, Rochester, MN) is maintained routinely in our laboratory and was cultured in Iscove's modified Dulbecco's medium/10% fetal calf serum with refreshment of the medium upon reaching a cell density of 5×10^6cells/ml. Cord blood and peripheral blood were obtained upon signed consent of the donors, and the entire experimental plan was reviewed and approved by the Hadassah Hospital Helsinki Committee for Human Experimentation.

Activation System and Mediator Assays—For mediator assays, cells were activated in Immunolon-2HB 96-well plates (ThermoLabsystems, MA). The plates were coated with sheep anti-mouse $F(ab')_2$ (25 μg/ml) followed by anti-CD226 (clone F5), anti-CD112, anti-CD155, or isotype control (10 μg/ml, 2 h at 37 °C). Mast cells were sensitized with human myeloma IgE (5 μg/ml, 2 h at 37 °C) 5 days prior to activation. On activation, cell were washed with Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM L-glucose, 2 mM KCl, 0.3 mM Na_2HPO_4, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$), added to the plate on ice simultaneously with mouse anti-human IgE antibody (5 μg/ml), and the plate was incubated at 37 °C for 30 min. Activation was stopped by removing supernatants from cells by centrifugation and freezing of both fractions for analysis. Tryptase was analyzed by a chromogenic assay as described (16). IL-4 was analyzed by a commercial Duoset enzyme-linked immunosorbent assay kit (Diaclone, Besançon, France). Prostaglandin D_2 was analyzed by a commercial enzymatic immunoassay kit (Cayman, Ann Arbor, MI).

For intracellular flow cytometry, antibody incubations (specific and sheep anti-mouse antibody) and activation were performed with cells in suspension (30 min on ice for each antibody incubation). Cells were fixed at the stated time points (see below). For calcium mobilization, antibody incubations (specific and sheep anti-mouse antibody) were performed with cells in suspension (30 min on ice for each incubation), and cells were loaded with Calcium Green-1AM prior to activation (see below).

Flow Cytometry—For surface cytometry, cells were washed once with cold HBA buffer (0.1% w/v bovine serum albumin, 0.05% w/v NaN_3 in Hanks' solution), and both primary and secondary antibody incubations were performed in HBA buffer (30 min on ice with two washes after each incubation). For intracellular cytometry, cells were fixed (2% v/v formaldehyde in Hanks' solution, 10 min on ice) at the stated time points (see Results), permeabilized and blocked (5% w/v bovine serum albumin, 1% v/v human serum, 0.1% w/v saponin, 10 mM HEPES, 0.05% w/v NaN_3 in Hanks' solution, 15 min on ice), and transferred to incubation buffer (0.1% w/v bovine serum albumin, 0.1% w/v saponin, 10 mM HEPES, 0.05% w/v NaN_3 in Hanks' solution). Primary and secondary antibody incubations were performed in incubation buffer (30 min on ice with two washes after each incubation). Data were acquired in a BD Biosciences FACScalibur and analyzed using a CellQuest software on a Mac-based work station.

Intracellular Ca^{2+} Mobilization—After antibody incubations, cells were loaded with Calcium Green-1AM (5 μM, 45 min, 37 °C) in α-MEM (2% v/v fetal calf serum), washed, and resuspended in 300 μl of Tyrode's gelatin buffer (Tyrode's buffer containing 0.1% w/v gelatin) prewarmed to 37 °C. The cells were allowed to flow freely in the cytometer for 30 s. At this time, mouse anti-human IgE antibody (5 μg/ml) was added. Changes in the FL-1 geo mean were recorded for a total of 3 min.

Western Blot and Immunoprecipitation—After antibody incubations and activation, cells (10^6/group) were lysed using M-PER lysis buffer (Pierce) supplemented with protease inhibitor mix (Sigma). Cell lysates were run on SDS-PAGE, trans-

ferred to polyvinylidene difluoride membrane, and blotted *versus* specific proteins (see Results). Immunoprecipitations were performed with a commercial kit (Sieze^{TM} Classic Mammalian Kit, Pierce) according to the manufacturer's instructions, using 7×10^6 cells/group. The antibodies used to precipitate CD226 were clone F22, which do not interfere with CD226 binding by clone F5 antibodies (see above). In some cases, cells were treated with 4 mM Na_3VO_4 (Sigma) for 10 min before lysis. Blotted membranes were developed using ECL Plus (Amersham Biosciences) according to the manufacturer's instructions.

Inhibition Assay—The bispecific antibody linking CD300a to IgE ($IE1^H$) was generated as described elsewhere (19). $IE1^H$ was used to treat mast cells activated with IgE and anti-CD226 as described above.

Mast Cell-Eosinophil Co-culture—Freshly isolated eosinophils were blocked with 10% v/v human serum in RPMI 1640 (10 min on ice) followed by 2.4G2 Fc receptor blocking reagent (10 min on ice), incubated with anti-CD226, anti-CD112, anti-CD155, or isotype control (10 μg/ml, 10 min on ice), and washed. Other groups were further treated with GM-CSF (50 ng/ml, 20 min at 37 °C) and washed extensively. Eosinophils were then added to mast cells that were treated as described above (activation system) to the plate simultaneously with anti-human IgE antibody. Activation was terminated, and mediators were analyzed as described above.

Statistical Analysis—Mediators were measured in triplicates and in at least three different sets of experiments or donor batches. Data are always the mean ± S.D. and were analyzed by analysis of variance followed by paired Student's *t* test assuming equal variances.

RESULTS

CD226 (DNAM-1), CD112 (Nectin-2), and CD155 (PVR) have been shown to mediate and modulate lymphocyte costimulation and effector functions such as tumor cell lysis (20, 21). Moreover, a recent study reported that these receptors are expressed on dendritic cells, thus having important consequences on dendritic cell-lymphocyte interactions (22).

We hypothesized that these molecules may modulate mast cell effector functions in allergic inflammation as well, with a strong emphasis on the manner by which these molecules can mediate information flow between mast cells and eosinophils. We were first set, therefore, to investigate whether CD226, CD112, and CD155 are expressed on mast cells and eosinophils. For this, human mast cells and eosinophils were screened using monoclonal antibodies recognizing CD226, CD112, and CD155. Flow cytometric analysis revealed that mast cells expressed high levels of CD226 and CD112 and negligible levels of CD155. Eosinophils expressed only high levels of CD112 and low levels of CD226 and CD155. We next examined the expression of these receptors on HMC-1 cells in comparison with their expression on primary mast cells. HMC-1 expressed high levels of all three receptors, including CD155, as opposed to the pattern observed on mast cells or eosinophils (Fig. 1).

Once expression of these receptors on mast cells was established, we wished to discern whether they are capable of modulating signal transduction and activation in these cells. For this

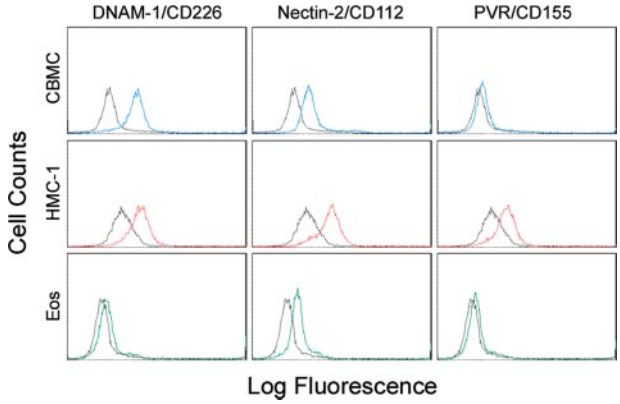

FIGURE 1. **Human mast cells and eosinophils express CD226, CD112, and CD155.** Human cord blood-derived mast cells (*CBMC*), HMC1 mast cell line, and freshly isolated human peripheral blood eosinophils (*Eos*) were stained with monoclonal antibodies recognizing CD226, CD112, and CD155, and expression levels were analyzed by FACS. Negative isotype control was used as background staining (*black peaks*). This figure is representative of three experiments.

purpose, mast cells were activated by FcεRI-IgE cross-linking, simultaneously with antibody-mediated engagement of CD226, CD112, and CD155. We have also activated cells with combinatorial linking (CD226+CD112, CD226+CD155, and CD112+CD155).

Upon engagement of CD226, a substantial increase in FcεRI-induced release of tryptase and IL-4 (Fig. 2*A* and data not shown, respectively) and in eicosanoid synthesis (Fig. 2*B*) was observed. This phenomenon was not shared by CD112 and CD155 or by the isotype control antibodies. Furthermore, the combinatorially linked cells displayed augmented degranulation only when CD226 was engaged.

The mechanism by which CD226 operates in lymphocytes has been studied (23, 24). However, it is still not known whether this mechanism is common regarding all the receptors that collaborate with CD226. Furthermore, mechanisms of mast cell costimulation have been reported that involve integrins, tumor necrosis factor superfamily receptors, chemokine receptors, and others. Thus, no particular hypothesis directed this stage of the study, and we adopted a screening approach of several signaling molecules critical within the mast cell activation cascade.

We have examined the phosphorylation states of syk, LAT, ERK (p44/42 MAPK), PLCγ1, and PLCγ2 in FcεRI-activated/CD226-engaged mast cells. At $t = 15$ min, CD226 augmented the phosphorylation of LAT and PLCγ2 but not of PLCγ1 (Fig. 3*A*). ERK phosphorylation was increased in CD226-engaged cells at $t = 30$ min. Syk phosphorylation levels remained unchanged in all experimental groups.

Degranulation is ultimately induced by a cytosolic calcium influx. We have therefore examined the effect of CD226 on the mobilization of intracellular calcium. We found that CD226 induced a significantly increased calcium influx with an immediate onset (Fig. 3*B*).

As a substrate of protein kinase activity, CD226 has been shown to possess a serine residue mediating its recruitment into lipid rafts and a tyrosine residue mediating its costimulatory activity (24). Our next aim was therefore to investigate whether CD226 on mast cells undergoes the same changes as a

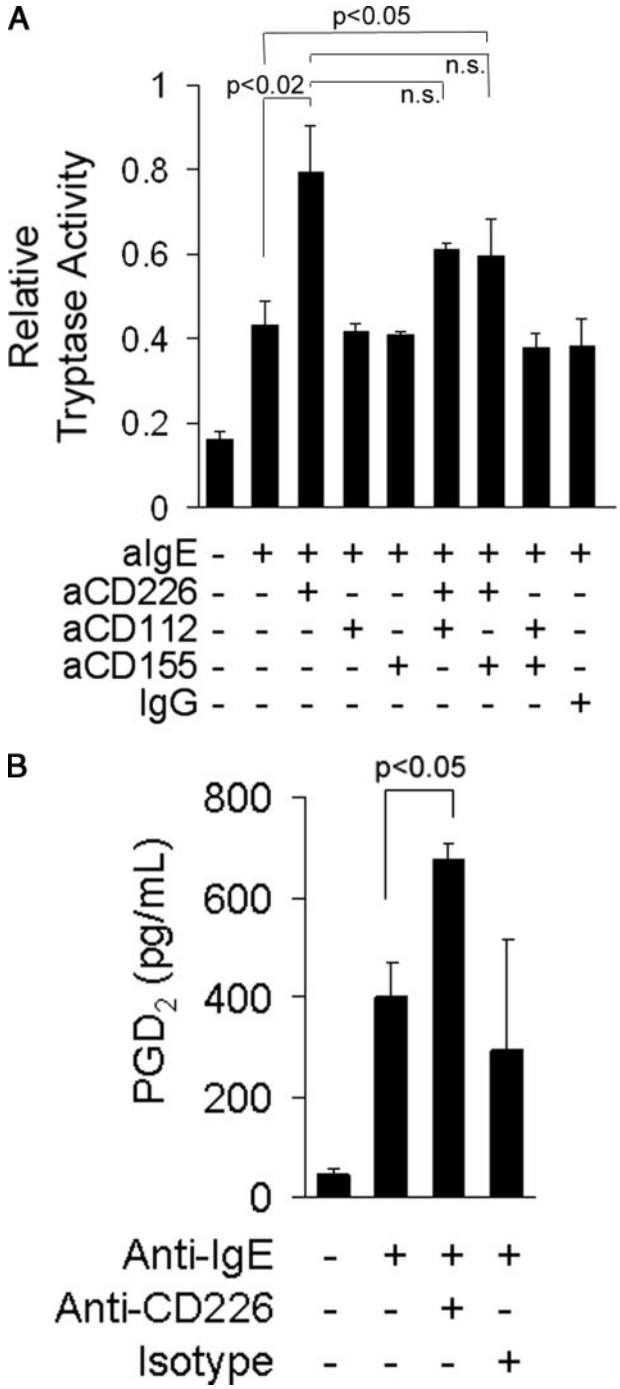

FIGURE 2. **Engagement of CD226 augments FcεRI-induced mast cell activation.** IgE-sensitized mast cells were activated by anti-IgE antibody, simultaneously with cross-linking of CD226, CD112, CD155, CD226+CD112, CD226+CD155, or CD112+CD155. *A*, tryptase levels were measured by a chromogenic assay. *B*, prostaglandin D_2 was measured by enzymatic immunoassay. This figure is representative of four experiments. *NA*, not activated.

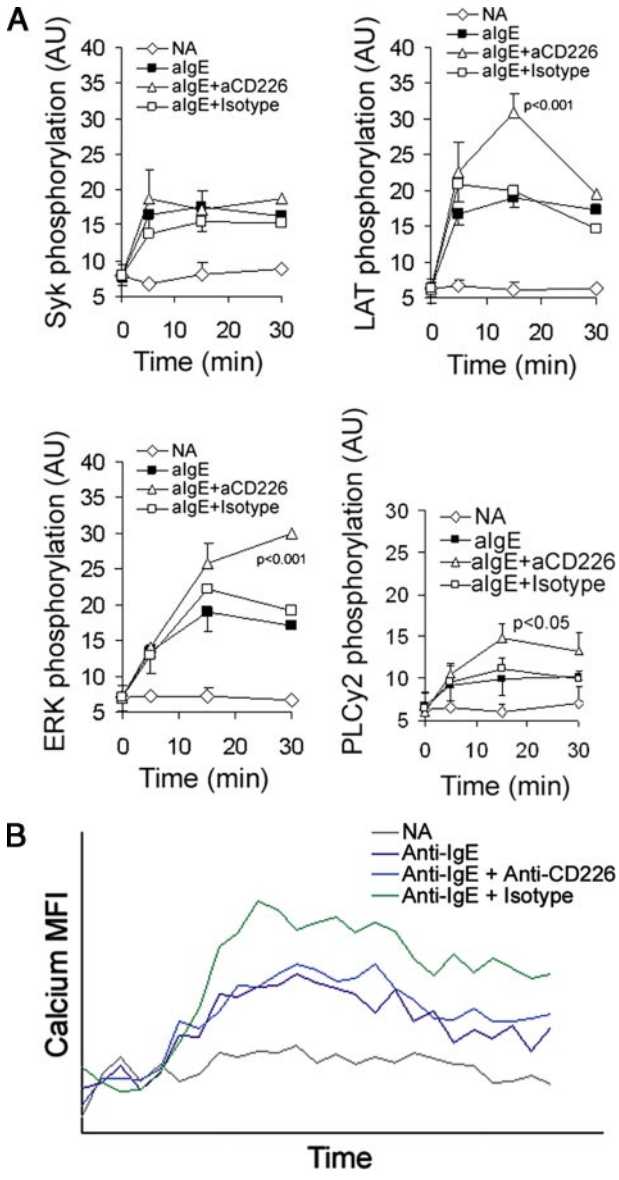

FIGURE 3. **Engagement of CD226 induces a distinct signaling pathway.** *A*, IgE-sensitized mast cells were activated by anti-IgE antibody following cross-linking with CD226 or isotype control. Cells were fixed, and phosphorylated signaling molecules were stained using specific antibodies and evaluated by intracellular FACS analysis. This figure is representative of three experiments. *B*, IgE-sensitized, CD226-engaged mast cells were loaded with Calcium Green-1AM and activated by anti-IgE antibody during flow cytometry at FL-1 *versus* time. This figure is representative of three experiments. Calcium *MFI*, mean fluorescent intensity at FL-1. *NA*, not activated. *AU*, arbitrary units.

part of its signaling mechanism. We have immunoprecipitated CD226 from FcεRI-activated/CD226-engaged mast cells and used Western blot to detect phosphorylated serine. Indeed, as shown (Fig. 4), CD226 is serine-phosphorylated upon its engagement.

To identify the signaling events induced by CD226 in activated mast cells, we have screened for proteins with increased tyrosine phosphorylation level that occur upon CD226 engagement. Immunoprecipitation *versus* CD226 and Western blot analysis showed that in FcεRI-activated/CD226-engaged cells, tyrosine-phosphorylated proteins that bind to CD226 include the following species (in kDa): ~150, ~125, ~65, ~55, and ~45 (Fig. 5*A*). The p65 species correlate with CD226 itself since Syk signaling was not increased following CD226 engagement. On the other hand, LAT phosphorylation was shown to be augmented by CD226, and therefore, it is most likely to correlate to the p45 species.

Based on reports concerning the mechanism of CD226 activity in other cells, we hypothesized that the p55 and p125/p150 species correlate to Fyn and β integrins, respectively, both of which have been shown to associate physically with CD226 in

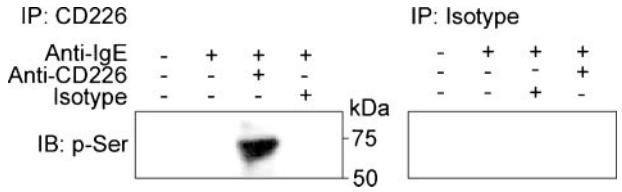

FIGURE 4. **Engagement of CD226 induces serine phosphorylation.** IgE-sensitized, CD226-engaged mast cells were activated by anti-IgE antibody, CD226 was immunoprecipitated (*IP*), and Western blot analysis was performed using anti-phosphoserine (*p-Ser*) antibodies. This figure is representative of three experiments. *IB*, immunoblot.

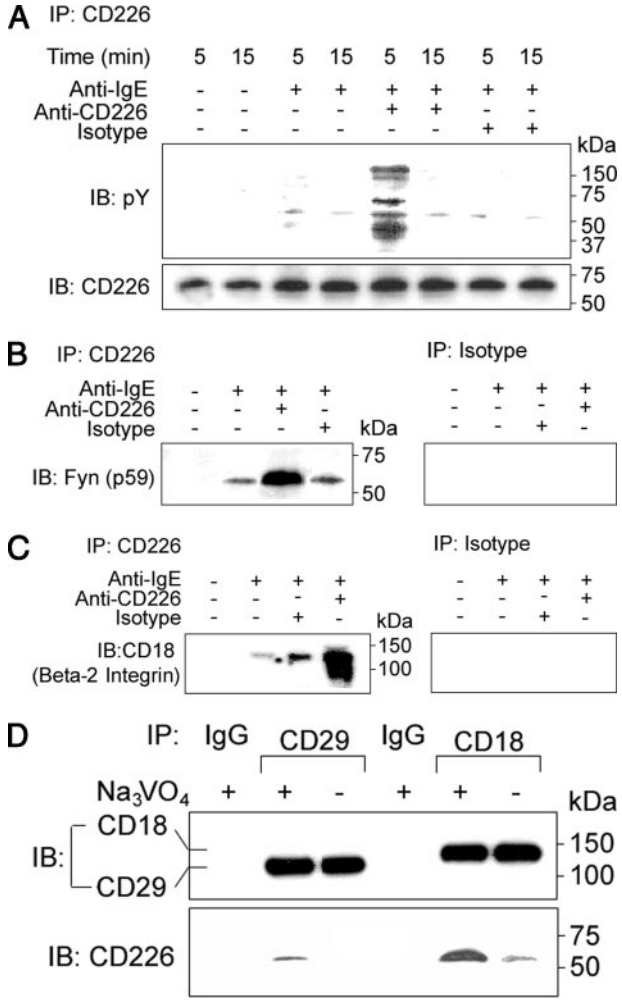

FIGURE 5. **CD226 associates with Fyn and CD18.** IgE-sensitized, CD226-engaged mast cells were activated by anti-IgE antibody, CD226 was immunoprecipitated (*IP*) at 5 and 15 min, and Western blot analysis was performed using anti-phosphotyrosine antibodies (*pY*) (*A*). *IB*, immunoblot. Western blot analysis was performed on the precipitated CD226 using anti-Fyn antibodies (*B*) and anti-CD18 (*C*). Immunoprecipitation of CD29 and CD18 or with isotype control (IgG) was performed after treatment of mast cells with Na_3VO_4, and Western blot analysis was performed using anti-CD226 antibodies (*D*). This figure is representative of three experiments.

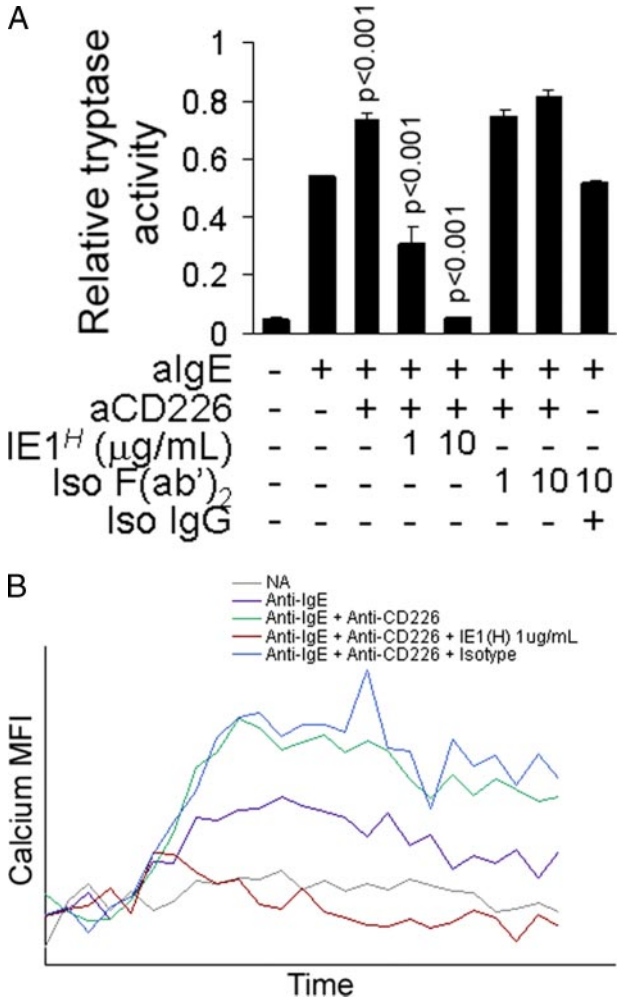

FIGURE 6. **CD226-induced costimulation is blocked by linking IgE and CD300a.** IgE-sensitized mast cells were activated by anti-IgE antibody following cross-linking with CD226 or isotype control. A bispecific antibody fragment linking IgE with CD300a ($IE1^H$) or isotype (*Iso*) $F(ab')_2$ was added with the anti-IgE antibody at the indicated concentrations. Tryptase levels were measured using a chromogenic assay (*A*). Mast cells were activated during flow cytometry at FL-1 *versus* time, simultaneously with $IE1^H$ or isotype (Iso) $F(ab')_2$, after loading with Calcium Green-1AM (*B*). This figure is representative of three experiments. *Calcium MFI*, = mean fluorescent intensity at FL-1. *NA*, not activated. *AU*, arbitrary units.

lymphocytes. To test this hypothesis, we first blotted immunoprecipitated CD226 *versus* Fyn and found that Fyn is physically associated with CD226 already upon FcεRI-induced activation, but this association is substantially increased upon CD226 engagement (Fig. 5*B*).

Second, we have blotted CD226 *versus* CD29 and CD18 and observed that CD226 was strongly associated with CD18, but not with CD29, upon its engagement, although a lesser extent of association was also evident upon FcεRI-induced activation (Fig. 5*C*). To discern whether this is due to inability of CD29 to bind to CD226 in mast cells, we have precipitated CD29 and CD18 from Na_3VO_4-treated cells and blotted them *versus* CD226. Indeed, CD226 associated preferentially to CD18, although a weak CD29 association was also observed (Fig. 5*D*).

We next aimed at determining the susceptibility of CD226 costimulatory signaling to inhibition by immune inhibitory receptors. To test this, we have treated cells with a bispecific antibody linking CD300a to IgE, which was shown to inhibit mast cell degranulation (19) at varying concentrations prior to FcεRI-activation. The augmented activation resulting from CD226 engagement was significantly reduced at 1 μg/ml bispecific antibody and was completely blocked at 10 μg/ml (Fig. 6*A*). The FcεRI/CD226-induced cytosolic calcium influx followed this exact inhibitory pattern at 1 μg/ml (data not shown) and 10 μg/ml (Fig. 6*B*).

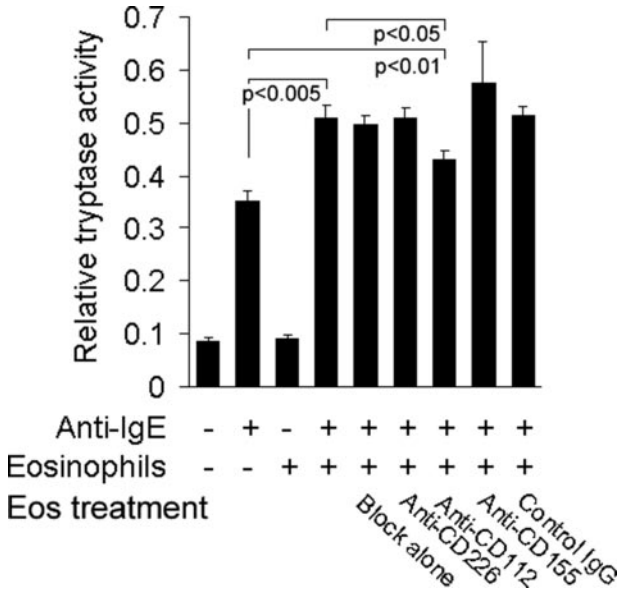

FIGURE 7. **Eosinophils (*Eos*) enhance FcεRI-induced mast cell activation through CD226/CD112 interactions.** CD226, CD112, or CD155 was neutralized on freshly isolated eosinophils using monoclonal antibodies. IgE-sensitized mast cells were activated by anti-IgE antibody in the presence of neutralized eosinophils. Tryptase levels were measured using a chromogenic assay. This figure is representative of three experiments.

Finally, we examined whether CD226 is involved in cell-cell interactions between mast cells and eosinophils. For this, we have co-cultured mast cells with freshly isolated eosinophils after neutralization of CD226, CD112, and CD155 only on the eosinophils and induced FcεRI activation. Moreover, some of the eosinophils were primed with GM-CSF prior to co-culture.

We found that neutralization of CD112 on eosinophils, but not of CD226 or CD155, normalized activation level to that observed without co-culture (Fig. 7). The isotype control did not elicit any significant effects. Preincubation with GM-CSF did not significantly alter this pattern (data not shown).

DISCUSSION

Mast cell-eosinophil cross-talk is a central axis in allergic inflammation, and understanding the signals mediating this cross-talk is crucial for understanding the allergic response. Although many studies have focused on pathways that modulate mast cell and eosinophil activity in allergic and other settings, surprisingly little is known about the reciprocal communication between these two effector cells. This issue extends beyond academic interest since preventing these cells from sharing information may be a promising approach in combating allergic diseases such as asthma, atopic dermatitis, allergic rhinitis, etc., which are constantly on the rise (25).

CD226 (DNAM-1) is a molecule mediating tumor-directed cytotoxicity and adhesion (20) in natural killer and T lymphocytes. CD112 (Nectin-2) and CD155 (PVR) were shown to be functional ligands of CD226 (17, 26). To date, most of the data regarding CD226 were deduced from lymphocyte studies. Recently, dendritic cells were shown to express CD226 (22, 27). Other than this, no other functions for these molecules have been observed, and no functions in cells other than lymphocytes were assigned for them.

In this study, we report that CD226 (DNAM-1) functions as a costimulatory receptor on human mast cells. Moreover, we report that CD226 is engaged by CD112 on human eosinophils to elicit its costimulatory effect. Based on these two lines of evidence, we present a novel interface between human mast cells and eosinophils that may constitute an important component in allergic disorders.

Expression analysis revealed that mast cells express high levels of CD226 and CD112, whereas expressing very low levels of CD155. However, only CD112 was expressed in high levels on eosinophils. This observation may provide an important hint as to the structure of the network between mast cells and eosinophils.

Strikingly, leukemic HMC-1 cells expressed very high levels of CD155, supporting previously described observations (22, 28). CD155 can be also down-regulated, for example by cytomegalovirus UL141 (29). This plasticity raised the question regarding the capability of various mediators to modulate CD226, CD112, and CD155 on mast cells. However, no mediator we have tested so far could induce a change in any of these molecules on human mast cells (data not shown).

It has been recently shown that CD226 augments the activation induced by several receptors containing an ITAM, *e.g.* CD16 and NKp46, as well as by receptors containing an ITSM, *e.g.* 2B4 (CD244) (30). We therefore hypothesized that FcεRI-mediated signaling will also be enhanced by cross-linking with CD226. Indeed, we have observed a costimulatory effect of CD226 on FcεRI, a multiple ITAM-bearing receptor. This phenomenon could mean that mast cells *in vivo* require CD226 to develop a full intensity allergic response. Conversely, CD226 engagement alone was not sufficient to induce mast cell activation, suggesting only a modulatory role on mast cells.

CD226 exerts its effect through a distinct signaling pathway. Analysis of signaling molecule phosphorylation indicated that Syk is not included in this pathway. On the other hand, LAT and PLCγ2 were substantially more phosphorylated upon CD226 engagement. This is also in support of previously described observations (7, 8, 31, 32). Other kinases, such as Btk (8, 9), could also be involved in this pathway. Intriguingly, maximal phosphorylation of CD226 was observed at 5 min, whereas LAT and ERK phosphorylation peaked at 15 min. This accounts for the fact that LAT and ERK are both downstream and independent of CD226, and although these signals propagate (15 min), CD226 rapidly reverts back to its standby state.

CD226 on T cells was shown to associate with LFA-1, a complex involving CD18 (23). In addition, LFA-1 mediates costimulation of mast cells by T cells (6). We therefore anticipated a β2-associated mechanism. Indeed, CD226 on mast cells bound preferentially to CD18, although it displayed an ability to bind to CD29 as well. This versatility could indicate that no limitations exist for synergy between activating pathways, in the sense that different cascades will not have to compete over a limited pool of common signaling molecules.

Based on our hypothesis that CD226-induced costimulation might contribute to a fully developed allergic responses *in vivo*, we view CD226 as a valuable target in allergic diseases. As we have shown previously, mast cells express CD300a, a potent inhibitory receptor capable of blocking FcεRI-induced degran-

ulation. Our findings here demonstrate that CD226-induced signaling is prone to blockade by ITIM-bearing inhibitory receptors, and therefore, this could be considered in the future as a new approach in allergy therapy as well (33).

A central question raised by our study is whether eosinophils communicate with mast cells through costimulatory receptor/ligand interactions. This question is crucial in allergic diseases since mast cells and eosinophils together are the key effector cells in these settings. Yet the data regarding their interactions, in any setting, are very scarce.

Eosinophils and mast cells may mutually activate and enhance each other's functions by a variety of signals, most of them secreted. We initiated a large scale screening of surface molecules to identify candidate receptor/ligand pairs that might mediate cell-cell interactions between mast cells and eosinophils and partially reported these findings (16, 18). Eosinophils, but not mast cells, express 2B4 (CD244), an ITSM-bearing CD2-subfamily receptor, that is capable of activating human eosinophils *in vitro* (34). The high affinity ligand of 2B4 is the glycosyl-phosphatidylinositol-anchored protein CD48, which is abundantly expressed throughout the immune system. CD48 is highly expressed on the mast cell surface, where it was recently shown to be a functional receptor for the fimbrial adhesion molecule FimH (35). CD48 also mediates phagocytosis of Gram-negative bacteria by mast cells (36, 37). We found that in co-culture with eosinophils, mast cell activation is enhanced and that this effect is partially 2B4/CD48-dependent.[3] Similarly, CD226/CD112 may contribute to this interface as a costimulatory signaling switch. Other switches could provide, for example, inhibitory signals (inhibitory receptor/ligand pairs). Our findings raise the fascinating possibility that a wide network of signaling exists between mast cells and eosinophils, which has to be further elucidated.

In conclusion, we show here a novel interface between mast cells and eosinophils, with important possible consequences on chronic allergic processes and other diseases on which these two cells are associated. As mentioned, blocking this interface may have a critical value in future therapy of allergic diseases.

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[3] I. Bachelet and A. Munitz, unpublished results.

Mast Cell Costimulation by CD226/CD112 (DNAM-1/Nectin-2): A NOVEL INTERFACE IN THE ALLERGIC PROCESS

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