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***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 pro-inflammatory 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 IKK β 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* (*E. 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 IKK β 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.

Keywords

Mast cell; SNAREs; degranulation; bacterial infection; *Escherichia coli*; SNAP23; Syntaxin4; VAMP8; intracellular trafficking; membrane fusion

Introduction

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 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, Fc ϵ RI, on the surface of

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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 *E. 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 hyper-reactivity of mast cells to circulating allergen. 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 suggests 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 TNFα 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 2h. Then the kinetics of β-hexosaminidase secretion was assessed for anti-DNP IgE sensitized RBLs stimulated with DNP-BSA. For comparative purposes, the amount of β-hexosaminidase released at 60min in the control population was

considered 100%. As shown in Figure S1A, *E. coli* inhibits FcεRI-mediated β-hexosaminidase release in a dose-dependent manner. A significant effect is observed at an MOI of 1,000 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), while 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 only achieved at an MOI of 10,000 (Fig 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 60min in control cells, while 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* (Fig 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 (Fig S1C, left and center panels). However, *E. coli*-conditioned medium alone does not inhibit degranulation (Fig S1C, right panel). These data suggest at least two possibilities: 1) 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 2) 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 2h either once, twice or three times, with each incubation occurring 24h apart. The MOI of 10,000 was chosen because it induced the maximal effect (Fig 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 (Fig 1A & B). After 60min 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. In fact, 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 2h 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 (Fig S2B, left panel) or thrice (Fig 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 (Fig S1B). Under certain conditions however, FcεRI surface expression has been shown to 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 4h 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* (Fig 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 2h, prior to stimulation with anti-DNP IgE/DNP-BSA or PMA/Ionomycin for 60min. 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 has been shown to correlate with degranulation in RBL-2H3 mast cells (34, 35). In control cells stimulated by either IgE/DNP-BSA (Fig 2A, top panel) or PMA/Ionomycin (Fig 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 (Fig 2A and 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 (Fig 2C and 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 or PMA/Ionomycin stimuli (Fig 2C and 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 reduction in p80 levels compared with the non-exposed control cells. At t=15min in *E. coli*-exposed cells stimulated with IgE/DNP-BSA and PMA/Ionomycin,

p80 surface levels were reduced by 15% and 26%, respectively (Fig 2C and 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**

Since 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 (Fig 3A). The impact of *E. coli* on β -hexosaminidase release in these stable RBL populations was assessed following multiple exposures and stimulation with PMA/Ionomycin, as described above. For comparative purposes, the amount of β -hexosaminidase released after 60min of stimulation for each unexposed population was considered as 100%. Similar to non-transfected RBLs (Fig 1B), *E. coli* co-culture additively inhibits β -hexosaminidase secretion in the GFP control population (Fig 3B). At 60min, 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 three times with *E. coli* (~39% inhibition at 60min). Interestingly, degranulation in cells overexpressing SNAP23-GFP is not significantly impaired by incubation with *E. coli* (Fig 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 (Fig 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 (Fig 3D). After 60min of stimulation, four exposures to *E. coli* reduces 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 Syntaxin 4 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 sub-domains. To do so, lipid rafts were isolated by density centrifugation. Lipid rafts were identified by CTB-HRP labeling which 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 mis-localization of SNAP23.

Upon stimulation, SNAP23 is phosphorylated at serine residues 95 and 120 by IKK β (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 will be 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 15min of stimulation with IgE/DNP-BSA or PMA/Ionomycin (Fig 4B and 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 60min post-stimulation with IgE/DNP-BSA (Fig 4B) and by ~25% at 60min with PMA/Ionomycin (Fig 4C). The differences in SNAP23 phosphorylation using IgE/DNP-BSA and PMA/Ionomycin stimulation correlates 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%, Fig S1A) compared with PMA/Ionomycin stimulation (47%, Fig S1B). Although we previously observed a significant inhibition of degranulation at 15min with PMA/Ionomycin stimulation (Fig 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 (Fig 4C), whereas degranulation was analyzed with adherent cells (Fig S1B). It has been previously shown that the kinetics of degranulation is delayed in suspension cells compared to 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 fact, in mast cells derived from IKK β ^{-/-} mice there is little to no stimulus-induced phosphorylation of SNAP23 suggesting that IKK β is the major kinase responsible for SNAP23 phosphorylation and that PKC acts upstream of IKK β (29).

We then determined whether SNAP23 hypophosphorylation was due to changes in IKK β activity. In addition to phosphorylating SNAP23, IKK β is also the major kinase that phosphorylates I κ B α during NF κ B signaling in response to Fc ϵ RI-mediated activation as well as Toll-like receptor engagement (40-42). We then tested IKK β activity by monitoring I κ B α phosphorylation following co-culture with or without *E. coli* using Western blot. In cells cultured with *E. coli*, we observed an increase in I κ B α phosphorylation following stimulation compared with the control cells (Fig 4D), suggesting that IKK β is still enzymatically active following co-culture with *E. coli*. The enhanced phosphorylation of I κ B α may be due to Toll-like receptor (TLR) engagement during the culture period, which would activate the NF κ B pathway resulting in I κ B α phosphorylation.

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

Upon stimulation, IKK β interacts with and phosphorylates SNAP23. Therefore, we investigated whether the reduction in SNAP23 phosphorylation was due to impairment in

the interaction between SNAP23 and IKK β . Initially, we tested whether IKK β recruitment into lipid rafts containing SNAP23 was impaired. However, we were unable to visualize IKK β in the rafts, likely due to the lack of sensitivity of the antibody (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 IKK β by Western blot. As shown in Fig 5A (control), IKK β co-immunoprecipitates with SNAP23, in agreement with Suzuki et al (29). However, in cells cultured with *E. coli*, we observed a 40% reduction in the amount of IKK β that co-immunoprecipitates with SNAP23 (Fig 5A and B). Together, these data suggest that *E. coli* exposure impairs the interaction between IKK β 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 since 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). Since SNAP23 phosphorylation in mast cells is significantly reduced following incubation with *E. coli* (Fig 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, we treated the cells with N-Ethylmaleimide (NEM) prior to cell 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 Fig 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 (Fig 6A and 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% (Fig 6A and B, VAMP8). However, similar amounts of VAMP2 co-immunoprecipitate with SNAP23 whether the cells are cultured with or without *E. coli* (Fig 6A and 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 (Fig 6C). Altogether, these results suggest that *E. coli* exposure specifically impairs the formation of VAMP8-dependent exocytic SNARE complexes.

Discussion

Mast cells play an integral role in the development of allergic inflammation through the rapid release of prestored pro-inflammatory 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 hypo-phosphorylation 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). In fact, 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 the present study, we showed that SNAP23 hypo-phosphorylation 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, while VAMP2-containing complexes are not affected. Since 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. In fact, 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). Since not all exocytic events are similarly affected by SNAP23 hypo-phosphorylation, 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 towards an immune response against the invading pathogen (53, 54).

Although the bacterial component(s) that initiates the inhibitory cascade and impairs the IKK β SNAP23 interaction and SNAP23 phosphorylation remains to be identified, the enhanced phosphorylation of I κ B α that we observed in *E. coli* exposed cells (Fig 4D) may provide some insight. In addition to inducing degranulation, Fc ϵ RI crosslinking also activates the NF κ B pathway, which involves the IKK β -dependent phosphorylation of I κ B α (40). Furthermore, bacterial pattern recognition receptors also activate the NF κ B pathway (42). Both degranulation and the NF κ B pathway require IKK β activity (29). Our data suggests that the reduced interaction between IKK β and SNAP23 may be due to the fact that IKK β is titrated away from the “degranulation pathway” and towards the NF κ B pathway. Thus, less IKK β would be available to phosphorylate SNAP23. Given that the increased I κ B α phosphorylation is stimulus-dependent, it is possible that bacterial exposure may “prime” the NF κ B pathway for antigen-mediated activation. The signaling pathway induced by Toll-like receptor (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 Fc ϵ RI-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 IKK β 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 (Fig S1C, left panel) suggesting that heat stable antigens like LPS 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 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 mast cell 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 previously described (27, 38). Briefly, RBL-2H3 cells were maintained in complete DMEM (DMEM supplemented with 15mM HEPES, 10% fetal bovine serum, 2mM L-glutamine, 100 units/ml penicillin and 100µg/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 previously 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 Cholera toxin subunit B and Goat anti-mouse IgG₁-AlexaFluor488 conjugated antibody was 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/well in 100µl of complete DMEM in 96 well flat bottom plates 24h prior to the assay. For single exposure experiments, *E. coli* was grown to the stationary phase, washed and diluted in DMEM without antibiotics to multiplicities of infection (MOI) of 100, 1,000 and 10,000 per 200µl of medium. *E. coli*-conditioned medium was generated by culturing *E. coli* at MOIs 10,000 (1×) and 20,000 (2×) in DMEM without antibiotics for 2h, followed by centrifugation at 100,000×g for 1h and filtration (0.2µm). *E. coli* was heat-killed for 45min at 70°C. The cells were washed with DMEM without antibiotics then 200µl of the appropriate MOI was added to the cells followed by incubation at 37°C for 2h. Control cells received 200µl of DMEM without antibiotics. To inhibit

protein translation, 100 μ g/ml chloramphenicol was added during the 2h incubation. The wells were then thoroughly washed to remove extracellular bacteria. For Fc ϵ RI-mediated stimulation, the cells were sensitized with 200 μ l of 100ng/ml anti-dinitrophenol (DNP) IgE (Sigma) in DMEM supplemented with 100 μ g/ml gentamicin for 1h at 37°C. Cells to be stimulated with phorbol myristate acetate (PMA) and Ionomycin received 200 μ l of DMEM supplemented with 100 μ g/ml gentamicin and were incubated for 1h at 37°C. The cells were then washed three times with β -hex medium (DMEM without phenol red containing 500 μ g/ml bovine serum albumin (BSA) and 2mM L-glutamine). For IgE-mediated stimulation, Fc ϵ RI was cross-linked with 200 μ l of 100ng/ml DNP-BSA (Sigma) in β -hex medium and the cells were incubated for 15, 30 or 60min at 37°C. For PMA/Ionomycin stimulation, the cells were stimulated with 200 μ l of 1 μ M PMA (Fisher) and 1 μ M Ionomycin (Fisher) in β -hex medium and incubated for 15, 30 or 60min 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 10cm tissue culture plates 24h prior to the first exposure. For each exposure, the cells were co-cultured with *E. coli* as described above for 2h at an MOI of 10,000 in 10ml of DMEM without antibiotics.

The cells were then washed thoroughly to remove extracellular bacteria and incubated overnight with DMEM supplemented with 100 μ g/ml gentamicin at 37°C. Each exposure occurred 24h apart. After the final exposure, the cells were incubated overnight at 37°C in DMEM containing 100 μ g/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 30min until they adhered to the plate. The cells were then sensitized with 200 μ l of 100ng/ml anti-DNP IgE or media alone (for PMA/Ionomycin stimulation) for 2h at 37°C followed by stimulation as described above.

Flow cytometry

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

Immunofluorescence microscopy

5×10^4 RBL-2H3 cells were seeded onto coverslips 24h prior to the experiment in complete DMEM. Cells were cultured with or without *E. coli* at an MOI of 10,000 for 2h.

Extracellular bacteria were removed by thorough washes with PBS. To assess surface levels of FcεRI, cells were fixed in 2% PFA for 30min, quenched with 50mM ammonium chloride for 15min, and stained as described below. For FcεRI-mediated stimulation the cells were sensitized with 100ng/ml anti-DNP IgE for 1h at 37°C following exposure. The cells were stimulated with 100ng/ml DNP-BSA for 60min at 37°C, then fixed and quenched. For PMA/Ionomycin stimulation, following *E. coli* exposure the cells were incubated at 37°C for 1h, stimulated with 1μM PMA/1μM Ionomycin for 60min 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 1h and incubated with primary antibodies diluted in blocking buffer as indicated in the Figure legends for 1h. The coverslips were washed and incubated with goat anti-mouse IgG₁ AlexaFluor488-conjugated antibody diluted in blocking buffer for 1h. The cells were washed and nuclei were labeled with 1μg/ml Hoechst (Invitrogen). Coverslips were mounted with Prolong Gold Anti-fade 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

1.6×10^7 RBL-2H3 cells were cultured with or without *E. coli* at an MOI of 10,000 for 2h in 150mm plates. Following the exposure, the cells were harvested using Cellstripper (Invitrogen) and resuspended to 2×10^6 cells/ml in DMEM containing 100μg/ml gentamicin at 37°C for 1h. For FcεRI-mediated stimulation, the cells were incubated with 800ng/ml of anti-DNP IgE during the hour recovery. The cells were washed three times by centrifugation with β-hex medium then resuspended to 2×10^6 cells/ml with either 1μM PMA/1μM Ionomycin or 800ng/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 N-Ethylmaleimide (NEM) as previously described (27) before cell lysis. Cells were lysed in lysis buffer (1% NP-40, 1mM EDTA, 1mM EGTA in TBS, pH 7.4) at a concentration of 3×10^7 cells/ml for 1h at 4°C followed by centrifugation at 14,000rpm for 20min. 3×10^7 cells were used for immunoprecipitation. 1mg of total protein was incubated with 30μl of BSA-blocked ProteinG Plus Agarose beads (Millipore) bound with 10μg 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 5min 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 PVDF membrane (Bioexpress). The membranes were blocked with wash buffer (25mM Tris, 250mM Sodium chloride, 0.2% Tween-20, pH 7.6) containing 5% milk for 1h and immunoblotted with primary antibodies diluted in wash buffer containing 5% BSA as indicated in the Figure legends for 1h. The membranes were then washed with wash buffer and incubated with HRP-conjugated secondary antibodies diluted in wash buffer containing 5% milk for 1h. 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 2h, then harvested and stimulated in suspension with 1 μ M PMA/1 μ M Ionomycin for 30min at 7 \times 10⁶ cells/ml. The cells were washed with cold PBS and resuspended to 2.5 \times 10⁸ cells/ml in cold MBS (25mM MES, pH 6.5 and 150mM NaCl) containing 1% Triton-X100, 1mM Na₃VO₄, 10mM NaF, 2mM PMSF, 5 μ g/ml Leupeptin, 2 μ g/ml Pepstatin A for 20min on ice followed by 20 passages in a dounce homogenizer. 400 μ l of lysate was mixed with 400 μ l of 80% sucrose and overlaid with 2.2ml of 30% and 1.4ml of 5% sucrose in a 4.2ml centrifuge tube. All sucrose solutions were prepared in MBS. The gradient was then centrifuged for 18h at 200,000 \times g at 4°C. 400 μ l fractions were collected from the top of the gradient and analyzed by Western blot.

Statistical analysis

The Students t-test (two-tailed) was used to compare the means of different populations. Data are considered statistically significant at *p* values \leq 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Synopsis

Mast cell degranulation plays a major role in allergic diseases. Here, we report that co-culturing mast cells with *E. coli* inhibits FcεRI-mediated degranulation by impacting the function of the exocytic SNARE fusion machinery. In particular, *E. coli* co-culture reduces the IKKβ-dependent phosphorylation of the t-SNARE SNAP23, thus impairing the formation of ternary exocytic SNARE complexes and ultimately mediator release.

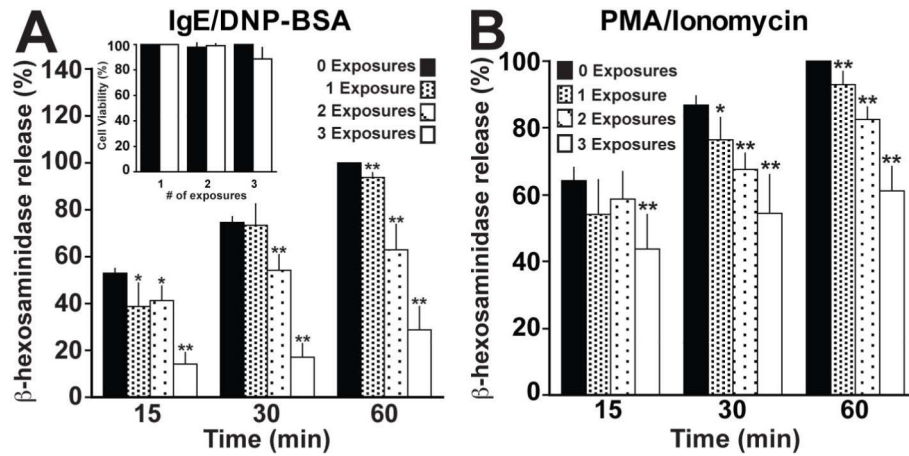


Figure 1. *E. coli* exposures additively inhibits mast cell degranulation

(A) 5×10^5 RBL-2H3 cells were exposed to *E. coli* multiple times for 2h at an MOI of 10,000. Each exposure occurred 24h apart. The cells were then transferred to 96 well plates at a density of 5×10^4 viable cells per well. Following sensitization with 100ng/ml anti-DNP IgE for 2h, the cells were stimulated with 100ng/ml DNP-BSA for the indicated times. Cell viability prior to stimulation was assessed by trypan blue exclusion (inset). (B) RBLs were treated as in A, but stimulated with $1 \mu\text{M}$ PMA/ $1 \mu\text{M}$ Ionomycin. The amount of β -hexosaminidase released at 60min in the control population was considered 100%. Graphs depict the mean \pm the standard deviation of 5 independent experiments conducted in duplicate. In all graphs, asterisks denote statistically significant *p* values, where * 0.05 and ** 0.01.

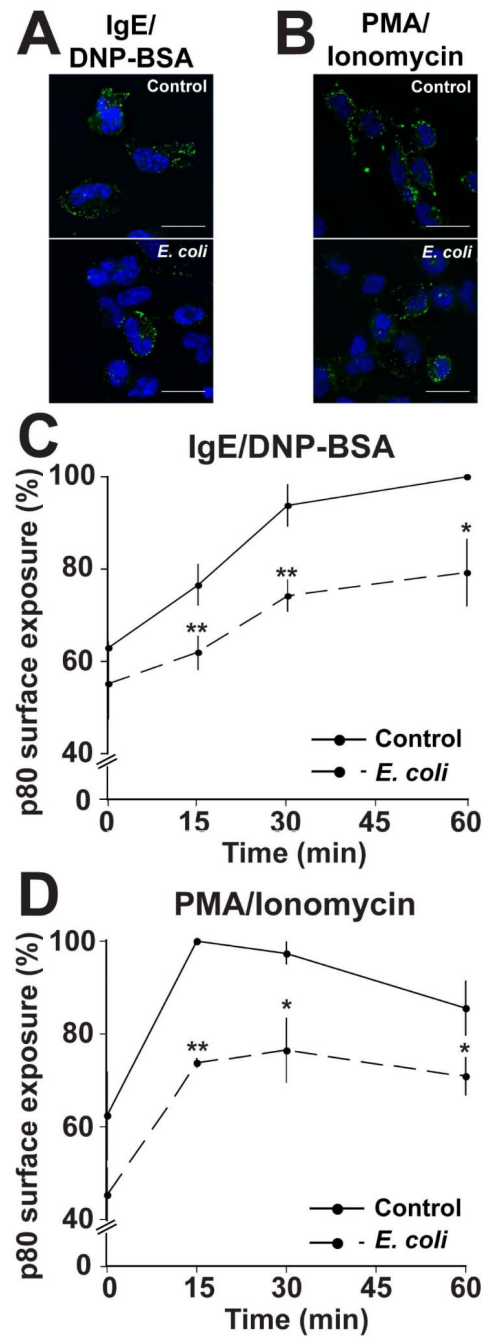


Figure 2. *E. coli* exposure impairs membrane fusion during degranulation

(A) RBLs were plated on coverslips at a density of 5×10^4 cells/coverslip 24hr prior to being incubated with (lower panel) or without (top panel-control) *E. coli* at an MOI of 10,000 for 2h. The cells were then sensitized with 100ng/ml anti-DNP IgE and stimulated with 100ng/ml DNP-BSA for 1h. 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 μ m. Representative of 3 independent experiments. (B) RBLs were treated as described in A, but stimulated with 1 μ M

PMA/1 μ M Ionomycin. Scale bars = 20 μ m. Representative of 3 independent experiments. (C) 4 \times 10⁶ RBLs were plated in 10cm tissue culture plates 24h prior to being incubated with or without *E. coli* at an MOI of 10,000 for 2h. The cells were harvested, sensitized with 800ng/ml anti-DNP IgE at a concentration of 2 \times 10⁶ cells/ml and stimulated with 800ng/ml DNP-BSA for 0, 15, 30 or 60min. The cells were fixed and labeled as described in A then analyzed by flow cytometry. (D) RBLs were treated as described in C, but stimulated with 1 μ M PMA/1 μ M Ionomycin. Graphs depict the mean fluorescence intensity of p80 staining \pm the standard deviation of 3 independent experiments. The maximal p80 fluorescence intensity in the control population was considered 100% (t=60 for IgE/DNP-BSA and t=15min for PMA/Ionomycin). In all graphs, asterisks denote statistically significant *p* values, where * 0.05 and ** 0.01.

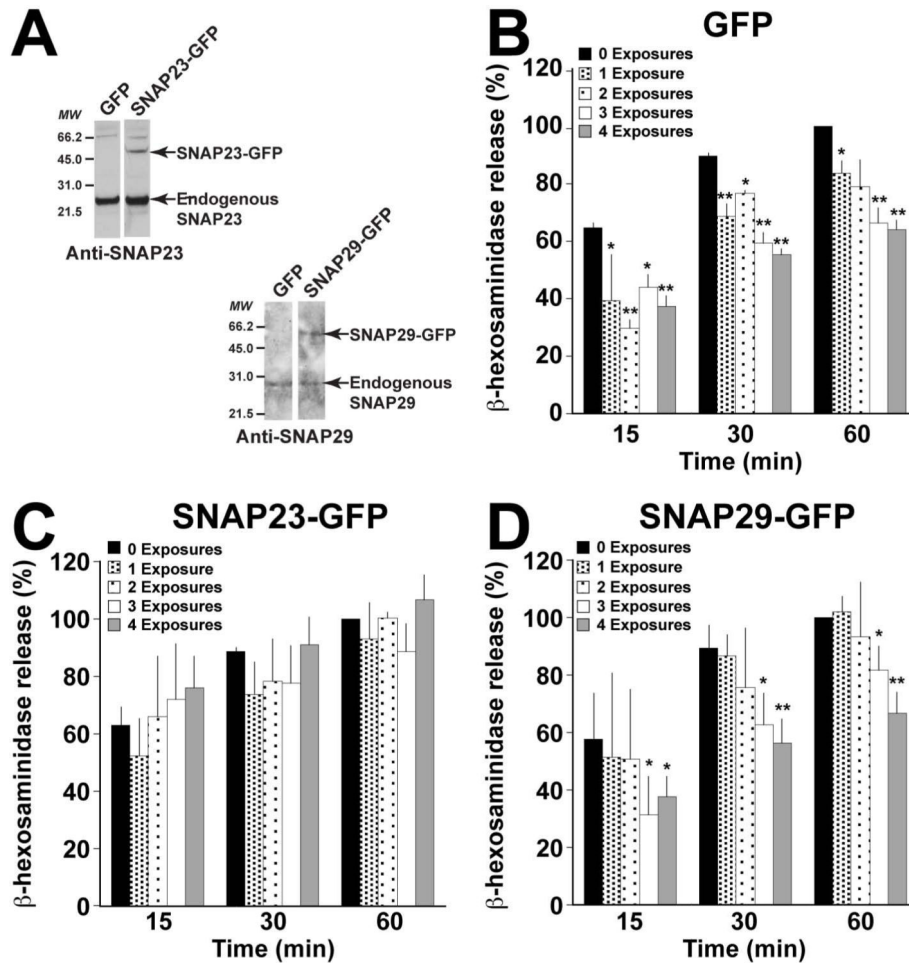


Figure 3. Overexpression of SNAP23 compensates for the inhibitory effect of *E. coli* exposure (A) Stable populations of RBL-2H3 cells overexpressing GFP, SNAP23-GFP or SNAP29-GFP were generated. The expression of SNAP23-GFP and SNAP29-GFP were analyzed by Western blot using anti-SNAP23 and anti-SNAP29 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). Stable populations expressing GFP (B), SNAP23-GFP (C) and SNAP29-GFP (D) were cultured with or without *E. coli*, as described in Fig 1A, either 1, 2, 3, or 4 times with each exposure occurring 24h apart. After each exposure, 5×10^4 viable cells were plated in 96 well plates in a volume of 100 μ l per well and allowed to recover for 2h at 37°C. The cells were then stimulated with 1 μ M PMA/1 μ M Ionomycin for the indicated times and β -hexosaminidase release was quantified. The amount of β -hexosaminidase released at 60min in each unexposed population was considered 100%. Graphs represent the mean \pm the standard deviation of 5 independent experiments conducted in duplicate. In all graphs, asterisks denote statistically significant *p* values, where * 0.05 and ** 0.01.

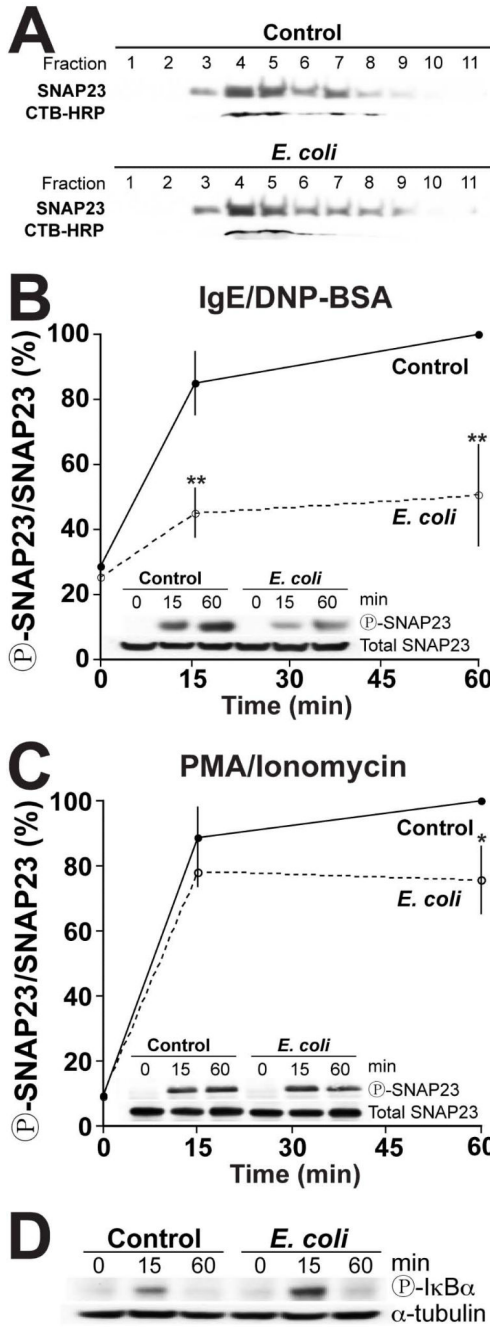


Figure 4. SNAP23 is hypo-phosphorylated in *E. coli* exposed mast cells, while IKK β remains functional
 (A) RBL-2H3 cells cultured with or without (control) *E. coli* for 2h, followed by stimulation in suspension with 1 μ M PMA/1 μ M Ionomycin for 30min. Lipid rafts were isolated by density centrifugation as described in the Methods. 20 μ l of each fraction was analyzed by Western blot using anti-SNAP23 antibody. Lipid rafts were identified using cholera toxin subunit B-HRP (CTB). Representative of 3 independent experiments. (B and C) 2 \times 10⁶ RBLs/ml cultured with or without (control) *E. coli* at an MOI of 10,000 for 2h. The cells were harvested and stimulated with 800ng/ml anti-DNP IgE and 800ng/ml DNP-BSA (B) or

with 1 μ M PMA/1 μ M Ionomycin (C) for 0, 15 or 60min. The cells were then lysed and SNAP23 was immunoprecipitated from 1mg of total protein for each condition. Samples were analyzed by Western blot using anti-SNAP23 and anti-SNAP23¹²⁰ antibodies (insets). The band intensities were determined by densitometry. Graphs represent the mean band intensity ratio of SNAP23¹²⁰ to total SNAP23 \pm the standard deviation of 3 independent experiments. The SNAP23¹²⁰ to total SNAP23 ratio of the control population at 60min 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 2h. The cells were then harvested and stimulated in suspension with 1 μ M PMA/1 μ M Ionomycin for 0, 15 or 60min before being lysed. 50 μ g of total protein from exposed and control whole cell lysates were analyzed by Western blot using anti-phospho-I κ B α and anti-alpha tubulin antibodies. Representative of 3 independent experiments.

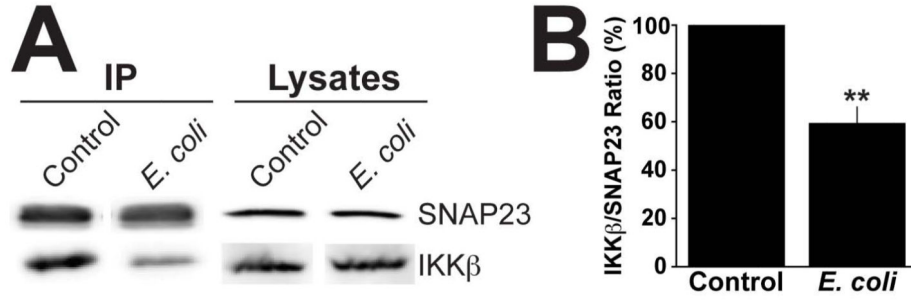


Figure 5. Binding of IKK β to SNAP23 is reduced following *E. coli* exposure

(A) RBLs were incubated with or without (control) *E. coli* at an MOI of 10,000 for 2h. The cells were harvested and stimulated for 30min with 1 μ M PMA/1 μ M Ionomycin at a concentration of 2×10^6 cells/ml, lysed and SNAP23 was immunoprecipitated from 1mg of total protein. Immunoprecipitated samples and 40 μ g of whole cell lysates were analyzed by Western blot with anti-SNAP23 and -IKK β antibodies. (B) Graph represents the mean band intensity ratio of IKK β to total SNAP23 \pm the standard deviation of 3 independent experiments. The IKK β /SNAP23 ratio of the control population was considered 100%. Asterisks denote statistically significant *p* values, where ** = 0.01.

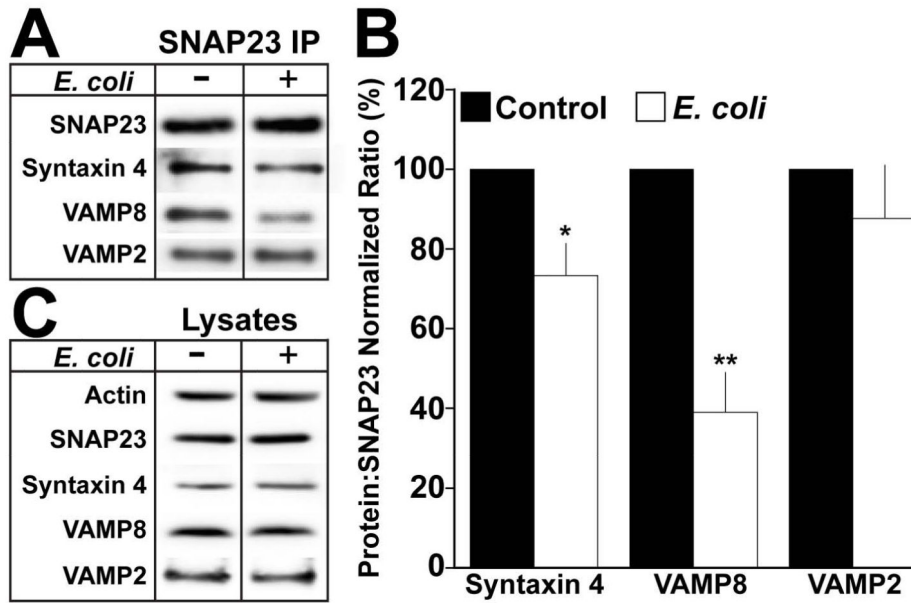


Figure 6. Exocytic SNARE complex formation is impaired in *E. coli* exposed mast cells
 (A) RBLs were cultured with or without (control) *E. coli* for 2h at an MOI of 10,000. The cells were harvested and stimulated for 30min with 1 μ M PMA/1 μ M Ionomycin at a concentration of 2×10^6 cells/ml. Cells were treated with 1mM NEM for 15min. Next, NEM was inactivated with 2mM DTT for 15min on ice. NEM-treated cells were washed and lysed. 1mg 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 3 independent experiments. (B) Quantification of the mean band intensity ratio's of the indicated protein to SNAP23 \pm the standard deviation of at least 3 independent experiments. The protein:SNAP23 ratio of the control population was considered 100%. Asterisks denote statistically significant *p* values, where * 0.05 and ** 0.01. (C) 40 μ g of total protein from exposed or control whole cell lysates was analyzed by Western blot as described in A.