E-Cadherin restricts mast cell degranulation in mice

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Crosslinking of FcεRI-bound IgE triggers the release of a large number of biologically active, potentially anaphylactic compounds by mast cells. FcεRI activation ought to be well-controlled to restrict adverse activation. As mast cells are embedded in tissues, adhesion molecules may contribute to limiting premature activation. Here, we report that E-Cadherin serves that purpose. Having confirmed that cultured mast cells express E-Cadherin, a mast-cell-specific E-Cadherin deficiency, Mcpt5-Cre E-Cdhfl/fl mice, was used to analyze mast cell degranulation in vitro and in vivo. Cultured peritoneal mast cells from Mcpt5-Cre E-Cdhfl/fl mice were normal with respect to many parameters but showed much-enhanced degranulation in three independent assays. Soluble E-Cadherin reduced the degranulation of control cells. The release of some newly synthesized inflammatory cytokines was decreased by E-Cadherin deficiency. Compared to controls, Mcpt5-Cre E-Cdhfl/fl mice reacted much stronger to IgE-dependent stimuli, developing anaphylactic shock. We suggest E-Cadherin-mediated tissue interactions restrict mast cell degranulation to prevent their precocious activation.

Keywords: degranulation · E-Cadherin · IgE · inflammatory cytokines · mast cells

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Mast cells may be best known for their critical role in allergic disease [1–5]. However, mast cells were reported to orchestrate several immune responses by the release of multiple mediators (reviewed in [6–13]), although the dimension of these biological contributions is debated [14] and continues to be actively researched. Mast cells are critical effectors in the response to venoms such as bee or snake venoms [15, 16]. There are distinct mast cell types and in mice, they are classified as connective tissue and mucosal mast cells (MMC) due to their mediator content and histochemical and functional properties [17].

Among the surface receptors expressed by mast cells, the high-affinity receptor for IgE (FcεRI) is a key survival and activation
receptor (reviewed in [18, 19]). Crosslinking of the IgE-bound FcɛRI by a specific antigen leads to exocytosis of prestored content of granules in an immediate reaction responsible, for example, for the type I hypersensitive reaction [20]. More than 200 compounds released from the granules include bioactive amines, such as histamine and serotonin, enzymes like hexosaminidase, proteases, lipid mediators like leukotrienes and others [19]. Minutes thereafter, mast cells start synthesizing proinflammatory cytokines which are secreted within the next hours, known as the late-phase response. These proinflammatory cytokines serve, for example, as chemoattractants for other immune cells in support of the ensuing immune response.

Under physiological conditions, mast cells interact with a variety of cell types. This interaction can be facilitated by adhesion molecules. Dudeck et al. [21] showed that immature mast cells adhere to endothelial cells via E- and P-selectin, VCAM-1, and PECAM-1, but the effect on mast cell activation was not reported. Besides these adhesion molecules, mast cells also express integrins and cadherins on the cell surface. The integrins are members of the RGD-integrin family, including α5β1, αvβ3, and αIIβ3 integrins, which are supporting mast cell degranulation induced by mechanical stress [22, 23]. The α2β1 integrin also provides a costimulatory function for mast cell activation and cytokine production in response to infections [24]. The surface expression of several Cadherins by mast cells was only scarcely described and no functional insights were reported. Bone marrow-derived mast cells (BMMC), as well as cultured peritoneal mast cells (CPMC), were reported to express epithelial E-Cadherin (Cdh1) on their surface, and the neuronal Cadherin (N-Cadherin) was shown to be expressed by BMMC [25]. E-Cadherin was described to promote adhesion to a cell layer of cultured E-Cadherin expressing fibroblasts [26, 27]. However, the impact of E-Cadherin on IgE-mediated mast cell activation remained unknown.

Cadherins belong to a superfamily of transmembrane glycoproteins which classically mediate homophilic, calcium-dependent cell-cell adhesion, development, and morphogenesis [28]. E-Cadherin (Cadherin-1) consists of five extracellular domains, a short transmembrane domain, and a cytoplasmic domain (reviewed in [29]). Intracellularly, E-Cadherin is linked to the underlying actin cytoskeleton via catenins [30]. Mechanical disruption of homotypic, E-Cadherin-dependent contacts between DCs was demonstrated to induce maturation leading to a tolerogenic DC phenotype [31]. In another setting, monocye-derived inflammatory DCs featuring E-Cadherin as a distinct marker were shown to accumulate in LNs and colon and to promote intestinal inflammation [32]. Further, heterotypic E-Cadherin-mediated interactions between DCs and NK, CD8+ T cells, Tregs, and IEL via either KLRG1 or αEβ7 integrin with E-Cadherin were reported (reviewed in [33]). Thus, indications accumulated in recent years for a role of E-Cadherin in regulating immune cell biology.

In this study, we examined the hitherto nondescribed functional role of E-Cadherin on mast cells by creating a mast cell-specific E-Cadherin deficiency in mice. The results indicate that E-Cadherin dampens IgE-mediated degranulation and, thus, the allergic response in vivo and in vitro. Further, E-Cadherin deficiency impairs the secretion of IL-2 and TNF-α. We propose that E-Cadherin-mediated tissue interactions protect the organism from overshooting immediate mast cell reactions and fine tune mast cell cytokine production.

**Results and discussion**

**Expression of E-Cadherin by mast cells and general features of E-Cadherin-deficient mast cells**

The expression of Cadherins by mast cells has been little described. In 2000, Tegoshi et al. reported E-Cadherin expression on the surface of cultured BMMC and, at lower levels, of CPMC [26]. We confirmed expression of E-Cadherin by murine BMMC cultured in the presence of IL-3, and showed E-Cadherin expression by CPMC, cultured in the presence of IL-3 and SCF (Fig. 1A and B; Supporting information Fig. S1A). The addition of SCF (c-kit ligand) to BMMC cultures did not change expression levels of E-Cadherin (not shown). Tryptase expression, which was recently reported to be higher in human odontogenic keratocyst and radicular cysts-associated mast cells expressing lower levels of E-Cadherin [34], was not affected by the E-Cadherin deficiency (Supporting information Fig. S1B).

As we speculated that E-Cadherin modulates activation of mast cells, we generated mice featuring mast cell-specific deficiency in E-Cadherin. Scholten et al. described efficient and specific Cre-mediated recombination in peritoneal and skin mast cells in Mcpt5-Cre transgenic reporter mice [35, 36]. To inactivate E-cadherin in mast cells, Mcpt5-Cre mice were crossed to E-cadherin<sup>Δβ1</sup> mice, which carry loxP-sites flanking Exon 3 of Cdh1. Mast cell-specific inactivation of the Cdh1 gene was demonstrated by semiquantitative PCR of the floxed locus in BMMC and CPMC from E-cadherin<sup>Δβ1,Δβ3-Mcpt5-Cre<sup>+</sup></sup> and control mice (Fig. 1A). The cells were taken from 6- to 18-week-old mice and cultured for approximately 5 weeks. The CPMC showed efficient excision of Exon 3 of Cdh1. BMMC, which are widely used in studies of mast cells, cannot be easily assigned to an in vivo correlate and may resemble a more immature phenotype of the MMC type [37, 38]. Excision of Exon 3 of Cdh1 by Mcpt5-Cre in BMMCs was not as efficient, estimated at about 50%. Therefore, CPMC were used for the subsequent experiments. The almost complete absence of E-Cadherin protein was verified by western blotting (Fig. 1B). The weak remaining E-Cadherin (consider that even more protein was loaded for the Cre-positive sample) may reflect few nondeleted cells or may not originate from mast cells in these cultures, but from the few (<3 %) other cell types present. FACS analysis showed the loss of E-Cadherin-positive mast cells in peritoneal lavage of adult, Cre-positive mice (Fig. 1C). Expression of c-kit and FcɛRI, as well as proliferation and apoptosis of CPMC deficient in E-Cadherin (hereafter named Cdh1<sup>Δβ1</sup> CPMC), was not different from that of control cells (Supporting information Fig. S2A-C).
Figure 1. Characterization of E-cadherin expression and of E-cadherin-deficient mast cells. (A) Semiquantitative RT-PCR showing excision of Cdh1 Exon 3 in mast cells of E-CadherinFL/FLMcpt5-Cre+ (Cdh1ex) mice. BMMCs and CPMC were generally analyzed prior to the other experiments. Representative figure from one experiment out of three experiments is shown (three mice), the uncropped blots are shown as Supporting information Fig. S5. (B) Western Blot analysis of E-Cadherin in E-CadherinFL/FLMcpt5-Cre+ and control CPMC cultured for 5 weeks (three control and three Cdh1ex mice, representative of three experiments, one mouse per genotype per experiment); β-tubulin served as a loading control, the uncropped gel images are shown as Supporting information Fig. S6. (C) Percentage of peritoneal mast cells (peritoneal MC) positive in flow cytometry for E-Cadherin in control and Cdh1ex mice (three control and three Cdh1ex mice, from three experiments, one mouse per genotype per experiment). (D) Representative CPMC analyzed by EM. Scale bars, 2.5 μm (three control and three Cdh1ex mice, representative of three experiments, one mouse per genotype per experiment). (E, F) Control (n = 10) and Cdh1ex (n = 13) CPMC analyzed by EM were quantified for size (E) and granule numbers per micrometer square (F). (G) Quantification by flow cytometry of mast cells from peritoneal lavage (peritoneal MC) in control (n = 9) and Cdh1ex (n = 9) mice (data from five experiments; 1–2 mice per genotype per experiment). (H) Quantification by flow cytometry of the skin mast cells in control (n = 7) and Cdh1ex (n = 6) mice (data from five experiments; 1–2 mice per genotype per experiment). Median with 95% CI is indicated and Mann–Whitney test was used in 1 E. Mean and SEM are indicated and unpaired two-tailed Student’s t-test was used in 1 C, F, G, and H. Not significant (ns), p ≤ 0.01 (**).

We also analyzed the ultrastructure of control and Cdh1ex CPMC by EM (Fig. 1D; Supporting information Fig. S2D) and observed no difference. The average size of the CPMC (Fig. 1E) and the number of granules per micrometer square (Fig. 1F) were not significantly different between control and Cdh1ex CPMC. Furthermore, the loss of E-Cadherin did not affect the numbers of mast cells in the peritoneal lavage (Fig. 1G, Supporting information Fig. S3A) or in the skin (Fig. 1H; Supporting information Fig. S3B, where also the full gating strategy is shown).

Loss of E-Cadherin in mast cells enhances anaphylactic reactions in mice

Mast cells are known as key effector cells in anaphylaxis and allergy and can be triggered to degranulate by a wide range of stimuli [1-5, 19]. To determine the role of E-Cadherin in mast cell-dependent anaphylactic reactions in vivo, passive anaphylaxis was tested in two models. In one, passive systemic anaphylaxis (PSA) [39, 40] was performed by sensitizing control and Cdh1ex mice with anti-DNP IgE 24 h before the mice were challenged...
Figure 2. Loss of E-Cadherin in mast cells enhances IgE-induced anaphylaxis in vivo and in vitro. (A) PSA was induced by sensitizing control (grey) and Cdh1<sup>ex</sup> mice (black) with anti-DNP IgE 24 h before DNP challenge with 20 μg DNP (five control and five Cdh1<sup>ex</sup> mice, data from two experiments, 2–3 mice per genotype per experiment) or 30 μg DNP (five control and three Cdh1<sup>ex</sup> mice, three experiments, 1–3 mice per genotype per experiment). Rectal temperature was measured every 10 min (B). Local PCA was quantified by measuring the OD of Evans blue extracted from ear tissue after the anti-DNP IgE/DNP-BSA challenge (six control and six Cdh1<sup>ex</sup> mice, data from three experiments; 1–3 mice per genotype per experiment). Mean ± SEM is indicated; unpaired two-tailed Student's <i>t</i>-test was used for the statistical analysis; <i>p</i> ≤ 0.05 (*), <i>p</i> ≤ 0.01 (**). (C) Degranulation of control (n = 3) and E-Cadherin-deficient (n = 3) CPMC was measured by quantifying the released β-hexosaminidase as the percentage of total β-hexosaminidase present in cell lysates (three control and three Cdh1<sup>ex</sup> mice, from three experiments, one mouse per genotype per experiment). Mean ± SEM is shown, and the unpaired two-tailed Student's <i>t</i>-test was used for the statistical analysis; not significant (ns), <i>p</i> ≤ 0.05 (*), <i>p</i> ≤ 0.01 (**). (D) Surface exposure of CD107a upon degranulation was analyzed by flow cytometry at the indicated time points, dotted lines indicate stimulation with ionomycin and solid lines indicate stimulation by 10 ng/mL DNP-BSA (four control and four Cdh1<sup>ex</sup> mice, data from four experiments, one mouse per genotype per experiment). Data are shown as Means ± SEM; <i>p</i>-values are shown as a result of the unpaired two-tailed Student's <i>t</i>-test between the control and Cdh1<sup>ex</sup> stimulated with DNP-BSA; <i>p</i> < 0.05 (*). (E) Soluble recombinant E-Cadherin (100 ng/mL) was added together with anti-DNP IgE overnight before degranulation was triggered by 100 ng/mL DNP-BSA and CD107a surface expression was measured by flow cytometry (six control and six Cdh1<sup>ex</sup> mice, data from three experiments, one mouse per genotype per experiment). Mean ± SEM is shown; paired two-tailed Student's <i>t</i>-test was used for the statistical analysis; not significant (ns), <i>p</i> ≤ 0.05 (*). (F) Visualization of degranulation of live CPMC through binding of AvidinA488 to surface heparin exposed by degranulation. Progressive formation of a corona of the stained surface is shown as a time course, starting at the time of addition of 5 ng/mL DNP-BSA (0 min). Signal quantification is shown as MFI (29 cells from three control mice and 29 cells from three Cdh1<sup>ex</sup> mice, data from three experiments, one mouse per genotype per experiment). Median with 95% CI are shown; Mann–Whitney test was used for the statistical analysis; not significant (ns), <i>p</i> ≤ 0.05 (*), <i>p</i> ≤ 0.01 (**), <i>p</i> ≤ 0.001 (***) , <i>p</i> ≤ 0.0001 (****).

with 20 or 30 μg DNP-BSA. PSA is an IgE-mediated type I immediate hypersensitivity reaction and causes a drop in body temperature. Body temperature was monitored by recording the rectal temperature every 10 min. Twenty micrograms of DNP-BSA was not sufficient to induce significant PSA in control mice. However, this suboptimal dose clearly induced PSA in Cdh1<sup>ex</sup> mice (Fig. 2A), indicating hypersensitivity of these mice. To induce PSA also in control mice, we increased the DNP-BSA dose to 30 μg. Control and Cdh1<sup>ex</sup> mice showed a strong response (Fig. 2A). The small difference in loss of body temperature was not statistically significant but may indicate a trend consistent with data using 20 μg DNP-BSA. Thus, Cdh1<sup>ex</sup> mice are hyper-reactive in IgE-dependent systemic anaphylaxis.

To independently confirm the increased sensitivity of Cdh1<sup>ex</sup> mice, we induced local immediate passive cutaneous anaphylaxis (PCA) in the ear [40, 41]. Animals were sensitized with anti-DNP IgE by injections into the pinna of one ear. The other ear was injected with PBS for control. Twenty-four hours later, an intraorbital challenge with DNP-BSA in Evans blue was applied. The vascular leakage of Evans blue in the ear was determined by measuring the dye that has extravasated into the ear skin. Consistent with the PSA reaction, the mast cell-specific loss of E-Cadherin increased the PCA reaction (Fig. 2B).

In summary, mast cell-specific E-Cadherin deficiency enhances anaphylactic reactions in mice.
Increased IgE-mediated degranulation of E-Cadherin-deficient cultured mast cells

Upon IgE-mediated FceRI crosslinking, cultured mast cells release preformed mediators including β-hexosaminidase. Measurement of β-hexosaminidase activity in the mast cell supernatant provides a frequently used read-out for mast cell degranulation. Consistent with the in vivo observations, Cdh1ex CPMC showed increased β-hexosaminidase release compared to controls if 10 ng/mL DNP was used as antigen (Fig. 2C). The suboptimal condition of 1 ng/mL DNP resulted in twice as much secretion but the difference was not statistically significant. As expected, IgE loading on mast cells itself does not induce degranulation. Treating Cdh1ex CPMC with the Ca2+ ionophore ionomycin, we observed a similar β-hexosaminidase release compared to control. Thus, Cdh1ex CPMC contain the same amount of this mediator and upon massive Ca2+ stimulation release it like control cells.

Upon fusion of the secretory granules with the mast cell cytoplasmic membrane, CD107a (LAMP-1) was exposed on the cell surface and can be used as an additional marker for degranulation [42]. In this second degranulation assay distinct from measuring β-hexosaminidase release, we observed approximately twofold increased degranulation-associated upregulation of CD107a on the cell surface of Cdh1ex CPMC (Fig. 2D) compared to controls. While the reaction started with the same kinetics in mutant and control, at every time point the loss of E-Cadherin caused much higher CD107a cell surface exposure on Cdh1ex CPMC. Thus, under these conditions, the reaction is not merely delayed, but control does not reach Cdh1ex values later and, thus, is reduced at all times. The ionomycin triggered CD107 exposure was not different between control and Cdh1ex CPMC indicating specificity of the observed phenotype.

Degranulation-associated upregulation of CD107a on the cell surface of WT but not of Cdh1ex CPMC could be partially suppressed by the addition of soluble, recombinant E-Cadherin protein representing the extracellular portion of the mature protein (Fig. 2E). The corresponding results, reduction of WT CPMC degranulation, were obtained using the β-hexosaminidase release assay (Supporting information Fig. S4B). This indicates that even though E-Cadherin on these cultured control mast cells already restricts degranulation, additional presence of E-Cadherin ligand further reduces it as more surface E-Cadherin may become occupied. The effect of soluble E-Cadherin may also reflect the more intense E-Cadherin-mediated interactions of mast cells happening in a tissue as opposed to a cell culture.

To directly visualize the time course of mast cell degranulation, we also employed live-cell imaging. Here, the fusion of the granules triggered by anti-DNP IgE/DNP-BSA is monitored by fluorescent avidin, which binds to heparin exposed on the mast cell surface upon degranulation [43] (Fig. 2F; Supporting information Fig. S4). Three different amounts of antigen were tested. With the two lower amounts of 5 and 10 ng DNP-BSA, the Cdh1ex CPMC again showed strongly enhanced degranulation. At the highest amount of 30 ng DNP-BSA, the difference was rarely observed.

Together, three distinct mast cell degranulation assays showed clearly increased degranulation of E-Cadherin-deficient CPMC in vitro.

Decreased proinflammatory cytokine production in activated E-Cadherin-deficient mast cells

Following the immediate reaction, mast cells begin the de novo synthesis and release of additional cytokines within hours after IgE-mediated activation [11, 19, 44]. To test the effect of E-Cadherin deficiency on this late-phase response, we sensitized CPMC with anti-DNP IgE and challenged them with DNP-BSA for 6 h. The secreted cytokines and chemokines in the culture supernatant were initially quantified by densitometric analysis of a dot-blot cytokine array (Fig. 3A). This revealed decreased levels of proinflammatory cytokines TNF-α and IL-6 and a trend toward lower GM-CSF, IL-13, and CCL3. ELISA experiments confirmed reduced production of IL-6 and showed a trend but no statistically significant difference between control and Cdh1ex CPMC for TNF-α, GM-CSF, and IL-13 (Fig. 3B).

Thus, E-Cadherin also supports the proper expression of proinflammatory cytokines by CPMC. As these cytokines are released after the initial phase of mast cell activation and degranulation, the support of cytokine gene expression through this adhesion receptor may ensure the full availability of cytokines needed in an acute immune response in the respective tissue. One may speculate that inflamed tissue supports mast cells in their cytokine production via interaction through adhesion receptors, such as E-Cadherin, to acquire a balanced situation where overshooting degranulation is prevented but a sufficient cytokine response is achieved.

Concluding remarks

While there are many mechanisms known that trigger and support mast cell degranulation, counter-regulatory principles limiting this response have not been as extensively described. Yet, balancing mast cell activation is important for a healthy tissue as premature or chronic inflammation caused by overly sensitive mast cells or acutely overshooting degranulation can be very damaging, not to the least causing anaphylaxis.

There are other mechanisms dampening mast cell activation such as through receptors or other surface molecules. For example, a deficiency in TRPM4, a cation channel enhances mast cell degranulation in vitro and in vivo [45], G-protein coupled receptors were described to modulate mast cell degranulation in both directions [46], and human IgG4 is known to reduce mast cell degranulation although the mechanism, likely through binding to FcyRIIb, is not fully understood [47]. However, the mechanisms in these cases are quite different as neither ion channels, GPCRs, nor Fc receptors are adhesion receptors. In contrast, E-Cadherin-mediated dampening, we suggest is a tissue-mediated, adhesion-based safety guard. Whether the reduction of degranulation and the support of cytokine expression are independent effects of
E-Cadherin or whether they reflect a coupled and balanced biological response remains to be elucidated. The recent report on a negative correlation between tryptase-expressing mast cells and E-Cadherin-expressing human odontogenic cysts [34] suggests an E-Cadherin-associated interaction and, thus, supports the idea that the findings reported here in mouse models may be relevant for humans.

Mast cells act at the very front of the immune defense. Quick, powerful, and efficient reactions are needed to combat invading pathogens and venoms. However, to cite S. J. Galli: “...it seems likely that sustaining the beneficial functions of this "allergy module" of immunity critically requires regulatory mechanisms that can keep this potentially dangerous effector mechanism under tight control” [48]. Tissue-mediated interactions, such as through E-Cadherin, are very likely just like that.

Materials and methods

Mice

The E-Cadherin<sup>fl/fl</sup> (strain EPD0036_1_G01, EUCOMM) and the Mcpt5-Cre (Scholten et al. [35]) strains on a C57BL/6 background were bred and housed at the Experimental Center, Medical Faculty Carl Gustav Carus, TU Dresden, under specific pathogen-free conditions. All animal experimentation was performed according to institutional guidelines and was approved by the Landesdirektion Dresden (Az No. D24-5131/354/56).

Cell culture

CPMC were obtained by culturing peritoneal cell suspensions in Hybridoma Medium (Gibco Inc.) containing 2% heat-inactivated FCS, 100 U/mL penicillin, 100 U/mL streptomycin, 10% culture supernatant of the mIL3-transformed cell line X63Ag8-653, and 2% of CHO transfectants secreting murine SCF (generated by S. Lyman, Immunex, Seattle, and kindly provided by P. Dubreuil, Marseille). After 4–5 weeks, approximately 96% of the cells expressed high levels of FcεRI and CD117. BMMC cultures were established as described before [49].

RT-PCR

mRNA was extracted from 5 weeks old CPMC using RNAeasy kit (QIAGEN). cDNA synthesis was performed with M-MLV Reverse Transcriptase (Promega) according to the manufacturer’s protocol.
Western blot

Cells were lysed in lysis buffer (Tris HCl pH 7.4, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM sodium vanadate, 10 mM NaF; 1% Triton X-100) supplemented with 0.1 mM benzamidine, 10 μg aprotinin, 10 μg leupeptin, and 2 μg pepstatin A. After standard SDS page and semidry blotting, nitrocellulose membrane was probed with anti-E-Cadherin (Cell Signaling Inc.) or anti-β-tubulin (Clone E7, Developmental Studies Hybridoma Bank).

Quantification of ear skin mast cells

The ears were collected and split in ventral and dorsal halves. Then, the ear skins were digested with 0.05 mg/mL Liberase (Roche), 792 U/mL DNase (Roche), and 1 mg/mL Hyaluronidase (Sigma) for 1.5 h at 37°C. The samples were run through a 40 μm sieve and collected by centrifugation. Cells were quantified by flow cytometry (BD LSR II flow cytometer).

Electron microscopy

Three-week-old CPMC were attached to either a fibronectin- (10 μg/mL) or poly-L-Lysine (0.1%) coated ACLAR surfaces (Plano, Germany) for 60 min at 37°C. The cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature and then postfixed in osmium tetroxide (1% in ddH2O, w/v) for 90 min. Following three washing steps with double-distilled water, the cells were dehydrated through a graded series of ethanol (70, 80, 90, 96, 2 × 100%; each step 30 min) and infiltrated with Epon/Araldite (EMS, USA) using increasing concentrations of resin (resin:ethanol: 1:3, 1:1, 3:1, then pure resin) for 1 h each step at room temperature. Samples were infiltrated with pure resin overnight and polymerized at 60°C for 48 h. Ultrathin sections (70 nm) were collected on Formvar-coated copper grids poststained with 2% w/v uranyl acetate in 70% v/v methanol, followed by 0.4% w/v lead citrate (Science Services, USA) in double-distilled water. Finally, CPMC were imaged using a TECNAI T12 Biotwin transmission electron microscope (Thermo Fisher Scientific, USA) operated at 120 kV and equipped with F214 CCD camera (TVIPS GmbH, Germany). Electron micrographs were acquired at a magnification of 2900× with a pixel size of 3.761 nm. Image analysis was performed using Fiji [50].

Degranulation assays

CPMC were incubated with 1 μg/mL IgE anti-DNP for 60 min on ice in 500 μL culture medium and then stimulated for 30 min at 37°C with indicated DNP-BSA concentrations in 100 μL Tyrode's buffer, pH 7.4. Culture supernatants from both stimulated and unstimulated cells were collected and assayed for β-hexosaminidase as previously described [40]. For analysis of the total β-hexosaminidase cell content, cells were lysed with 0.5% Triton X-100 in Tyrode's buffer. The percentage of degranulation was measured at 405 nm and calculated as follows: (absorbance of culture supernatant × 100)/(absorbance of culture supernatant + absorbance of total cell lysate).

For testing the effect of soluble E-Cadherin, 4-week-old CPMC were sensitized with 0.1 μg/mL anti-DNP IgE in 1 mL of cytokine-free medium overnight at 37°C with addition of 100 ng/mL of recombinant E-Cdh (BioLegend, #78004) for the indicated samples. The cells were stimulated with 100 ng/mL DNP-BSA in 100 μL of Tyrode's buffer and stained with 1:500 dilution of anti-CD107a FITC for 30 min on ice. After two washing steps, the stained cells were analyzed by FACS. In an additional approach, 100 ng/mL of recombinant mouse E-Cdh-human IgG Fc chimera protein (R&D Systems, #748-EC) was added during the incubation with 1 μg/mL anti-DNP IgE for 60 min on ice. PCMC were then stimulated with 30 ng/mL of DNP-BSA for 30 min at 37°C. The further procedure to determine the amount of β-hexosaminidase was done as described.

For measuring CD107a on the mast cell surface, CPMC were incubated with 1 μg/mL anti-DNP IgE for 60 min on ice in 500 μL culture medium and then stimulated for indicated time points at 37°C with 10 ng/mL DNP-BSA in 100 μL Tyrode's buffer, pH 7.4. The cell pellet was collected and the surface was stained with 1 of 500 dilution of anti-CD107a FITC for 25 min on ice followed by two washing steps. The stained cells were analyzed by FACS.

For live-cell imaging, a four-chamber cell culture dish (Greiner Bio-One 627871) was coated with 600 μL of 10 μg/mL fibronectin in PBS solution overnight at 4°C. On the next day, coated chambers were washed three times with 600 μL PBS. One million CPMC were resuspended in 800 μL of 10 μg/mL fibronectin containing culture medium and thereof 200 μL was taken for each fibronectin-coated chamber and 300 μL of culture medium was added. Following 60 min adhesion of the cells at 37°C, the chambers were carefully washed with 600 μL warm
PBS and 500 μL of a 15 μg/mL Avidin-A488 solution in warm Tyrode’s Buffer was added. DNP-BSA was added at time point 0 of time-lapse recording in the concentrations indicated and the samples were then subjected to fluorescent live-cell microscopy for 20 min (Nikon Eclipse TE2000-E; NIS-Elements AR 4.10.01 software).

PCA

Mice were locally sensitized by intradermal injection of 20 ng IgE anti-DNP (Sigma Aldrich) in 20 μL PBS into the left ear pinna and as a control with PBS in the right ear pinna. Twenty-four hours later, mice were challenged with 50 μg DNP-BSA (Sigma Aldrich) in 140 μL 1% Evans blue in 0.9% NaCl. After 30 min, mice were sacrificed, and ear tissues were incubated with 500 μL formamide at 50°C for 24 h. Extracted dye was quantified photometrically at 620 nm.

PSA

Mice were sensitized by i.p. injection of 12.5 μg IgE anti-DNP. Twenty-four hours later, mice were challenged by i.p. injection of indicated amounts of DNP-BSA (Sigma Aldrich) in PBS or PBS alone. The temperature drop was assessed using a rectal thermometer (Bioseb Inc).

FACS analysis

Cells were resuspended in PBS/5% FCS and were stained for 30 min at 4°C with the antibodies against the following markers listed below. Cells suspensions were washed once and were resuspended in PBS/5% FCS. To determine cell viability, Annexin V (Invitrogen) and PI were used. For E-Cadherin assessment, the cells were fixed in 20% ethanol. Analysis was done using a BD LSR II Flow Cytometer according to the guidelines stated in [51]. Antibodies used were:

| Specificity | Target cells | Host/isotype | Clone | Dye | Final dilution | Commercial source |
|-------------|--------------|--------------|-------|-----|---------------|-------------------|
| FcRI Mast cells/basophils | armenan hamster IgG | MAR-1 | APC, FITC, PE | 1:750 | eBioscience |
| c-Kit (CD117) Mast cells/ basophils | Rat IgG2b κ | 2B8 | A488, PE-Cy7, A700 | 1:750 | eBioscience |
| CD107a Mast cells/ basophils | Rat IgG2b κ | 1D4B | FITC | 1:500 | eBioscience |
| F4/80 Macrophages | Rat IgG2b κ | BM8 | PE | 1:200 | eBioscience |
| CD45 Hematopoietic cells | Rat IgG2b κ | 30-F11 | A700 | 1:200 | eBioscience |
| E-cadherin Mast cells/basophils | rabbit IgG | 24E10 | A488 | 1:50 | Cell signaling |
| Annexin V Apoptotic cells | rabbit IgG | 24E10 | FITC | 1:50 | Biolegend |

Cytokine protein array and ELISA

CPMC were incubated with 1 μg/mL IgE anti-DNP (Sigma-Aldrich) for 60 min at 37°C in 1.5 mL culture medium and then stimulated with 30 ng/mL DNP-BSA (Sigma-Aldrich) in 1.5 mL Tyrode’s buffer for 6 h at 37°C. Supernatant from both stimulated and unstimulated cells was collected. Mouse protein cytokine arrays were performed according to the manufacturer’s instructions (R&D Systems, ARY006).

TNF-α, GM-CSF, IL-13, and IL-6 protein levels in supernatant of CPMC cultures incubated with 1 μg/mL anti-DNP IgE for 60 min at 37°C and with 30 ng/mL DNP-BSA for the indicated time at 37°C were determined by ELISA (BD Bioscience) as recommended by the manufacturer’s protocol.

Statistical analyses

Statistical analyses were performed using the Graph Pad Prism 9. Shapiro–Wilks and Kolmogorov–Smirnov tests were used to test normality of distribution. Based on the results either Student’s t-test (paired or unpaired) or Mann–Whitney or Wilcoxon test was employed. p values ≤0.05 were considered statistically significant.

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Abbreviations: BMMC: bone marrow-derived mast cells · CPMC: cultured peritoneal mast cells · MMC: mucosal mast cells · PCA: passive cutaneous anaphylaxis · PSA: passive systemic anaphylaxis

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