Escherichia coli Exposure Inhibits Exocytic SNARE-Mediated Membrane Fusion in Mast Cells

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Abstract

Mast cells orchestrate the allergic response through the release of proinflammatory mediators, which is driven by the fusion of cytoplasmic secretory granules with the plasma membrane. During this process, SNARE proteins including Syntaxin4, SNAP23 and VAMP8 play a key role. Following stimulation, the kinase IKKβ interacts with and phosphorylates the t-SNARE SNAP23. Phosphorylated SNAP23 then associates with Syntaxin4 and the v-SNARE VAMP8 to form a ternary SNARE complex, which drives membrane fusion and mediator release. Interestingly, mast cell degranulation is impaired following exposure to bacteria such as *Escherichia coli*. However, the molecular mechanism(s) by which this occurs is unknown. Here, we show that *E. coli* exposure rapidly and additively inhibits degranulation in the RBL-2H3 rat mast cell line. Following co-culture with *E. coli*, the interaction between IKKβ and SNAP23 is disrupted, resulting in the hypophosphorylation of SNAP23. Subsequent formation of the ternary SNARE complex between SNAP23, Syntaxin4 and VAMP8 is strongly reduced. Collectively, these results demonstrate that *E. coli* exposure inhibits the formation of VAMP8-containing exocytic SNARE complexes and thus the release of VAMP8-dependent granules by interfering with SNAP23 phosphorylation.

Microbiota play a major role in regulating immune responses (1,2). Interestingly, changes in microbiota have been observed in patients with allergic diseases (3,4). Moreover, allergic inflammation is exacerbated in germ-free mice suggesting that the presence of bacteria may influence the allergic response, during which mast cells play a central role (5).

Mast cells are localized at the host–environment interface in connective and mucosal tissues, in particular the gut where the prevalence of bacteria is high (6,7). During the first exposure to an allergen, IgE is produced and binds its high-affinity receptor, FcεRI, on the surface of mast cells. As the allergen is reintroduced, it binds directly to IgE on the surface of mast cells, thus cross-linking FcεRI. FcεRI-mediated activation results in the rapid release of proinflammatory mediators including histamine and proteases as well as the production of cytokines (6,8,9). Based on their localization in microbe-rich tissues, mast cells may be a crucial link to understanding how bacterial exposure or stimulation influences the development of allergic inflammation.

Several studies have established that bacteria differentially regulate the secretion of mast cell-derived mediators (10). While some bacteria such as *Mycobacterium tuberculosis* (11), *Mycoplasma pneumonia* (12) and *Streptococcus pneumonia* (13) activate mast cells to secrete cytokines and induce degranulation, other bacteria such as probiotics (14–17) and *Escherichia coli* inhibit degranulation in human and mouse mast cells (18,19). Although *E. coli* exposure reduces serotonin and β-hexosaminidase secretion, it induces the release of histamine in mouse models (20,21). Thus, bacterial exposure may play a

regulatory role by which certain bacteria selectively modulate the hyperreactivity of mast cells to circulating allergens. However, the molecular mechanisms involved in this phenomenon are unclear.

Mast cell degranulation is largely mediated by the exocytic SNARE proteins. In addition to the t-SNAREs SNAP23 and Syntaxin4, several v-SNAREs have been implicated in this process including VAMP2, VAMP8 and VAMP7. However, their function is dependent on mast cell subsets and types of granules (22–25). Data suggest that VAMP8 regulates the release of a subset of secretory granules in rodent mast cells, where VAMP8 significantly colocalizes with serotonin and cathepsin D, but is absent from histamine-containing granules (26,27). Additionally, while bone marrow-derived mast cells generated from VAMP8-deficient mice have profound defects in β-hexosaminidase, serotonin and cathepsin D release, they exhibit no defect in histamine or tumor necrosis factor-α secretion (26). Although VAMP2 interacts with Syntaxin4 and SNAP23 in a stimulus-dependent manner, a functional role for this particular v-SNARE in mediator release has yet to be determined (23,28,29). In contrast, both VAMP8 and VAMP7 are required for degranulation in cord blood-derived human mast cells (25).

Here, we demonstrate that co-culturing mast cells with *E. coli* induces a profound decrease in SNAP23 phosphorylation and ternary SNARE complex assembly, both of which are required for exocytosis, resulting in the inhibition of FcϵRI-dependent degranulation.

Results

Translationally active *E. coli* rapidly and additively inhibit RBL-2H3 mast cell degranulation

We investigated the impact of *E. coli* exposure in the RBL-2H3 (RBL) rat mast cell line, a commonly used model to study the mechanisms of mast cell function (27,28,30).

First, we determined whether *E. coli* interfered with RBL degranulation and which multiplicity of infection (MOI) would induce the optimal effect. RBLs were co-cultured with increasing MOIs of *E. coli* for 2 h. Then the kinetics of β-hexosaminidase secretion was assessed for anti-dinitrophenyl (DNP) IgE-sensitized RBLs stimulated

with DNP-BSA. For comparative purposes, the amount of β-hexosaminidase released at 60 min in the control population was considered 100%. As shown in Figure S1A, Supporting Information, *E. coli* inhibits FcϵRI-mediated β-hexosaminidase release in a dose-dependent manner. A significant effect is observed at an MOI of 1000 and becomes maximal at an MOI of 10 000. This result is consistent with the effect of a single co-culture with *E. coli* observed in mouse mast cell lines and in primary peritoneal mast cells (18). To further test *E. coli*-induced inhibition, we bypassed FcϵRI and directly stimulated RBLs using phorbol myristate acetate (PMA) and ionomycin. PMA activates protein kinase C (PKC), whereas ionomycin releases the intracellular calcium pool, which are two critical signals for mast cell degranulation (31). In these conditions, RBL degranulation is still inhibited following a single exposure, with significant inhibition achieved only at an MOI of 10 000 (Figure S1B). This could be due to the fact that PMA/ionomycin is a potent stimulant and small differences may be undetectable. Consistent with this possibility, FcϵRI-mediated stimulation results in ∼35% secretion at 60 min in control cells, whereas PMA/ionomycin stimulation induces ∼78% total β-hexosaminidase release (data not shown). Alternatively, an FcϵRI-dependent signaling component may be impacted by *E. coli* exposure as PMA/ionomycin bypasses these proximal signaling events. The reduction in β-hexosaminidase secretion was not due to differences in the total intracellular pool of β-hexosaminidase between control cells and those incubated with *E. coli* (Figure S1A, inset), suggesting that *E. coli* did not induce mast cell degranulation during the exposure.

Interestingly, we observed that only translationally active *E. coli* is able to inhibit secretion (Figure S1C, left and center panels). However, *E. coli*-conditioned medium alone does not inhibit degranulation (Figure S1C, right panel). These data suggest at least two possibilities: (i) the inhibition of mast cell degranulation requires *E. coli* to be translationally active and any bacterial factor(s) responsible for the effect are locally secreted, probably in close contact with mast cells, and/or (ii) a membrane factor is responsible for the inhibition, but is denatured during the heat inactivation process. Further investigation will be required to distinguish between these possibilities.

Next, we examined the extent to which multiple *E. coli* exposures would affect degranulation. RBLs were incubated with *E. coli* at an MOI of 10 000 for 2 h either once, twice or thrice, with each incubation occurring 24 h apart. The MOI of 10 000 was chosen because it induced the maximal effect (Figure S1A,B). The cells were then stimulated with either anti-DNP IgE/DNP-BSA or PMA/ionomycin. In these conditions, we observed an exposure-dependent inhibition of β hexosaminidase release (Figure 1A,B). After 60 min of stimulation with IgE/DNP-BSA, one exposure results in ~7% inhibition, while two exposures lead to a decrease of ~37% and three exposures to a decrease of ~71%. A similar decrease is observed in cells stimulated with PMA/ionomycin. This inhibition is not due to reduced cell viability as shown in Figure 1A (inset).

Collectively, these results demonstrate that not only does co-culture with *E. coli* rapidly inhibit mast cell degranulation but also that repetitive exposures enhance the inhibitory effect. This is consistent with the impact of *E. coli* on peritoneal mast cells, in that it takes at least 5 days to regain normal levels of degranulation following a single co-culture with *E. coli* (18).

FcεRI surface expression is not affected by *E. coli* exposure

One possible explanation for the reduction in FcεRI-mediated degranulation in mast cells co-cultured with *E. coli* is changes in the surface levels of FcεRI. By using human mast cells, Kulka et al. observed a significant reduction in FcεRI surface levels following overnight incubation with *E. coli* (19). To test this possibility, FcεRI surface levels in RBLs cultured in the presence or absence of *E. coli* for 2 h were examined using immunofluorescence microscopy. As shown in Figure S2A, the surface expression of FcεRI is similar in RBLs cultured with or without *E. coli*. This observation was confirmed using flow cytometry. Irrespective of being exposed once (Figure S2B, left panel) or thrice (Figure S2B, right panel) to *E. coli*, FcεRI expression remains constant. These data suggest that the inhibition of FcεRI-mediated degranulation following incubation with *E. coli* is not due to changes in the surface expression of FcεRI. These observations are consistent with the fact that RBLs exposed to *E. coli* still secrete less when stimulated by PMA/ionomycin (Figure S1B). Under certain conditions, however, FcεRI surface expression has been shown to
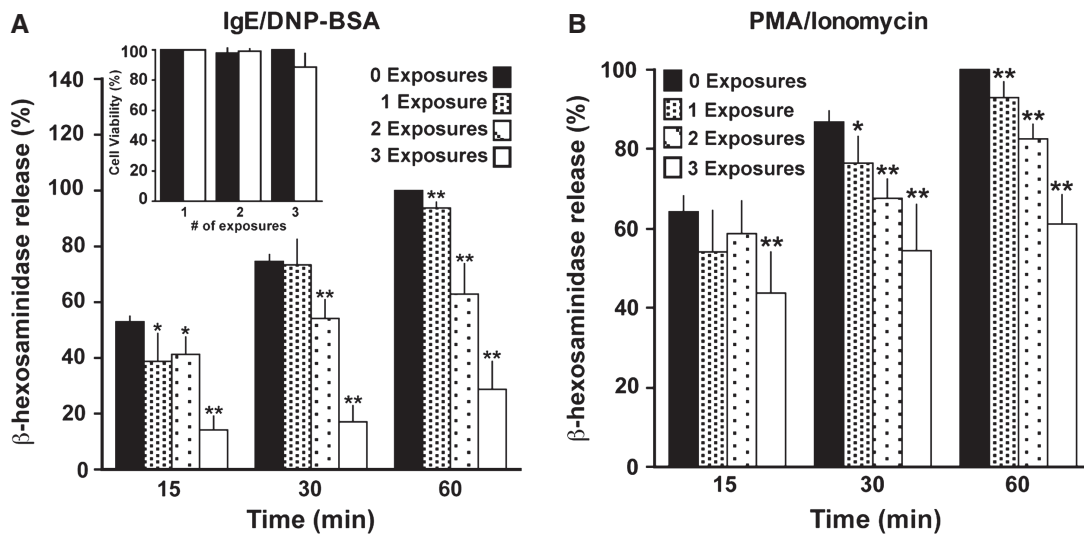

Figure 1: *Escherichia coli* exposures additively inhibit mast cell degranulation. A) RBL-2H3 cells were exposed to *E. coli* multiple times for 2 h at an MOI of 10 000. Each exposure occurred 24 h apart. Following sensitization with anti-DNP IgE for 2 h, the cells were stimulated with DNP-BSA for the indicated times. Cell viability prior to stimulation was assessed by trypan blue exclusion (inset). B) RBLs were treated as in panel A, but stimulated with PMA/ionomycin. The amount of β-hexosaminidase released at 60 min in the control population was considered 100%. Graphs depict the mean ± standard deviation of five independent experiments conducted in duplicate. In all graphs, asterisks denote statistically significant p values, where * ≤ 0.05 and ** ≤ 0.01.

be reduced (19,32,33). This discrepancy may be due to differences in the exposure conditions as reductions in FcεRI expression are reported when mast cells are co-cultured for 4 h or longer with bacteria (19,32,33).

E. coli exposure impacts exocytic membrane fusion in mast cells

Given that equivalent pools of β-hexosaminidase are available for release, yet secretion is reduced following incubation with *E. coli* (Figure S1A, inset), granule–plasma membrane fusion is likely compromised. This event constitutes the final step in the degranulation process, and blocking it will result in reduced secretion. To test this hypothesis, we cultured RBLs with or without *E. coli* for 2 h, prior to stimulation with anti-DNP IgE/DNP-BSA or PMA/ionomycin for 60 min. Next, the presence of the protein p80 on the cell surface was measured by immunofluorescence microscopy. p80 is an integral membrane protein found in the lumen of mast cell secretory granules (34), which becomes available on the cell surface upon stimulation. The presence of p80 on the cell surface correlates with degranulation in RBL-2H3

mast cells (34,35). In control cells stimulated by either IgE/DNP-BSA (Figure 2A, top panel) or PMA/ionomycin (Figure 2B, top panel), p80 was observed as uniform spots on the cell surface. In contrast, when RBLs were co-cultured with *E. coli*, a noticeable reduction in p80 surface levels was observed (Figure 2A,B, lower panels). Not only is the amount of p80 per cell reduced but also fewer cells were labeled with p80. To quantify the levels of p80, we analyzed p80 surface expression by flow cytometry. In unstimulated cells cultured in the absence (control) or presence of *E. coli*, low levels of p80 surface expression were recorded, likely due to constitutive exocytosis (Figure 2C,D, $t = 0$) (34). Note that at $t = 0$, lower levels of spontaneous release are observed in *E. coli*-treated cells compared with the control, suggesting that *E. coli* also interferes with constitutive secretion of β-hexosaminidase in mast cells. Upon stimulation, we observed a significant increase in p80 surface expression in control cells using both anti-DNP IgE/DNP-BSA and PMA/ionomycin stimuli (Figure 2C,D, solid lines). In cells co-cultured with *E. coli*, although stimulation induces the appearance of p80 on the surface, we observed a rapid and significant
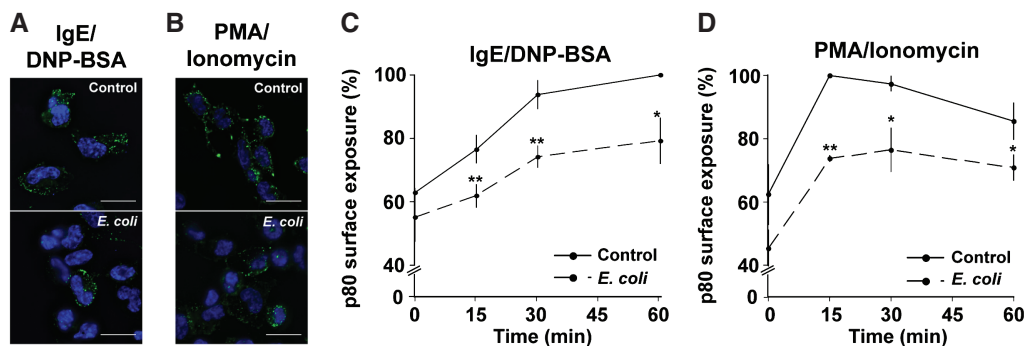
Figure 2: *E. coli* exposure impairs membrane fusion during degranulation. A) RBLs were plated on coverslips 24 h prior to being incubated with (lower panel) or without (top panel, control) *E. coli* at an MOI of 10 000 for 2 h. The cells were then sensitized with anti-DNP IgE and stimulated with DNP-BSA for 1 h. Cells were fixed and stained with anti-p80 and anti-mouse IgG₁ AlexaFluor488-conjugated antibodies. Nuclei were stained with Hoechst. Coverslips were analyzed by immunofluorescence microscopy. Scale bars = 20 μm, representative of three independent experiments. B) RBLs were treated as described in panel A, but stimulated with PMA/ionomycin. Scale bars = 20 μm, representative of three independent experiments. C) RBLs were plated in 10-cm tissue culture plates 24 h prior to being incubated with or without *E. coli* at an MOI of 10 000 for 2 h. The cells were harvested, sensitized with anti-DNP IgE and stimulated with DNP-BSA for 0, 15, 30 or 60 min. The cells were fixed and labeled as described in panel A and then analyzed by flow cytometry. D) RBLs were treated as described in panel C, but stimulated with PMA/ionomycin. Graphs depict the mean fluorescence intensity of p80 staining ± standard deviation of three independent experiments. The maximal p80 fluorescence intensity in the control population was considered 100% ($t = 60$ for IgE/DNP-BSA and $t = 15$ min for PMA/ionomycin). In all graphs, asterisks denote statistically significant p values, where * ≤ 0.05 and ** ≤ 0.01.
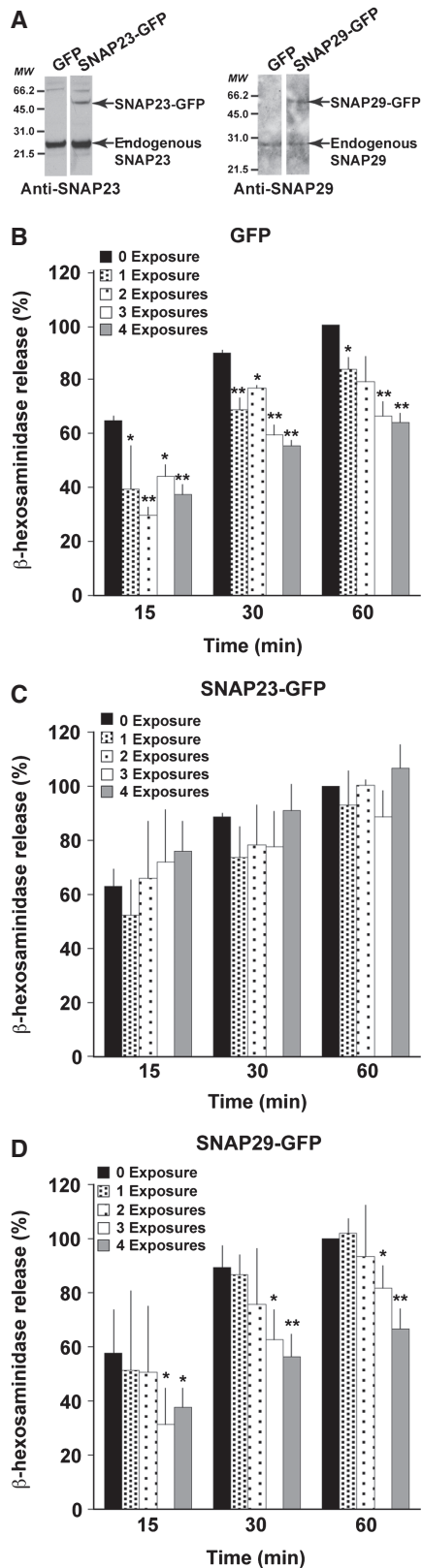
Figure 3: Legend on Next column.

reduction in p80 levels compared with the non-exposed control cells. At $t = 15$ min in *E. coli*-exposed cells stimulated with IgE/DNP-BSA and PMA/ionomycin, p80 surface levels were reduced by 15 and 26%, respectively (Figure 2C,D, dashed lines). These data suggest that membrane fusion is inhibited in mast cells co-cultured with *E. coli*.

E. coli exposure specifically influences the function of the exocytic SNARE SNAP23

Because incubating mast cells with *E. coli* results in a reduction of membrane fusion, we anticipate that the exocytic SNARE machinery is affected in this process. The mast cell secretion machinery is composed of Syntaxin4, SNAP23 and VAMP8 in which SNAP23 is a critical component (23,29,36,37). One way to inhibit membrane fusion is by reducing the amount of functional exocytic SNAREs, in particular the amount of functional SNAP23 (28,29). To assess this possibility, we generated stable RBL clones overexpressing SNAP23-GFP and tested whether this overexpression was able to reverse the secretion phenotype caused by *E. coli* exposure. As a control, we overexpressed GFP alone. The overexpression of SNAP23-GFP was confirmed by western blot (Figure 3A). The impact of *E. coli* on β-hexosaminidase release in these

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Figure 3: Overexpression of SNAP23 compensates for the inhibitory effect of *E. coli* exposure. A) Stable populations of RBL-2H3 cells overexpressing GFP, SNAP23-GFP or SNAP29-GFP were generated. The expression of SNAP23-GFP and SNAP29-GFP was analyzed by western blot using anti-SNAP23 and anti-SNAP29 antibodies. In each population, two bands were detected for SNAP23 and SNAP29, corresponding to the endogenous protein (lower band) and the GFP-fusion protein (upper band). Cells expressing GFP (B), SNAP23-GFP (C) and SNAP29-GFP (D) were cultured with or without *E. coli*, as described in Figure 1A, either one, two, three or four times with each exposure occurring 24 h apart. After each exposure, the cells were stimulated with PMA/ionomycin for the indicated times and β-hexosaminidase release was quantified. The amount of β-hexosaminidase released at 60 min in each unexposed population was considered 100%. Graphs represent the mean ± standard deviation of five independent experiments conducted in duplicate. In all graphs, asterisks denote statistically significant p values, where $* \leq 0.05$ and $** \leq 0.01$.

stable RBL populations was assessed following multiple exposures and stimulation with PMA/ionomycin. For comparative purposes, the amount of β-hexosaminidase released after 60 min of stimulation for each unexposed population was considered 100%. Similar to non-transfected RBLs (Figure 1B), *E. coli* co-culture additively inhibits β-hexosaminidase secretion in the GFP control population (Figure 3B). At 60 min, one exposure leads to ∼17% inhibition, while three and four exposures inhibit ∼34 and 37%, respectively. This amount of inhibition is comparable to non-transfected RBLs exposed thrice with *E. coli* (∼39% inhibition at 60 min). Interestingly, degranulation in cells overexpressing SNAP23-GFP is not significantly impaired by incubation with *E. coli* (Figure 3C). Even with four exposures, we were unable to detect a difference in β-hexosaminidase release between SNAP23-GFP-expressing cells cultured with or without *E. coli* (107% versus 100%). To confirm that this phenotype was specific to the exocytic SNARE SNAP23, we measured the impact of *E. coli* co-culture on degranulation in SNAP29-GFP-expressing cells (Figure 3A). Previously, we have shown that SNAP29, a homolog of SNAP23, is not involved in mast cell exocytosis (38), and therefore is an appropriate control for SNAP23. When SNAP29-GFP-expressing cells are incubated with *E. coli*, degranulation is inhibited in an exposure-dependent manner (Figure 3D). After 60 min of stimulation, four exposures to *E. coli* reduce β-hexosaminidase secretion by ∼35%, similar to the GFP control. These data suggest that *E. coli* co-culture with mast cells specifically impacts the exocytic SNARE SNAP23.
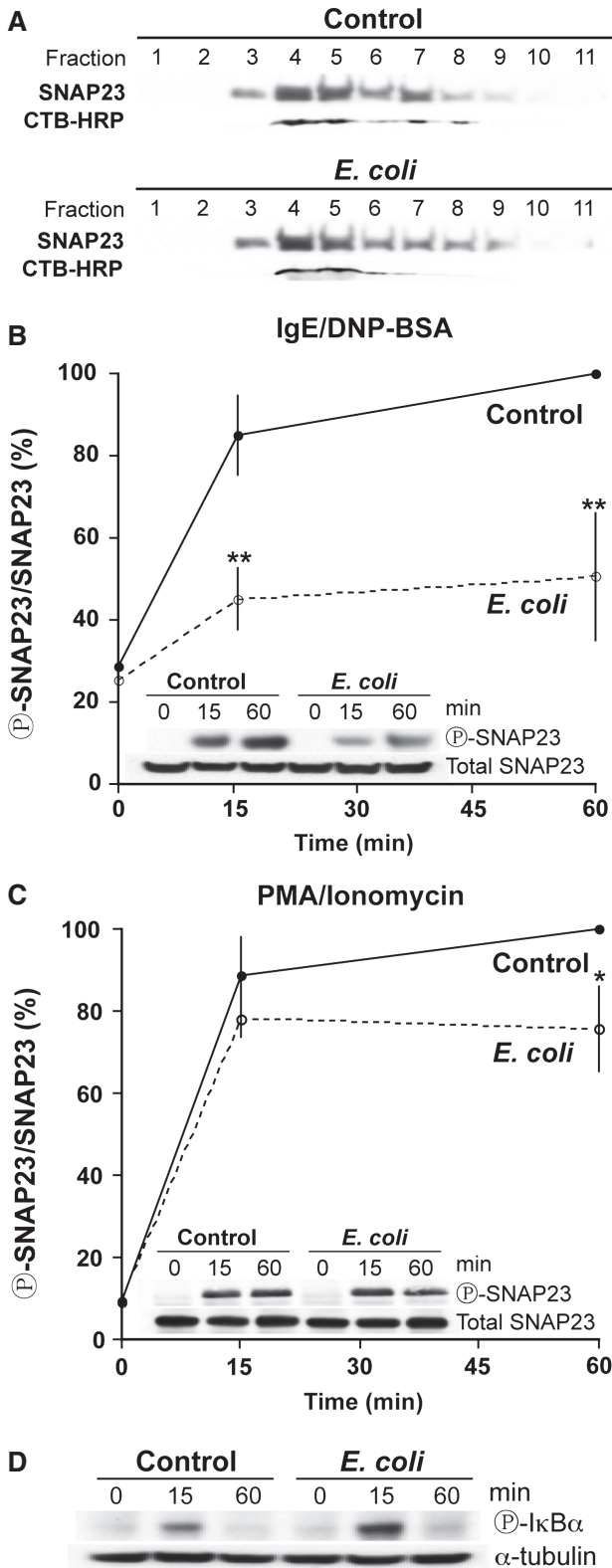
E. coli exposure impairs SNAP23 phosphorylation during stimulation without affecting its localization in lipid rafts

SNAP23 is significantly localized in lipid rafts within the plasma membrane, where it triggers the association with Syntaxin4 to mediate membrane fusion (23,29). Thus, we assessed whether the reduced degranulation was caused by changes in the association of SNAP23 with these particular membrane subdomains. To do so, lipid rafts were isolated by density centrifugation. Lipid rafts were identified by cholera toxin subunit B (CTB)-horseradish peroxidase (HRP) labeling that specifically localizes to these domains (23,29). As seen in Figure 4A, we did not observe any changes in the association of SNAP23 with lipid rafts.

SNAP23 is enriched in fractions 4 and 5 with CTB-HRP in both control and *E. coli*-treated cells, suggesting that the inhibition of mast cell degranulation is not due to the mislocalization of SNAP23.

Upon stimulation, SNAP23 is phosphorylated at serine residues 95 and 120 by IKKβ (28,29). *In vivo* and *in vitro*, this phosphorylation event is essential for degranulation to occur (29). If SNAP23 phosphorylation is compromised, mast cell degranulation is reduced (28). Therefore, we investigated whether co-culturing mast cells with *E. coli* interfered with this event. To do so, SNAP23 was immunoprecipitated from stimulated RBLs cultured with or without *E. coli*. SNAP23 phosphorylation at serine residue 120 was determined using western blot. In control cells, SNAP23 is rapidly phosphorylated within 15 min of stimulation with IgE/DNP-BSA or PMA/ionomycin (Figure 4B,C, control), consistent with previous observations (28,29). Interestingly, when cells are incubated with *E. coli*, SNAP23 phosphorylation is significantly reduced by ∼50% at both 15 and 60 min post-stimulation with IgE/DNP-BSA (Figure 4B) and by ∼25% at 60 min with PMA/ionomycin (Figure 4C). The differences in SNAP23 phosphorylation using IgE/DNP-BSA and PMA/ionomycin stimulation correlate with the level of β-hexosaminidase secretion observed previously, where *E. coli* exposure inhibited secretion about twice as much with IgE/DNP-BSA stimulation (87%, Figure S1A) compared with PMA/ionomycin stimulation (47%, Figure S1B). Although we previously observed a significant inhibition of degranulation at 15 min with PMA/ionomycin stimulation (Figure S1B), we did not observe a significant reduction in SNAP23 phosphorylation at the same time point. This discrepancy is likely due to the fact that SNAP23 phosphorylation was analyzed in cells that were stimulated in suspension (Figure 4C), whereas degranulation was analyzed with adherent cells (Figure S1B). It has been previously shown that the kinetics of degranulation is delayed in suspension cells compared with adherent cells (39).

Although PMA directly activates PKC, which has been shown to phosphorylate SNAP23 *in vitro* (28), PKC does not appear to be directly phosphorylating SNAP23 *in vivo*. In mast cells derived from IKKβ−/− mice there is little to no stimulus-induced phosphorylation of SNAP23,

Figure 4: SNAP23 is hypophosphorylated in *E. coli*-exposed mast cells, while IKKβ remains functional. A) RBL-2H3 cells cultured with or without (control) *E. coli* for 2 h, followed by stimulation in suspension with PMA/ionomycin for 30 min. Lipid rafts were isolated by density centrifugation as described in *Materials and Methods*; 20 μL of each fraction was analyzed by western blot using anti-SNAP23 antibody. Lipid rafts were identified using CTB-HRP; representative of three independent experiments. B and C) RBLs were cultured with or without (control) *E. coli* at an MOI of 10 000 for 2 h. The cells were harvested and stimulated with anti-DNP IgE and DNP-BSA (B) or with PMA/ionomycin (C) for 0, 15 or 60 min. The cells were then lysed and SNAP23 was immunoprecipitated from 1 mg of total protein for each condition. Samples were analyzed by western blot using anti-SNAP23 and anti-SNAP23[120] antibodies (insets). The band intensities were determined by densitometry. Graphs represent the mean band intensity ratio of SNAP23[120] to total SNAP23 ± standard deviation of three independent experiments. The SNAP23[120] to total SNAP23 ratio of the control population at 60 min was considered 100%. In all graphs, asterisks denote statistically significant p values, where * ≤ 0.05 and ** ≤ 0.01. D) RBLs were cultured with or without (control) *E. coli* (MOI 10 000) for 2 h. The cells were then harvested and stimulated in suspension with PMA/ionomycin for 0, 15 or 60 min before being lysed. Fifty micrograms of total protein from exposed and control whole-cell lysates was analyzed by western blot using anti-phospho-IκBα and anti-alpha tubulin antibodies; representative of three independent experiments.

suggesting that IKKβ is the major kinase responsible for SNAP23 phosphorylation and that PKC acts upstream of IKKβ (29).

We then determined whether SNAP23 hypophosphorylation was due to changes in IKKβ activity. In addition to phosphorylating SNAP23, IKKβ is also the major kinase that phosphorylates IκBα during nuclear factor kappa B (NFκB) signaling in response to FcεRI-mediated activation as well as Toll-like receptor (TLR) engagement (40–42). We then tested IKKβ activity by monitoring IκBα phosphorylation following co-culture with or without *E. coli* using western blot. In cells cultured with *E. coli*, we observed an increase in IκBα phosphorylation following stimulation compared with the control cells (Figure 4D), suggesting that IKKβ is still enzymatically active following

co-culture with *E. coli*. The enhanced phosphorylation of IκBα may be due to TLR engagement during the culture period, which would activate the NFκB pathway resulting in IκBα phosphorylation.

IKKβ binding to SNAP23 is decreased in mast cells exposed to *E. coli*

Upon stimulation, IKKβ interacts with and phosphorylates SNAP23. Therefore, we investigated whether the reduction in SNAP23 phosphorylation was due to impairment in the interaction between SNAP23 and IKKβ. Initially, we tested whether IKKβ recruitment into lipid rafts containing SNAP23 was impaired. However, we were unable to visualize IKKβ in the rafts, likely due to the lack of antibody sensitivity (data not shown). We then assessed the direct interaction between both of these proteins. To do so, we immunoprecipitated SNAP23 from stimulated RBLs cultured with or without *E. coli*, and analyzed the binding of IKKβ by western blot. As shown in Figure 5A (control), IKKβ co-immunoprecipitates with SNAP23, in agreement with Suzuki and Verma (29). However, in cells cultured with *E. coli*, we observed a 40% reduction in the amount of IKKβ that co-immunoprecipitates with SNAP23 (Figure 5A,B). Together, these data suggest that
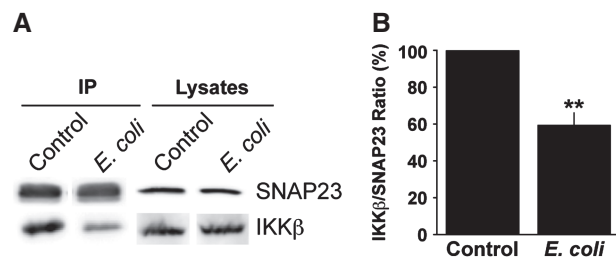
Figure 5: Binding of IKKβ to SNAP23 is reduced following *E. coli* exposure. A) RBLs were incubated with or without (control) *E. coli* at an MOI of 10 000 for 2 h. The cells were harvested and stimulated for 30 min with PMA/ionomycin, lysed and SNAP23 was immunoprecipitated from 1 mg of total protein. Immunoprecipitated samples and 40 µg of whole-cell lysates were analyzed by western blot with anti-SNAP23 and -IKKβ antibodies. B) Graph represents the mean band intensity ratio of IKKβ to total SNAP23 ± standard deviation of three independent experiments. The IKKβ/SNAP23 ratio of the control population was considered 100%. Asterisks denote statistically significant p values, where ** ≤ 0.01.

E. coli exposure impairs the interaction between IKKβ and SNAP23 resulting in the hypophosphorylation of SNAP23.

Ternary exocytic SNARE complex formation is inhibited in *E. coli*-exposed mast cells

The exocytic SNARE machinery plays a critical role in degranulation because it catalyzes membrane fusion. SNAP23, which is phosphorylated upon mast cell activation (23,28,29), binds its cognate t-SNARE Syntaxin4 to form the exocytic t-SNARE complex (23,27). The v-SNAREs VAMP2 and VAMP8 in turn interact with the t-SNARE complex to form ternary SNARE complexes (23,29,43). Because SNAP23 phosphorylation in mast cells is significantly reduced following incubation with *E. coli* (Figure 4B,C), ternary SNARE complex formation should also be impaired. To test this hypothesis, ternary SNARE complex formation was examined by co-immunoprecipitation and western blot analysis of stimulated RBLs cultured with or without *E. coli*. To stabilize SNARE complexes, the cells were treated with *N*-ethylmaleimide (NEM) prior to lysis. NEM blocks NEM-sensitive factor (NSF), which is responsible for SNARE complex disassembly. As a result, NEM enhances the accumulation of SNARE complexes (27, 43–45). As shown in Figure 6A, Syntaxin4 co-immunoprecipitates with SNAP23 in the absence of *E. coli* exposure. However, there is a 23% reduction in the co-immunoprecipitation of Syntaxin4 when the cells are cultured with *E. coli* prior to stimulation (Figure 6A,B, Syntaxin4), indicating that t-SNARE complex formation is impaired.

The assembly of a competent t-SNARE complex is essential for the formation of SNARE complexes with cognate v-SNAREs (46,47). Thus, we examined the interactions between SNAP23 and its cognate exocytic v-SNAREs VAMP2 and VAMP8. *E. coli* exposure profoundly impairs the interaction between SNAP23 and VAMP8 by ~59% (Figure 6A,B, VAMP8). However, similar amounts of VAMP2 co-immunoprecipitate with SNAP23 whether the cells are cultured with or without *E. coli* (Figure 6A,B, VAMP2). The reduction in SNARE complex formation is not due to differences in the expression of SNAP23, Syntaxin4 or VAMP8 as shown in the lysates (Figure 6C). Altogether, these results suggest that *E. coli* exposure specifically impairs the formation of VAMP8-dependent exocytic SNARE complexes.
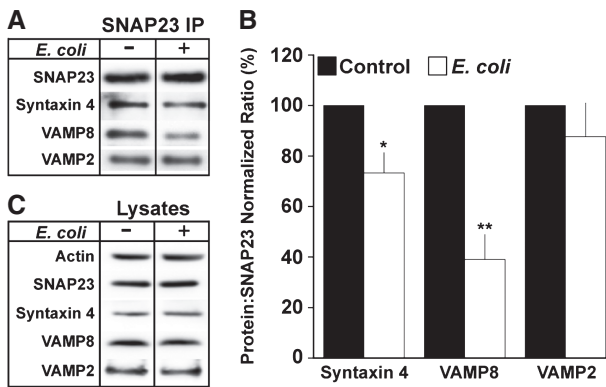
Figure 6: Exocytic SNARE complex formation is impaired in *E. coli*-exposed mast cells. A) RBLs were cultured with or without (control) *E. coli* for 2 h at an MOI of 10 000. The cells were harvested and stimulated for 30 min with PMA/ionomycin. Cells were treated with NEM for 15 min. Next, NEM was inactivated with DTT for 15 min on ice. NEM-treated cells were washed and lysed. One milligram of total protein was immunoprecipitated using anti-SNAP23 antibody. Samples were analyzed by western blot using anti-SNAP23, -Syntaxin4, -VAMP8 and -VAMP2 antibodies. Each blot is representative of at least three independent experiments. B) Quantification of the mean band intensity ratios of the indicated protein to SNAP23 ± standard deviation of at least three independent experiments. The protein:SNAP23 ratio of the control population was considered 100%. Asterisks denote statistically significant p values, where * $\leq$ 0.05 and ** $\leq$ 0.01. C) Forty micrograms of total protein from exposed or control whole-cell lysates was analyzed by western blot as described in panel A.

Discussion

Mast cells play an integral role in the development of allergic inflammation through the rapid release of prestored proinflammatory mediators (48). Importantly, different bacteria have been shown to interfere with mast cell degranulation (14–19, 32). *E. coli*, for instance, has been reported to inhibit FcϵRI-mediated secretion in primary mast cells (18,19). In this report, we demonstrate that, similar to primary mast cells and mouse mast cell lines, *E. coli* exposure inhibits FcϵRI-mediated and PMA/ionomycin-induced β-hexosaminidase secretion in RBL-2H3 rat mast cells (18). Increasing MOIs as well as repeated *E. coli* exposures inhibit mast cell degranulation in a cumulative manner, suggesting that both the magnitude (i.e. MOI)

and the chronicity of the infection are important factors in inhibiting mast cell degranulation. Chronic exposure to bacteria is important for regulating immune responses and inflammation, especially in the gut where exposure to the bacterial microflora is extensive (1). Collectively, these results suggest that the microenvironment within which mast cells reside modulates their activation (10–19).

The mechanism by which bacteria inhibit FcϵRI-mediated degranulation in mast cells is unknown. Here, we dissected the cascade of events leading to the inhibition of mast cell degranulation and the role of the exocytic SNARE machinery in this process. We discovered that culturing mast cells with *E. coli* reduces the interaction between SNAP23 and its kinase IKKβ, which in turn leads to SNAP23 hypophosphorylation and a decrease in exocytic SNARE complex formation following activation.

SNARE protein phosphorylation is an important signal in regulated exocytosis. In particular, SNAP23 phosphorylation plays a pivotal role in regulated secretion, and has been reported in a number of cell types (28, 49–51). In mast cells, SNAP23 phosphorylation on Ser95 and Ser120 represents an important step for exocytic SNARE protein function and complex assembly (28). Several kinases are able to phosphorylate SNAP23 *in vitro*, including PKC (28,49,50) and IKKβ (29), although only IKKβ has been shown to be involved in this process *in vivo* (29). Little to no phosphorylation of SNAP23 is detected in the absence of IKKβ (29). The phosphorylation of SNAP23 by IKKβ has also been observed during platelet secretion, thus further supporting a role for the SNAP23:IKKβ axis in regulated secretion (51). Here, we have shown that *E. coli* exposure impairs this critical checkpoint in SNARE complex formation, which has significant consequences for membrane fusion and mast cell degranulation.

In mast cells cultured with *E. coli*, SNAP23 phosphorylation is reduced following FcϵRI- or PMA/ionomycin-mediated stimulation. Less stringent inhibition was observed with PMA/ionomycin-induced than with FcϵRI-mediated stimulation following *E. coli* exposure, suggesting a possible compensatory mechanism controlled by PKC activation. However, the association of IKKβ with SNAP23 is still significantly reduced in mast cells co-cultured with *E. coli* and stimulated with

PMA/ionomycin, suggesting that even if PKC activation partially compensates for SNAP23 phosphorylation, the impairment in IKKβ recruitment plays a significant role in the inhibition of SNAP23 phosphorylation. Further experiments will be necessary to investigate the extent to which PKC is impacted during bacterial exposure.

Previous studies have shown that the phosphorylation of SNAP23 is essential for mast cell degranulation and SNARE complex assembly (28,29). In this study, we showed that SNAP23 hypophosphorylation induced by *E. coli* results in a decrease in SNARE complex formation. In particular, incubating mast cells with *E. coli* leads to the inhibition of VAMP8-containing SNARE complexes, whereas VAMP2-containing complexes are not affected. Because VAMP2 and VAMP8 are localized to different secretory granules (27), these data demonstrate that *E. coli* exposure interferes with the release of a subset of secretory granules, and does not block exocytosis as a whole. Only VAMP8 has been shown to be specifically involved in serotonin and β-hexosaminidase secretion (26). While VAMP8−/− mice are defective in β-hexosaminidase and serotonin release, VAMP2−/− mice show normal levels of degranulation. Interestingly, VAMP8−/− mice exhibited no defect in histamine (26) or cytokine secretion (43), suggesting that the fusion of subsets of secretory granules in mast cells are regulated by different SNARE proteins. Supporting this hypothesis, *E. coli* exposure appears to inhibit β-hexosaminidase (this study) and serotonin release (18), but induces histamine (20) and cytokine secretion (52). Because not all exocytic events are similarly affected by SNAP23 hypophosphorylation, our results also suggest that each VAMP-containing vesicle might fuse with different exocytic t-SNARE complexes that contain SNAP23. Alternatively, the requirement for SNAP23 phosphorylation might be different for each v-SNARE. Collectively, these data suggest that culturing mast cells with *E. coli* specifically blocks the fusion of VAMP8-containing β-hexosaminidase granules. In view of this evidence, one can hypothesize that *E. coli* exposure redirects the reactivity of mast cells toward an immune response against the invading pathogen (53,54).

Although the bacterial component(s) that initiates the inhibitory cascade and impairs the IKKβ/SNAP23 interaction and SNAP23 phosphorylation remains to be identified, the enhanced phosphorylation of IκBα that was observed in *E. coli*-exposed cells (Figure 4D) may provide some insight. In addition to inducing degranulation, FcεRI cross-linking also activates the NFκB pathway, which involves the IKKβ-dependent phosphorylation of IκBα (40). Furthermore, bacterial pattern recognition receptors also activate the NFκB pathway (42). Both degranulation and the NFκB pathway require IKKβ activity (29). Our data suggest that the reduced interaction between IKKβ and SNAP23 may be due to the fact that IKKβ is titrated away from the 'degranulation pathway' and toward the NFκB pathway. Thus, less IKKβ would be available to phosphorylate SNAP23. Given that the increased IκBα phosphorylation is stimulus-dependent, it is possible that bacterial exposure may 'prime' the NFκB pathway for antigen-mediated activation. The signaling pathway induced by TLR activation may explain this phenomenon. Mast cells express a number of TLRs, which play an important role in immune responses to bacterial infection (55). Surface-expressed TLR4 and TLR2 recognize bacterial lipopolysaccharide and peptidoglycan, respectively (55). Treatment of mast cells with TLR2/4 agonists prior to FcεRI-mediated stimulation inhibits mast cell degranulation (56) and dampens allergic inflammation (57–59) while inducing cytokine secretion (42). Of particular interest is the involvement of the kinase IKKβ in TLR signaling pathways and SNAP23 phosphorylation (29,55), which suggests that TLRs are strong candidates for initiating the inhibition of mast cell degranulation. Although TLR4 agonists alone inhibit degranulation, heat-inactivated *E. coli* do not inhibit mast cell secretion (Figure S1C, left panel), suggesting that heat-stable antigens such as lipopolysaccharide are not involved in this process. Whether other TLRs or surface receptors play a role in this inhibitory cascade requires further investigation.

In summary, these data present a novel mechanism by which *E. coli* exposures influence mast cell degranulation through specific changes in membrane fusion events. This effect most likely preserves the secretion of protective cytokines while reducing the release of mediators that drive allergic inflammation. It will be important to determine which events upstream of SNARE complex formation in mast cells are impaired as a result of bacterial exposure. In addition, identifying the bacterial component(s) that contributes to the inhibition of mast cell degranulation

will be essential for developing ways to artificially regulate activation and ultimately the development of allergy.

Materials and Methods

Cell line and bacterial strain

The rat basophilic leukemia (RBL-2H3) cell line was obtained from the ATCC (CRL-2256) and cultured as described (27,38). Briefly, RBL-2H3 cells were maintained in complete DMEM (DMEM supplemented with 15 mM HEPES, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin). The *E. coli* strain BL21 (DE3) (Invitrogen) was grown to the stationary phase overnight in Luria broth for all assays.

RBL-2H3 transfection and generation of stable cell lines

RBL-2H3 cells were transfected with SNAP23-EGFP-N3, SNAP29-EGFP-N3 or empty EGFP-N3 vectors as described (38). The expression of GFP fusion proteins was assessed by western blot.

Antibodies

Rabbit anti-SNAP23, -Syntaxin4, -VAMP8, -actin and mouse anti-alpha tubulin were purchased from Sigma. Rabbit anti-VAMP2 was obtained from Synaptic Systems. Anti-p80 (clone 5G10, mouse) and -phospho-SNAP23 (Serine 120, rabbit) were kind gifts from Drs J. Bonifacino and P. Roche, respectively. Anti-IKKβ (rabbit) was purchased from Cell Signaling and anti-FcεRIα (mouse) was obtained from Abcam. Mouse anti-SNAP23 and -phospho IκBα were purchased from Santa Cruz Biotechnology. HRP-conjugated CTB and goat anti-mouse IgG₁-AlexaFluor488 conjugated antibody were obtained from Invitrogen. Sheep anti-mouse and donkey anti-rabbit HRP-conjugated antibodies were purchased from GE Healthcare.

β-Hexosaminidase assay

RBL-2H3 cells were plated at 5×10^4 cells per well in 100 µL of complete DMEM in 96-well flat-bottom plates 24 h prior to the assay. For single exposure experiments, *E. coli* was grown to the stationary phase, washed and diluted in DMEM without antibiotics to MOIs of 100, 1000 and 10 000 per 200 µL of medium. *E. coli*-conditioned medium was generated by culturing *E. coli* at MOIs of 10 000 (1×) and 20 000 (2×) in DMEM without antibiotics for 2 h, followed by centrifugation at $100 000 \times \boldsymbol{g}$ for 1 h and filtration (0.2 µm). *E. coli* was heat-killed for 45 min at 70°C. The cells were washed with DMEM without antibiotics and then 200 µL of the appropriate MOI was added to the cells followed by incubation at 37°C for 2 h. Control cells received 200 µL of DMEM without antibiotics. To inhibit protein translation, 100 µg/mL chloramphenicol was added during the 2-h incubation. The wells were then washed to remove extracellular bacteria. For FcεRI-mediated stimulation, the cells were sensitized with 200 µL of 100 ng/mL anti-DNP IgE (Sigma) in DMEM supplemented with 100 µg/mL gentamicin for 1 h at 37°C. Cells to be stimulated with PMA and ionomycin received 200 µL of DMEM

supplemented with 100 µg/mL gentamicin and were incubated for 1 h at 37°C. The cells were then washed with β-hex medium [DMEM without phenol red containing 500 µg/mL bovine serum albumin (BSA) and 2 mM L-glutamine]. For IgE-mediated stimulation, FcεRI was cross-linked with 200 µL of 100 ng/mL DNP-BSA (Sigma) in β-hex medium and the cells were incubated for 15, 30 or 60 min at 37°C. For PMA/ionomycin stimulation, the cells were stimulated with 200 µL of 1 µM PMA (Fisher) and 1 µM ionomycin (Fisher) in β-hex medium and incubated for 15, 30 or 60 min at 37°C. At each time point a sample of the supernatant was removed for analysis. β-Hexosaminidase was quantified as described (38).

To determine the effect of multiple *E. coli* exposures on β-hexosaminidase release, 5×10^5 RBL-2H3 cells were plated in 10-cm tissue culture plates 24 h prior to the first exposure. For each exposure, the cells were co-cultured with *E. coli* as described above for 2 h at an MOI of 10 000 in 10 mL of DMEM without antibiotics. The cells were then washed to remove extracellular bacteria and incubated overnight with DMEM supplemented with 100 µg/mL gentamicin at 37°C. Each exposure occurred 24 h apart. After the final exposure, the cells were incubated overnight at 37°C in DMEM containing 100 µg/mL gentamicin. The cells were then harvested and 5×10^4 viable cells (as determined by trypan blue exclusion) were plated in 96-well plates in DMEM and incubated at 37°C for 30 min until they adhered to the plate. The cells were then sensitized with 200 µL of 100 ng/mL anti-DNP IgE or media alone (for PMA/ionomycin stimulation) for 2 h at 37°C followed by stimulation as described above.

Flow cytometry

A total of 4×10^6 RBL-2H3 cells were cultured with or without *E. coli* at an MOI of 10 000 in 10-cm culture plates, harvested and stimulated in suspension with either 800 ng/mL anti-DNP IgE and 800 ng/mL DNP-BSA or 1 µM PMA/1 µM ionomycin at a concentration of 2×10^6 cells/mL. Following stimulation, cells were fixed with 2% paraformaldehyde (PFA) on ice for 30 min. The cells were washed with PBS, blocked for 30 min with FACS buffer [1% BSA, 1 mM ethylenediaminetetraacetic acid (EDTA) in PBS, pH 7.4] containing 20% goat serum and resuspended in primary antibodies indicated in the figure legends for 1 h on ice. Cell pellets were washed with FACS buffer by centrifugation and then stained with goat anti-mouse IgG₁ AlexaFluor488-conjugated antibody (Invitrogen) for 30 min on ice. Samples were washed, resuspended in FACS buffer and analyzed using the FACS Calibur Flow Cytometer (BD). Data were analyzed using FLOWJO software.

Immunofluorescence microscopy

A total of 5×10^4 RBL-2H3 cells were seeded onto coverslips 24 h prior to the experiment in complete DMEM. Cells were cultured with or without *E. coli* at an MOI of 10 000 for 2 h. Extracellular bacteria were removed by washes with PBS. To assess surface levels of FcεRI, cells were fixed in 2% PFA for 30 min, quenched with 50 mM ammonium chloride for 15 min and stained as described below. For FcεRI-mediated stimulation the cells were sensitized with 100 ng/mL anti-DNP IgE

for 1 h at 37°C following exposure. The cells were stimulated with 100 ng/mL DNP-BSA for 60 min at 37°C, then fixed and quenched. For PMA/ionomycin stimulation, following *E. coli* exposure the cells were incubated at 37°C for 1 h, stimulated with 1 μM PMA/1 μM ionomycin for 60 min, then fixed and quenched. To label surface proteins, the coverslips were blocked with blocking buffer (20% goat serum, 0.1% BSA in PBS, pH 7.4) for 1 h and incubated with primary antibodies diluted in blocking buffer as indicated in the figure legends for 1 h. The coverslips were washed and incubated with goat anti-mouse IgG₁ AlexaFluor488-conjugated antibody diluted in blocking buffer for 1 h. The cells were washed and nuclei were labeled with 1 μg/mL Hoechst (Invitrogen). Coverslips were mounted with Prolong Gold Antifade reagent (Invitrogen). Images were acquired using a Nikon TiE inverted immunofluorescence microscope with a 60× oil immersion lens and NIS Elements AR 3.2 software (Nikon). Image analysis was conducted using ImageJ (NIH).

Immunoprecipitation

A total of 1.6×10^7 RBL-2H3 cells were cultured with or without *E. coli* at an MOI of 10 000 for 2 h in 150-mm plates. Following the exposure, the cells were harvested using Cellstripper (Invitrogen) and resuspended to 2×10^6 cells/mL in DMEM containing 100 μg/mL gentamicin at 37°C for 1 h. For FcεRI-mediated stimulation, the cells were incubated with 800 ng/mL of anti-DNP IgE during the hour recovery. The cells were washed thrice by centrifugation with β-hex medium and then resuspended to 2×10^6 cells/mL with either 1 μM PMA/1 μM ionomycin or 800 ng/mL DNP-BSA in β-hex medium and incubated at 37°C for the indicated time. Ice-cold PBS was added to the cell suspension to stop degranulation and the cells were pelleted by centrifugation at 4°C. Stimulants were removed by washing the cell pellets with ice-cold PBS followed by centrifugation. To stabilize SNARE complexes, the cell pellets were treated with NEM as previously described (27) before cell lysis. Cells were lysed in lysis buffer (1% NP-40, 1 mM EDTA and 1 mM EGTA in TBS, pH 7.4) at a concentration of 3×10^7 cells/mL for 1 h at 4°C followed by centrifugation at $20\,000 \times g$ for 20 min. A total of 3×10^7 cells were used for immunoprecipitation. One milligram of total protein was incubated with 30 μL of BSA-blocked ProteinG Plus Agarose beads (Millipore) bound with 10 μg of anti-SNAP23 (mouse) antibody overnight at 4°C. The beads were then washed with lysis buffer, eluted with sodium dodecyl sulfate-containing sample buffer and boiled for 5 min at 95°C. Samples were analyzed by SDS–PAGE and western blot.

Gel electrophoresis and western blotting

Samples were run on 10% Bis-Tris gels (Invitrogen) and then transferred to polyvinylidene fluoride membrane (Bioexpress). The membranes were blocked with wash buffer (25 mM Tris, 250 mM sodium chloride and 0.2% Tween-20, pH 7.6) containing 5% milk for 1 h and immunoblotted with primary antibodies diluted in wash buffer containing 5% BSA as indicated in the figure legends for 1 h. The membranes were then washed with wash buffer and incubated with HRP-conjugated secondary antibodies diluted in wash buffer containing 5% milk for 1 h. Following several washes, the blots were revealed with WesternBright Quantum ECL (Bioexpress).

Images were captured using a FluorChem M imager with CCD camera (ProteinSimple) and band intensities were analyzed using ALPHAVIEW software (ProteinSimple).

Lipid raft preparation

RBL-2H3 cells were co-cultured with or without (control) *E. coli* at an MOI of 10 000 for 2 h, then harvested and stimulated in suspension with 1 μM PMA/1 μM ionomycin for 30 min at 7×10^6 cells/mL. The cells were washed with cold PBS and resuspended to 2.5×10^8 cells/mL in cold MBS (25 mM MES sodium salt, pH 6.5 and 150 mM NaCl) containing 1% Triton-X-100, 1 mM Na₃VO₄, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin and 2 μg/mL pepstatin A for 20 min on ice followed by 20 passages in a dounce homogenizer; 400 μL of lysate was mixed with 400 μL of 80% sucrose and overlaid with 2.2 mL of 30% and 1.4 mL of 5% sucrose in a 4.2-mL centrifuge tube. All sucrose solutions were prepared in MBS. The gradient was then centrifuged at $200\,000 \times \boldsymbol{g}$ for 18 h at 4°C. 400 μL fractions were collected from the top of the gradient and analyzed by western blot.

Statistical analysis

The Student's *t*-test (two-tailed) was used to compare the means of different populations. Data are considered statistically significant at p values ≤0.05.

Acknowledgments

Supporting Information

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

Figure S1. *E. coli*, **which must be translationally active, rapidly inhibits RBL-2H3 mast cell degranulation.** A) RBLs were plated in 96-well plates 24 h prior to being cultured with increasing MOIs of *E. coli* for 2 h. Following sensitization with anti-DNP IgE for 1 h and stimulation with DNP-BSA for the indicated times, β-hexosaminidase release was quantified. Total intracellular β-hexosaminidase content was determined after *E. coli* co-culture for each MOI (inset). B) RBLs were cultured with or without *E. coli* as described in panel A, but stimulated with 1 μM PMA/1 μM ionomycin and β-hexosaminidase release was quantified. Graphs depict the mean ± standard deviation of five independent experiments conducted in duplicate. The amount of β-hexosaminidase

released at 60 min in the control population was considered 100%. C) RBLs were plated in 96-well plates 24 h prior to being cultured with or without (control) heat-killed *E. coli* (left), chloramphenicol-treated *E. coli* (center) or *E. coli*-conditioned medium (right) for 2 h. The cells were then sensitized with anti-DNP IgE for 1 h, stimulated with DNP-BSA for 1 h and β-hexosaminidase release was quantified. The amount of β-hexosaminidase released at 60 min in the control population was considered 100%. Graphs depict the mean ± standard deviation of three independent experiments conducted in triplicate. In all graphs, asterisks denote statistically significant p values, where * ≤ 0.05 and ** ≤ 0.01.

Figure S2. FcεRI surface expression is not affected by *E. coli* exposure. A) RBLs were seeded on coverslips 24 hr prior to being incubated with (right panel) or without (left panel, control) *E. coli* at an MOI of 10 000 for 2 h. The cells were then washed, fixed and stained with anti-FcεRI and anti-mouse IgG₁ AlexaFluor488-conjugated antibodies. Nuclei were stained with Hoechst. Surface levels of FcεRI were determined using immunofluorescence microscopy. Scale bars = 20 μm, representative of three independent experiments. B) RBLs were cultured with or without *E. coli* at an MOI of 10 000 either once (left panel) or thrice (right panel), labeled as described in panel A and surface levels were quantified using flow cytometry. Graphs represent the mean fluorescence intensity of FcεRI staining from three independent experiments ± standard deviation.

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