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# The FcεRIβ homologue, MS4A4A, promotes FcεRI signal transduction and store-operated $Ca^{2+}$ entry in human mast cells

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## Abstract

Members of the membrane spanning 4A (MS4A) gene family are clustered around 11q12–13, a region linked to allergy and asthma susceptibility. Other than the known functions of FcεRIβ (MS4A2) and CD20 (MS4A1) in mast cell and B cell signaling, respectively, functional studies for the remaining MS4A proteins are lacking. We thus explored whether MS4A4A, a mast cell expressed homologue of FcεRIβ, has related functions to FcεRIβ in FcεRI signaling. We establish in this study that MS4A4A promotes phosphorylation of PLCγ1, calcium flux and degranulation in response to IgE-mediated crosslinking of FcεRI. We previously demonstrated that MS4A4A promotes recruitment of KIT into caveolin-1-enriched microdomains and signaling through PLCγ1. Caveolin-1 itself is an important regulator of IgE-dependent store-operated $Ca^{2+}$ entry (SOCE) and promotes expression of the store-operated $Ca^{2+}$ channel pore-forming unit, Orai1. We thus further report that MS4A4A functions through interaction with caveolin-1 and recruitment of FcεRI and KIT into lipid rafts. In addition to proximal FcεRI signaling, we similarly show that MS4A4A regulates Orai1-mediated calcium entry downstream of calcium release from stores. Both MS4A4A and Orai1 had limited effects with compound 48/80 stimulation, demonstrating some degree of selectivity of both proteins to FcεRI receptor signaling over Mas-related G Protein

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[7]Author Contributions

G.K.A. performed experiments, analyzed data, prepared figures and wrote the manuscript; L.C.E-H. performed experiments and analyzed data; D.B.S. performed experiments, analyzed data; C.J. isolated and cultured human mast cells; S.J.T. supervised procedures at UNC-CH, obtained funding, contributed to the manuscript; D.D.M. supervised experiments at NIH and edited the manuscript; G.C. conceived and designed the study, supervised experiments and directed the research, performed experiments, prepared figures, obtained funding and wrote the manuscript.

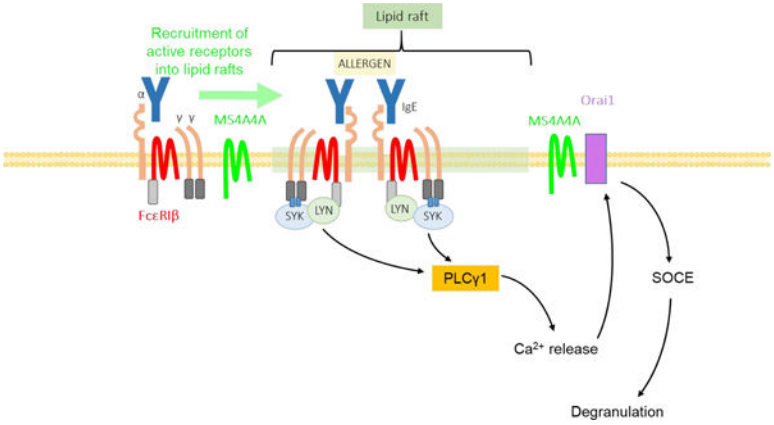


[6]Conflict of Interest

The authors declare no conflicts of interest related to the reported research.

coupled receptor X2 signaling. Overall, our data are consistent with the conclusion that MS4A4A performs a related function to the homologous FcεRIβ to promote PLCγ1 signaling, SOCE, and degranulation through FcεRI in human mast cells and thus represents a new target in the regulation of IgE-mediated mast cell activation.

## Graphical abstract




### Keywords

Mast cell; IgE receptor; MS4A; allergy; Orai1

## 1 Introduction

The membrane-spanning 4-subfamily A (MS4A) gene cluster are a family of at least 16 genes in humans that are related to *MS4A1* (the gene encoding CD20) and are expressed primarily in immune cells (1, 2). The full-length MS4A family proteins are 4-pass transmembrane proteins that have similar topology, but low homology to tetraspanins. Therefore, MS4A proteins may have related, albeit distinct functions from tetraspanins. Although little is known about the function of MS4A proteins, recent studies have implicated the gene family in multiple signal transduction pathways for various receptors (3–6). MS4A4A (also known as MS4A4) specifically, has been identified regulator of dectin-1 signaling in macrophages that promotes NK cell-mediated resistance metastasis and found to be associated with recruitment of MS4A4A and dectin-1 into lipid rafts (3). In addition, we previously reported that MS4A4A protein functions to promote signaling and recycling of the receptor tyrosine kinase, KIT, through the PLCγ1 pathway and MS4A4A facilitated recruitment of KIT into caveolin-1-enriched lipid rafts (6). Further, the mouse homologue of MS4A4A, Ms4a4b, acts as a membrane adaptor protein for the glucocorticoid-induced tumor necrosis factor receptor-related (GITR) protein and forms complexes in lipid rafts of T cells (7). Additionally, this mouse homologue directly interacts with the store-operated $Ca^{2+}$ channel pore-forming unit, Orai1 (CRACM1) (7). Collectively, these studies suggest that MS4A4A, in humans, could play an important role in lipid raft-associated signaling and complex formation of receptors and ion channels.

Contrary to the role that we and others have found for MS4A4A in promoting receptor multimeric complex formation and signaling in lipid rafts, the closely related mouse homologues of MS4A4A along with other Ms4a genes in mice, have been proposed to act directly as chemoreceptors in olfactory necklace sensory neurons where the Ms4a proteins recognize ligands including fatty acids and pheromones to trigger $Ca^{2+}$ responses (8). Therefore, some MS4A proteins may recognize ligands directly to trigger signaling, or act as adaptor proteins to regulate signaling of companion receptors through multimeric complex formation in membrane microdomains. Whether ligand recognition is direct or indirect, MS4A proteins clearly have diverse functions in cells that respond to environmental cues.

We, and others, have extensively studied the closely related MS4A gene family member *MS4A2*, which encodes the β subunit (FcεRIβ) of the high affinity IgE receptor, FcεRI, because of the critical role it plays in FcεRI function (5, 9–15). Crosslinking FcεRI on mast cells (MCs) by IgE-bound antigens culminates in the release of proinflammatory mediators by compound exocytosis, termed degranulation. Recently, we identified expression of MS4A4A in human MCs, validated shRNA knockdown of MS4A4A, and determined that MS4A4A is associated with lipid rafts in human MCs after stimulation with the growth factor receptor KIT (6). Given the critical role of FcεRIβ in FcεRI function, and the high homology between FcεRIβ and MS4A4A, we hypothesized that in addition to the effects that we reported for MS4A4A with the KIT receptor, MS4A4A also functions in FcεRI signaling.

In this study, we tested this hypothesis and show, for the first time, that MS4A4A plays an important role in IgE-mediated MC signaling, SOCE and degranulation. Mechanistically, MS4A4A plays a role in proximal FcεRI signaling through PLCγ1 phosphorylation, which we propose is due to recruitment into lipid rafts, thereby promoting $Ca^{2+}$ flux. In addition, our data support the hypothesis that MS4A4A promotes FcεRI and KIT crosstalk and synergy by promoting their interaction in lipid rafts. Further, we show that MS4A4A promotes store-operated $Ca^{2+}$ entry (SOCE) through regulation of Orai1 downstream of $Ca^{2+}$ release to drive MC degranulation. These mechanisms of MS4A4A function appear selective to anaphylactic, FcεRI-mediated degranulation, over anaphylactoid degranulation induced by compound 48/80 through Mas-related G Protein coupled receptor X2 (MRGPRX2) receptors. Taken together, our data sort important roles for MS4A4A in MC biology by regulating both proximal and distal IgE-mediated MC signaling pathways and thus identify a new target in the regulation of mast cell activation.

## 2 Materials and Methods

### 2.1 Cell cultures

LAD2 human MCs were cultured as described (6, 16) in StemPro-34 medium containing 13 ml supplement, penicillin (100 units/ml) and streptomycin (100 μg/ml) (all from Life Technologies, Paisley, UK), and 100 ng/ml recombinant human SCF (R&D, Abingdon, UK). Half of the medium was replaced with fresh medium and SCF every 7 d. CBMCs were cultured from de-identified, heparinized cord blood obtained from the Carolinas Cord Blood Bank at UNC Hospitals. Units with volumes or cell counts too low for use by the Cord Blood Bank for stem cell transplants were utilized and use for this research was determined

to be exempt from the approval by the Biomedical Institutional Review Board at the University of North Carolina at Chapel Hill. Differentiation of mast cell progenitors was achieved as described (17). Cells were cultured for 12 weeks and mast cell purity at 12 weeks was determined to be >95% as assessed by flow cytometry and surface KIT and FcεRIα expression.

### 2.2 shRNA-mediated knockdown of MS4A4A and Orai1

For gene silencing of MS4A4A and ORAI1, MISSION® shRNA constructs and lentiviruses were used (5, 6) (Sigma-Aldrich, St Louis, MO). The previously validated shRNA for MS4A4A and scramble control were used as described (6). For Orai1, the following constructs were purchased and tested using the same procedures as described (5, 6). Experiments were performed after 7 days of shRNA transduction as described (5, 6).

shOrai1v1: TRCN0000439096

CGGGAGTTACTCCGAGGTGATGAGCTCGAGCTCATCACCTCGGAGTAACTCTTTTTTG

shOrai1v2: TRCN0000413611

CCGGGAGCAACGTGCACAATCTCAACTCGAGTTGAGATTGTGCACGTTGCTCTTTTTTG

shOrai1v3: TRCN0000437946

CCGGGCACCTGTTTGCGCTCATGATCTCGAGATCATGAGCGCAAACAGGTGCTTTTTTG

shOrai1v4: TRCN0000165044

(CCGGGCAACGTGCACAATCTCAACTCTCGAGAGTTGAGATTGTGCACGTTGCTTTTTTG)

shOrai1v5: TRCN0000161221

(CCGGGAAACTGTCCTCTAAGAGAATCTCGAGATTCTCTTAGAGGACAGTTTCTTTTTTG)

### 2.3 Quantitative real-time RT-PCR

Total RNA was isolated using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, with inclusion of the QIAshredder step. For quantitative real-time RT-PCR (qPCR), first strand cDNA synthesis was conducted using a RevertAid kit (Thermo Fisher, Waltham, MA). Reactions were run on a MyiQ iCycler PCR system (Bio-Rad) with iQ5 software version 2 (Bio-Rad, Hercules, CA). Products were run on a 2% agarose gel to confirm appropriate product sizes and bands were then excised from the gel and sequenced.

### 2.4 Mediator release assays

LAD2 cells were cultured for 6 d after transduction of shRNA, and then sensitized with 100 ng/mL biotinylated human myeloma IgE and incubated overnight (16 h). Cells were subsequently activated by streptavidin (SA) (Sigma Aldrich), thapsigargin or compound 48/80 for 30 min. Degranulation was assayed by β-hexosaminidase release, and $PGD_2$ was measured as described (18). CBMCs were sensitized the same as LAD2 cells, but were activated by IgE crosslinking with anti-IgE.

### 2.5 Flow cytometry

Surface receptor expression was assessed using flow cytometry as described (5, 19).

### 2.6 Calcium signaling assay

Changes in cytosolic $Ca^{2+}$ levels were determined following loading of the cells with Fura-2 AM ester (Molecular Probes, Eugene, OR), as described (5, 20). LAD2 cells were transduced with shRNA as described above, and calcium signaling assays were conducted on Day 7. For IgE-mediated calcium mobilization, cells were sensitized with 100 ng/mL biotinylated human myeloma IgE on Day 6 and incubated overnight (16 h). Fluorescence was measured at two excitation wavelengths (340 and 380 nm) and an emission wavelength of 510 nm using a Biotek Synergy Neo2 multimode plate reader.

### 2.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

MC activation was performed with a dose-response of SA as for degranulation assays and immunoblotting was carried out as described (5, 6).

### 2.8 Cholesterol depletion and repletion

Experiments using cholesterol depletion and repletion were carried out as reported (21). In brief, LAD2 cells were split into 3 treatment conditions with all incubations carried out at 37°C. Untreated cells were incubated in BSA-containing buffered saline solution (BSA/BSS: 20 mM Hepes, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 1 mg/ml BSA) as used previously (21). Cholesterol depleted cells were incubated with 10 μM methyl-β-cyclodextrin (MβCD) (Sigma) in BSA/BSS for 1 hour. Additionally, LAD2 cells were subjected to cholesterol depletion with 10 μM MβCD in BSA/BSS for 1 hour followed by washing and cholesterol repletion (0.4 μM cholesterol in BSA/BSS incubation for 2 hours). After all incubations concluded, cells were washed and resuspended in BSA/BSS and then challenged against increasing doses of SA for β-hexosaminidase assays. All conditions were carried out concurrently and in parallel.

### 2.9 Confocal microscopy

Confocal microscopy on LAD2 cells was carried out within the Biological Imaging Section of the Research Technologies Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, on a Leica TCS SP8 confocal microscope.as described (6).

# 3 Results

## 3.1 MS4A4A functions in FcεRI-mediated degranulation

We hypothesized that MS4A4A has a related function to its homologue, FcεRIβ, in FcεRI function. Therefore, we employed shMS4A4A and studied degranulation in response to IgE and FcεRI crosslinking. Transduction of LAD2 MCs with shMS4A4A resulted in a significant reduction in MS4A4A protein expression (Supplemental Fig. 1) and IgE-dependent MC degranulation in response to streptavidin (SA) alone (Fig. 1A) or SA plus SCF co-stimulation (Fig. 1B). Given the effects of shMS4A4A on FcεRI-induced degranulation, we examined whether shMS4A4A treatment resulted in a downregulation of surface FcεRI expression. There was no significant reduction in surface FcεRI with shMS4A4A treatment (Fig. 1C), but as we have reported, surface KIT expression was markedly reduced (6). These data are consistent with the conclusion that MS4A4A is involved in FcεRI signaling rather than regulating expression of FcεRI.

Because MCs are activated by IgE-dependent and IgE-independent stimuli, we next examined the effects of shMS4A4A in response to compound 48/80, which activates MCs *via* the G protein-coupled MRGPRX2 receptors (22). There was a small inhibition of compound 48/80-induced degranulation with shMS4A4A (Fig. 1D), but this inhibition was not as marked as with IgE crosslinking (XL) with SA, suggesting a degree of specificity of MS4A4A function to FcεRI signaling over MRGPRX2 receptors. As an additional control, we used thapsigargin, because thapsigargin induces degranulation (23) by depleting the endoplasmic reticular pool of $Ca^{2+}$ and subsequently triggering $Ca^{2+}$ influx via SOCE (24, 25). This effectively bypasses both FcεRI and PLCγ1 to induce degranulation. We found that shMS4A4A had a significant inhibitor y effect on thapsigargin-induced degranulation (Fig. 1E). Because thapsigargin stimulation bypasses surface receptors, this suggests that MS4A4A may play a role in MC degranulation downstream of $Ca^{2+}$ release from stores.

We next tested $PGD_2$ production, which is $Ca^{2+}$-dependent, but secretory granule independent, and found that $PGD_2$ production in response to FcεRI stimulation was significantly inhibited in shMS4A4A treated MCs (Fig. 1F). This suggests that the defect in degranulation was not due to a secretory granule defect in trafficking. Calculation of the inhibition of degranulation with each stimuli demonstrated that SA stimulation was inhibited by ~50–60% (Fig 1G), SA + SCF degranulation was inhibited by >50% (Fig 1H), compound 48/80 degranulation was inhibited by <20% (Fig 1I) and thapsigargin degranulation was inhibited by <40% (Fig 1J). Taken together, these data indicate that MS4A4A promotes MC activation and degranulation preferentially, but not solely, through FcεRI and that the inhibition of secretion was not a result of reduced FcεRI expression.

## 3.2 MS4A4A promotes FcεRI-dependent PLCγ1 signaling

We have shown that MS4A4A actively participates in trafficking and signaling of the growth factor receptor KIT (6). MS4A4A promotes KIT recruitment into caveolin-1 enriched lipid raft domains, altering endocytosis and recycling of the receptor, and facilitating PLCγ1 signaling in response to ligand (6). Because PLCγ1 and $Ca^{2+}$ influx are critical drivers of MC degranulation (20, 26), we predicted that inhibition of MC degranulation with

shMS4A4A was through reduced PLCγ1 signaling. IgE-dependent activation of MCs triggered a dose-dependent phosphorylation of PLCγ1 $Y^{783}$, which was significantly inhibited by shMS4A4A (Fig. 2A & B). Phosphorylation of AKT $S^{473}$, was not inhibited (Fig. 2A & C), but phosphorylation of AKT was only weakly induced by stimulation and interference from SCF used to maintain human MCs in culture could interfere with basal AKT signaling. The downstream MAPK, ERK $T^{202}$-$Y^{204}$ was strongly induced by FcεRI stimulation and markedly reduced with shMS4A4A (Fig. 2A & D). In summary, these data indicate that the PLCγ1 pathway, rather than the PI3K/AKT pathway is responsible for the inhibition of MC degranulation by MS4A4A silencing.

### 3.3 MS4A4A promotes FcεRI-dependent and KIT-mediated $Ca^{2+}$ conductance promoting synergy in $Ca^{2+}$ responses

Activation of PLCγ1 in MCs leads to the production of inositol triphosphate ($IP_3$) that triggers the release of $Ca^{2+}$ from intracellular stores by binding to the $IP_3$ receptor on the ER membrane (26). $Ca^{2+}$ release from stores is critical for influx of extracellular $Ca^{2+}$ through SOCE. Therefore, we examined the effects of shMS4A4A on $Ca^{2+}$ conductance using Fura2 ratiometric assays. Addition of medium alone did not induce a $Ca^{2+}$ response and no difference was observed between scramble control and shMS4A4A (Fig. 3A). Addition of SCF induced a small but significant increase in intracellular $Ca^{2+}$ in scramble control, but not in shMS4A4A-treated MCs (Fig. 3B). Stimulation of MCs with SA to crosslink IgE bound to FcεRI resulted in a robust $Ca^{2+}$ influx, which was significantly reduced with shMS4A4A treatment and more evident at the higher SA dose (Fig. 3C & D). Compound 48/80 stimulation resulted in a trend for reduced $Ca^{2+}$ responses with shMS4A4A treatment, only significant at late time-points (Fig. 3E). There was no significant difference with thapsigargin stimulation (Fig. 3F). Taken together, these data are in agreement with the degranulation studies and further suggest that MS4A4A plays an important role in FcεRI and SCF global $Ca^{2+}$ flux, but has limited effects on compound 48/80 and thapsgargin stimulation.

An important crosstalk interaction exists between two key receptors in MCs, FcεRI and KIT, the receptor tyrosine kinase for stem cell factor (SCF) (26–28). SCF is a growth and survival factor for MCs (29, 30) and synergistically enhances antigen-induced degranulation (26, 31). Given the effects of shMS4A4A on FcεRI (Fig. 2) and KIT (6) signaling through PLCγ1, we postulated that MS4A4A functions to promote synergy between KIT and FcεRI, because the PLCγ1 pathway is an important convergent of FcεRI and KIT signaling (32). The effects of shMS4A4A on $Ca^{2+}$ conductance were more evident when the cells were co-stimulated with IgE crosslinking and SCF (Fig. 3G). Calculating the change in fluorescence ratio pre-stimulation to maximum ratio post-stimulation demonstrated that co-stimulation of FcεRI and KIT in scramble control cells resulted in a synergistic effect with fluorescence above the additive level (Fig. 3H). Conversely, in shMS4A4A treated MCs, co-stimulation of FcεRI and KIT resulted in an additive increase in $Ca^{2+}$ flux (Fig. 3I). These data are consistent with the conclusion that MS4A4A also functions to promote synergy between these two canonical MC receptors.

### 3.4 MS4A4A promotes KIT and FcεRI colocalization after co-stimulation of receptors

We have established that MS4A4A co-immunoprecipitates with caveolin-1 in MCs (6). We also showed that a proportion of KIT is recruited into lipid rafts after stimulation with SCF and that this recruitment is promoted by expression of MS4A4A (6). Phosphorylation of PLCγ1 in response to SCF is weak and could represent a small proportion of KIT that are recruited into lipid rafts that signal through the PLCγ1 pathway (6). Therefore, we tested whether MS4A4A promotes colocalization of FcεRI and KIT following activation of both receptors. IgE crosslinking alone in MCs did not induce colocalization of FcεRI and KIT (Fig. 4A&C). However, co-stimulation of MCs with IgE crosslinking and SCF induced colocalization of both receptors at or near the plasma membrane (Fig. 4B&C), suggesting that both receptors have the potential to be recruited into the same membrane microdomains. Colocalization between KIT and FcεRI was significantly reduced with shMS4A4A (Fig. 4C). Therefore, we propose that MS4A4A promotes KIT and FcεRI crosstalk by recruiting both receptors into lipid rafts.

### 3.5 Inhibition of degranulation with shMS4A4A is reduced with depletion of cholesterol

As further evidence of lipid raft involvement in MS4A4A function and FcεRI signaling, we chose to utilize depletion and repletion of cholesterol experiments. Cholesterol is a critical component of lipid rafts and depletion of cholesterol disrupts binding of lipid raft-associated proteins (21, 33). To deplete cholesterol, we used methyl-β-cyclodextrin (MβCD) as previously used for MCs (21). MβCD is water soluble and rapidly depletes cholesterol from the plasma membrane in living cells without incorporating itself or alternative lipid products into the membrane (34). Using this approach, we established that in mock treated cells, there was a significant inhibition of degranulation by shMS4A4A (Fig. 4D). Cholesterol depletion with MβCD resulted in marked inhibition of degranulation in response to IgE crosslinking, with reduced differences in degranulation between scramble control and shMS4A4A, which no longer reached significance (Fig. 4E). Although MβCD preferentially depletes cholesterol, it can result in depletion of other lipids from the plasma membrane. Therefore, we also examined cholesterol repletion, after depletion, to confirm that the effects of MβCD were due to cholesterol. With repletion of cholesterol, a complete restoration of phenotype was achieved (Fig. 4F). Overall, these data suggest that MS4A4A participates in proximal FcεRI signaling, most likely in lipid raft microdomains.

### 3.6 MS4A4A functions in SOCE

Having established that MS4A4A functions in proximal FcεRI signaling by promoting recruitment into lipid rafts and signaling through PLCγ1, we next asked whether MS4A4A was also functioning in distal FcεRI signaling. Knockdown of MS4A4A has an inhibitory effect on degranulation in response to thapsigargin (Fig. 1E), but has limited effects on global $Ca^{2+}$ particularly during early time-points (Fig. 3F). Thapsigargin strongly induces SOCE, which is downstream of $Ca^{2+}$ release from stores, and not measurable with global $Ca^{2+}$ flux assays. We have shown that MS4A4A and caveolin-1 coimmunoprecipitate (6). In addition to the roles of caveolin-1 in caveolae, lipid rafts and endocytosis, caveolin-1 also regulates SOCE and expression of Orai1 (35), where caveolin-1 and lipid rafts are critical for Orai1-STIM1 interaction (36). Since SOCE in human MCs is primarily driven by Orai1

(37), we predicted that the effects of shMS4A4A on degranulation of MCs with thapsigargin stimulation was due to Orai1 regulation by MS4A4A and caveolin-1 interactions. To examine SOCE, we stimulated MCs with either SA, compound 48/80 or thapsigargin in the absence of extracellular $Ca^{2+}$ followed by addition of $Ca^{2+}$ (Fig. 5A–C). Using this approach, we established that SOCE in response to SA and SCF co-stimulation (Fig. 5A) and thapsigargin stimulation (Fig. 5B) were both significantly reduced with shMS4A4A, but SOCE in response to compound 48/80 was not (Fig. 5C). To confirm that the effects of MS4A4A on $Ca^{2+}$ influx were indeed due to SOCE, we added the $Ca^{2+}$-release activated $Ca^{2+}$ channel (CRAC) blocker YM-58483 to the cells and examined SOCE. With the addition of this CRAC inhibitor, there was a marked reduction in SOCE and the differences between scramble and shMS4A4A were diminished (Fig. 5A–C). SOCE with compound 48/80 was inhibited with YM-58483, but not with shMS4A4 suggesting that while ion channels that carry $Ca^{2+}$ were involved in SOCE with compound 48/80 stimulation, MS4A4A was not playing a role. Taken together, these data support the conclusion that MS4A4A functions in $Ca^{2+}$ conductance and SOCE from SA and thapsigargin, but has limited impact on compound 48/80.

### 3.7 MS4A4A function in SOCE is through regulation of Orai1

Because of the reported role of caveolin-1 on promoting Orai1 function discussed above (35, 36), we predicted that MS4A4A was affecting degranulation and SOCE by regulating function of Orai1. We therefore examined the effects of MS4A4A knockdown with additional knockdown of Orai1. If MS4A4A was affecting Orai1 function, we would expect to see a similar phenotype if either protein was knocked down, and no additional effect of double knockdown. We tested five shRNA constructs for Orai1 and chose shOrai1v2, the most efficient shRNA construct (Fig. 6A). None of the knockdowns showed evidence of cytotoxicity over the course of the experiments, which was measured by trypan blue counts (not shown). shMS4A4A resulted in a significant reduction in MC degranulation in response to IgE crosslinking (Fig. 6B). shOrai1 resulted in greater reduction in degranulation than shMS4A4A, but double knockdown with shMS4A4A and shOrai1 was comparable to shOrai1 alone (Fig. 6B). Stimulation with compound 48/80 was considerably less affected by shMS4A4A and shOrai1 (Fig. 6C), suggesting that not only was MS4A4A less involved in compound 48/80 degranulation compared to IgE crosslinking, but that Orai1 was also playing less of a role in this pathway. In addition, thapsigargin-induced degranulation was inhibited by shMS4A4A and shOrail, but again there was no additional effect of shMS4A4A and shOrai1 double knockdown (Fig. 6D). Taken together, these data are consistent with the conclusion that MS4A4A and Orai1 are functioning in a common pathway, and that SOCE with MRGPRX2 GPCR activation in MCs is less dependent on both MS4A4A and Orail.

### 3.8 MS4A4A is expressed in primary human cord blood-derived MCs and its function is conserved

We next sought to confirm that MS4A4A expression and function was conserved in primary human MCs. We employed human cord blood-derived MCs (HCBDMCs) (17). HCBDMCs demonstrated low degranulation capacity in response to IgE crosslinking (XL) in the absence of IL-4 (Fig. 7A). However, with 7 days of 100 ng/ml IL-4 in culture, HCBDMCs demonstrated more robust degranulation in response to IgE XL (Fig. 7A). Ionomycin was

used as a positive control, and this was unaffected by IL-4 (Fig 7B). The low degranulation response appeared to be due to low FcεRIα expression on the surface of the HCBDMCs, which was upregulated with IL-4 priming (Fig. 7C & D). We next examined surface MS4A4A expression, as described (3) and found that surface MS4A4A was present on HCBDMCs, but expression was lower than in LAD2 cells (Fig. 7E). After we had reported the expression of MS4A4A by human MCs (6), MS4A4A was reported to be a macrophage tetraspan that acts as a marker of alternatively activated M2 macrophages (3, 38). Because IL-4 is a key regulator of the M2 macrophage phenotype, IL-4 has been examined and shown to induce or upregulate surface expression of MS4A4A in macrophages (3, 38). Therefore, we expected surface MS4A4A expression to be upregulated in MCs after IL-4 priming. However, we did not find any change in surface MS4A4A expression with IL-4 addition in either HCBDMCs or LAD2 cells (Fig. 7E), despite surface FcεRIα expression increasing in HCBMCs (Fig. 7D). These findings suggest that while MS4A4A expression modulates FcεRI function, MS4A4A does not appear to regulate surface FcεRI expression, which is in agreement with the data showing that knockdown of MS4A4A does not affect surface FcεRIα expression in LAD2 cells (Fig. 1C).

Finally, having established expression of MS4A4A in HCBDMCs we next confirmed that shMS4A4A also resulted in reduced degranulation of HCBDMCs (Fig. 7F). The effects of shMS4A4A on degranulation in HCBDMCs were significant, but less marked than in LAD2 cells (compare Fig. 7D to Fig. 1A). We propose that this was due to the lower expression of MS4A4A in HCBDMCs compared to LAD2 cells (Fig. 7E) and that higher expression of MS4A4A results in greater degranulation in response to IgE XL.

## 4 Discussion

The function of the human *MS4A4A* gene is unknown and this is the first study to identify that the four transmembrane protein product, MS4A4A (also named MS4A4), functions in MC degranulation and signaling through FcεRI. We have further established that MS4A4A functions to promote human MC degranulation through FcεRI crosslinking by two distinct mechanisms. Firstly, MS4A4A promotes proximal FcεRI signaling through the PLCγ1 pathway, which is likely due to promoting lipid raft association and interaction of the complex with adaptor proteins and signaling molecules. Secondly, MS4A4A promotes Orai1-mediated SOCE downstream of $Ca^{2+}$ release from ER stores.

MS4A4A is a homologue of FcεRIβ and thus we postulated that it could have related functions. We have shown that MS4A4A actively participates in trafficking and signaling of the growth factor receptor KIT (6). MS4A4A promotes KIT recruitment into caveolin-1 enriched lipid raft domains facilitating PLCγ1 signaling in response to ligand, altering endocytosis and recycling of the receptor (6). The result is that MS4A4A regulates KIT signaling pathways to alter MC phenotype. We now show that MC degranulation in response to antigen is significantly reduced with shMS4A4A and we propose that this effect is due to recruitment of crosslinked FcεRI into lipid rafts at the plasma membrane. We examined signaling in response to antigen and found that PLCγ1 signaling was significantly reduced and this correlated with reduction in $Ca^{2+}$ influx. Importantly, the effects of MS4A4A knockdown on $Ca^{2+}$ were more evident when the cells were co-stimulated with antigen and

SCF (the ligand for KIT) and act synergistically to promote MC functions (31). Since MS4A4A knockdown reduced PLCγ1 signaling for both receptors, we predict that MS4A4A plays a role in recruitment of both receptors into lipid rafts. Indeed, FcεRI and KIT colocalized to membrane domains only when co-stimulated and knockdown of MS4A4A reduced this colocalization, suggesting that MS4A4A function in lipid raft association of receptors is not limited to a single receptor and could have relevance in several immune cells that express MS4A4A.

In support of our hypothesis that MS4A4A functions to recruit receptors into lipid rafts in a promiscuous manner, recent studies have identified MS4A4A expression in alternatively activated macrophages (3, 38), which differ considerably from MCs in receptor expression. MS4A4A expression was identified in peripheral blood, which was principally in monocytes, and on the surface of M2, but not M1 macrophages (38). The precise role that MS4A4A plays in monocytes and M2 macrophages is not yet established, but it was shown that MS4A4A promotes dectin-1 signaling and both MS4A4A and dectin-1 were recruited into lipid rafts after zymosan engagement where they were shown to interact (3). These observations, taken together with our findings, lead us to propose that MS4A4A functions in signaling through multiple receptors by promoting complex formation within lipid rafts. The mechanisms underlying this function of MS4A4A are yet to be elucidated, but we have shown that MS4A4A traffics through caveolae and interacts with caveolin-1 (6), suggesting that this interaction could be critical. While it seems that signaling through several receptors are affected by MS4A4A, we show that MS4A4A provides some selectivity between receptors. For FcεRI signaling, phosphorylation of FcεRIγ ITAM occurs mainly by the Src family kinase, Lyn kinase, which is recruited to the non-canonical ITAM of FcεRIβ (39). Upon activation by crosslinking, FcεRI is rapidly recruited into lipid rafts enriched in glycosphingolipids, cholesterol, and glycosylphosphatidylinositol-anchored proteins, where it is phosphorylated by Lyn kinase (40, 41). The recruitment of FcεRI complexes into lipid rafts likely plays an important role in downstream signaling events. Downstream signaling through FcεRI is critically dependent on the linker protein, LAT (42), which is resident in lipid rafts and crucial for PLCγ1 phosphorylation (43). Bone marrow-derived MCs from LAT deficient mice exhibit intact proximal phosphorylation of FcεRI subunits and Syk in response to FcεRI aggregation, but phosphorylation of PLCγ1 and $Ca^{2+}$ influx were markedly reduced (42).

It has been demonstrated that while LAT is critical for complete FcεRI signal transduction, $Ca^{2+}$ influx and degranulation (42) in lipid rafts (43), LAT phosphorylation and downstream signaling can still occur independently from Lyn kinase translocation into lipid rafts (41). Lyn is critical for proximal FcεRI signaling, but these data suggest that signaling through LAT within lipid rafts can occur without Lyn translocation to lipid rafts. Indeed, FcεRI can be phosphorylated outside of lipid rafts (44), and thus signaling through FcεRI and LAT can occur without FcεRI or Lyn kinase translocation to lipid rafts. However, lipid raft translocation may amplify signaling, which could be evidenced by the reduced PLCγ1 phosphorylation we observed with shMS4A4A. We propose that by facilitating FcεRI recruitment into lipid rafts, MS4A4A promotes FcεRI signal transduction through PLCγ1 to promote distal signaling and $Ca^{2+}$ mobilization. However, we show in the present study that knockdown of MS4A4A reduces IgE-dependent LAD2 cell degranulation by around 50%,

and while PLCγ1 signaling is reduced, it still occurs, and is thus unlikely to be the only mechanism for the inhibitory effect of shMS4A4A on distal FcεRI signaling.

In addition to proximal FcεRI signaling, we also demonstrate that MS4A4A functions in distal FcεRI signaling downstream of $Ca^{2+}$ release from stores to promote SOCE through Orai1. MS4A1 and MS4A12 have been proposed to act as distinct $Ca^{2+}$ channels because ectopic expression induces a $Ca^{2+}$ current post-transfection (45, 46). We found that MS4A4A promotes $Ca^{2+}$ conductance and SOCE in human MCs suggesting that MS4A4A could function as a $Ca^{2+}$ channel. However, SOCE in human MCs is primarily driven by the $Ca^{2+}$ channel pore-forming unit, Orai1 (37). Thus, we examined MC degranulation with shRNA knockdown of MS4A4A and/or Orai1 and found that the phenotype with shMS4A4A closely resembled shOrai1 and knockdown of both Orai1 and MS4A4A had no additive effect. It is possible that MS4A4A forms a $Ca^{2+}$ channel that is dependent upon Orai1. However, we propose that it is more likely that MS4A4A is not acting as a $Ca^{2+}$ channel itself, but rather regulating Orai1 function. Caveolin-1 functions in SOCE by promoting Orai1 interaction with STIM1 and by regulating of expression of Orai1 (35, 36). Caveolin-1 also channels $Ca^{2+}$ microdomain signaling through distinct pathways to activate transcription factors where caveolin-1 and lipid rafts are critical for Orai1-STIM1 interactions (36). We propose that MS4A4A, most likely in the ER and juxtanuclear regions of the cells, functions in this pathway to regulate Orai1-mediated SOCE.

Strong supporting evidence in our study that MS4A4A functions in an Orai1 mediated SOCE pathway was the finding that human MC degranulation was less dependent upon either Orai1 or MS4A4A when the cells were activated by compound 48/80 when compared to IgE crosslinking. SOCE was not reduced by shMS4A4A with compound 48/80 treated cells, but blocking CRAC channels with compound YM-58483 did inhibit SOCE in both control and shMS4A4A treated cells. It has been established that Orai1 is the primary CRAC channel responsible for SOCE in human MCs with IgE-dependent degranulation (37), but this has been less characterized with MRGPRX2 in human MCs. Although blocking SOCE with compound 48/80 suggests CRAC channel involvement and YM-58483 potently blocks Orai1, it also blocks other ion channels, such as the non-selective cation TRPC channels (47). Indeed, compound 48/80-induced $Ca^{2+}$ influx in rat MCs is primarily driven by non-selective cation channels (48–51). We found that degranulation in response to compound 48/80 was significantly less sensitive to Orai1 knockdown than either IgE-crosslinking or thapsigargin stimulation. Our findings with MS4A4A knockdown also followed this same pattern. These observations, taken together, suggest that the primary ion channels that carry SOCE in IgE-mediated (anaphylactic) and MRGPRX2-mediated (anaphylactoid) human MC degranulation differ, with Orai1 driving IgE-mediated SOCE and another ion channel, perhaps a TRP channel, mediating MRGPRX2 SOCE.

## 5 Summary

In summary, we show that MS4A4A promotes MC degranulation by facilitating the PLCγ1 signaling pathway most likely within lipid rafts, as well as SOCE through Orai1. Further study of the mechanisms and functions of MS4A4A are warranted to establish whether MS4A4A contributes to the membrane and organelle trafficking during degranulation in

MCs and establish the role MS4A4A plays in allergic and inflammatory diseases. The MS4A family are clustered around chromosome 11q12-q13 (1, 2), a region previously linked to allergy and asthma susceptibility (52–54). Given the role of FcεRIβ in MCs, FcεRIβ is considered a candidate for the linkage of these genetic loci with allergy. However, given the high homology between FcεRIβ other MS4A cluster proteins, other members of the MS4A cluster, in particular MS4A4A, could well contribute to the development, and pathophysiology of allergy, and may represent a novel therapeutic target.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- Human mast cells express MS4A4A on the cell surface.
- MS4A4A functions in a related manner to its homologue, the β subunit of the high affinity IgE receptor (the *MS4A2* gene).
- MS4A4A functions in proximal IgE receptor signaling in a lipid raft-dependent manner to promote degranulation.
- MS4A4A also functions in distal IgE receptor signaling by promoting store-operated calcium entry through Orai1 in human mast cells.

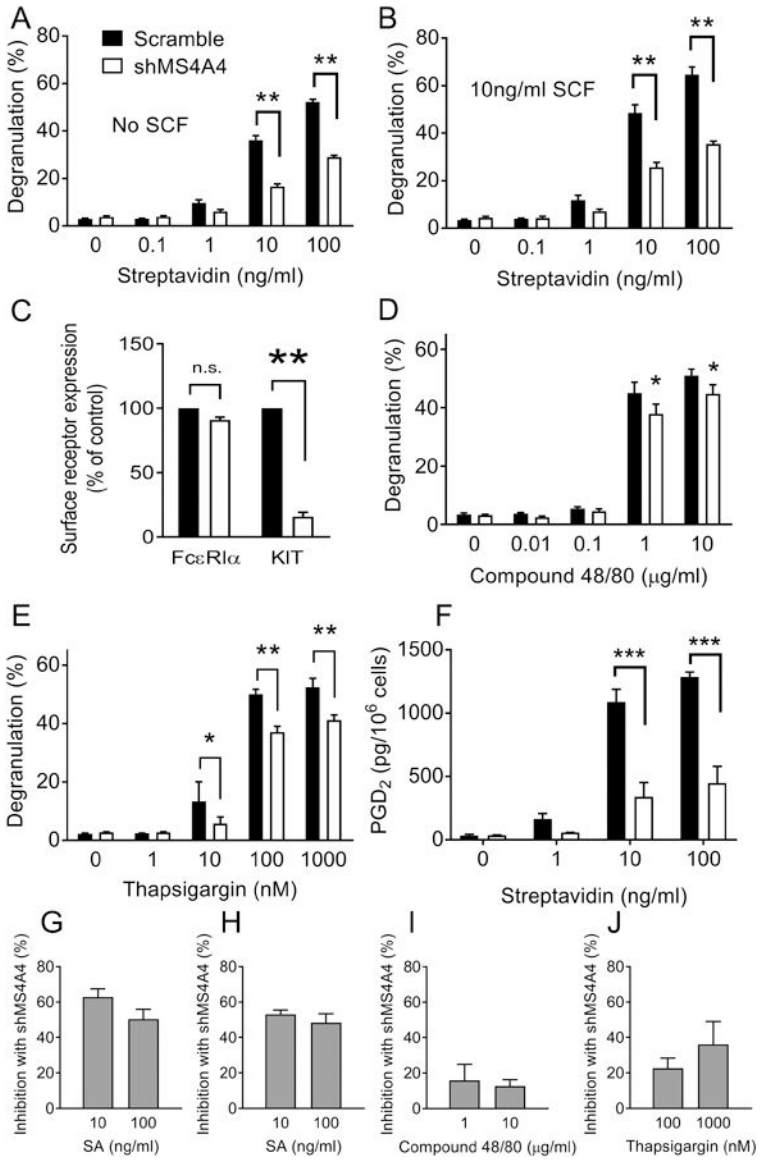


**Figure 1. MS4A4A functions in FcεRI-mediated degranulation.**

Transduction with shMS4A4A reduces release of β-hexosaminidase from LAD2 cells stimulated with (A) SA (n=11) and (B) SA and 10 ng/ml SCF co-stimulation (n=5). (C) Flow-cytometric analysis of surface FcεRIα and KIT in LAD2 cells after transduction with either scramble or shMS4A4A (n=3). (D) Compound 48/80-induced release of β-hexosaminidase from LAD2 cells was not significantly inhibited by shMS4A4A (n=3). (E) Transduction with shMS4A4A reduces release of β-hexosaminidase from LAD2 cells stimulated with thapsigargin (n=6). (F) $PGD_2$ release from LAD2 cells was inhibited by shMS4A4A (n=3). (G-J) Inhibition of degranulation with shMS4A4A for each stimulus was calculated as the percent inhibition at the top two concentrations. Data are the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, n.s. = not significant. ANOVA with Sidak's posttest.

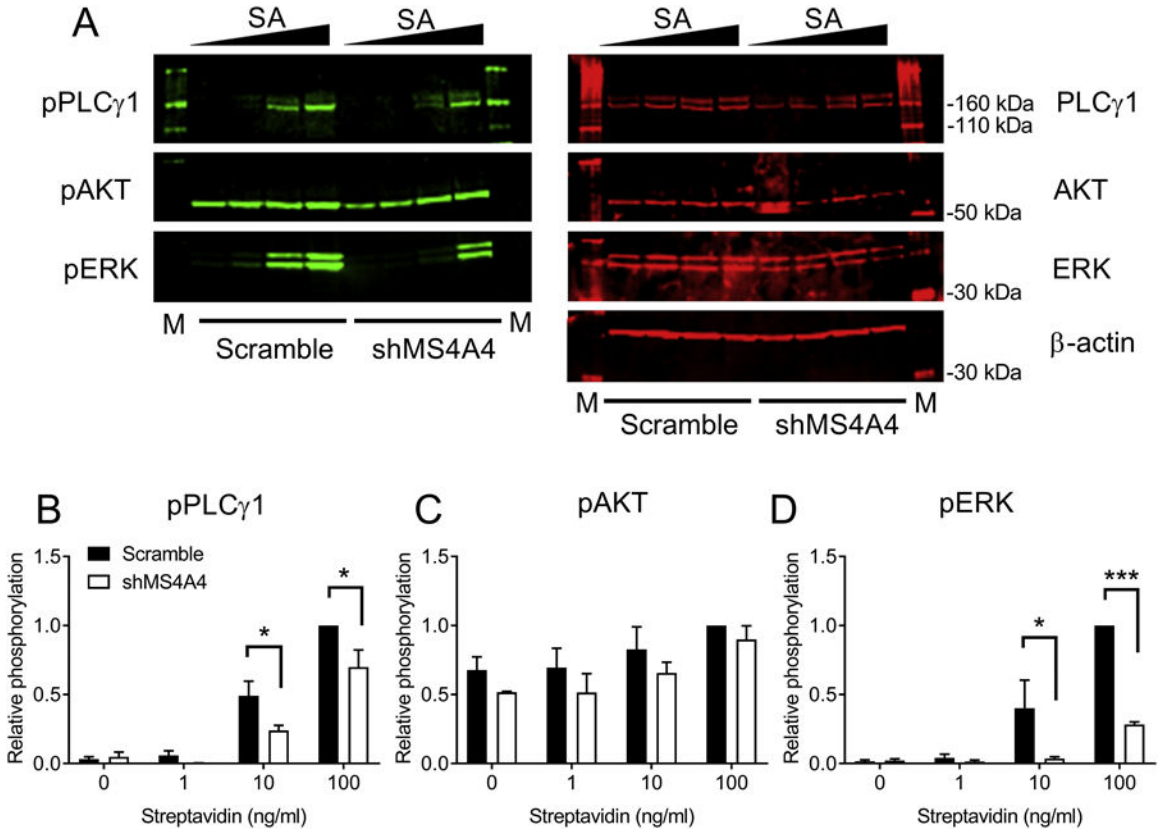


**Figure 2. MS4A4A promotes FcεRI-dependent PLCγ1 signaling.**

(A) Immunoblots from LAD2 cells transduced with either scramble or shMS4A4A lentiviruses. (B-D) Quantification of PLCγ1 (B), AKT (C), and ERK (D) phosphorylation for scramble or shMS4A4A-transduced LAD2 cells calculated as relative phosphorylation after correction against total protein and normalized to maximal phosphorylation for each phosphoprotein. Data are the mean ± SEM from three independent experiments. *$p<0.05$, ***$p<0.001$, ANOVA with Sidak's posttest.

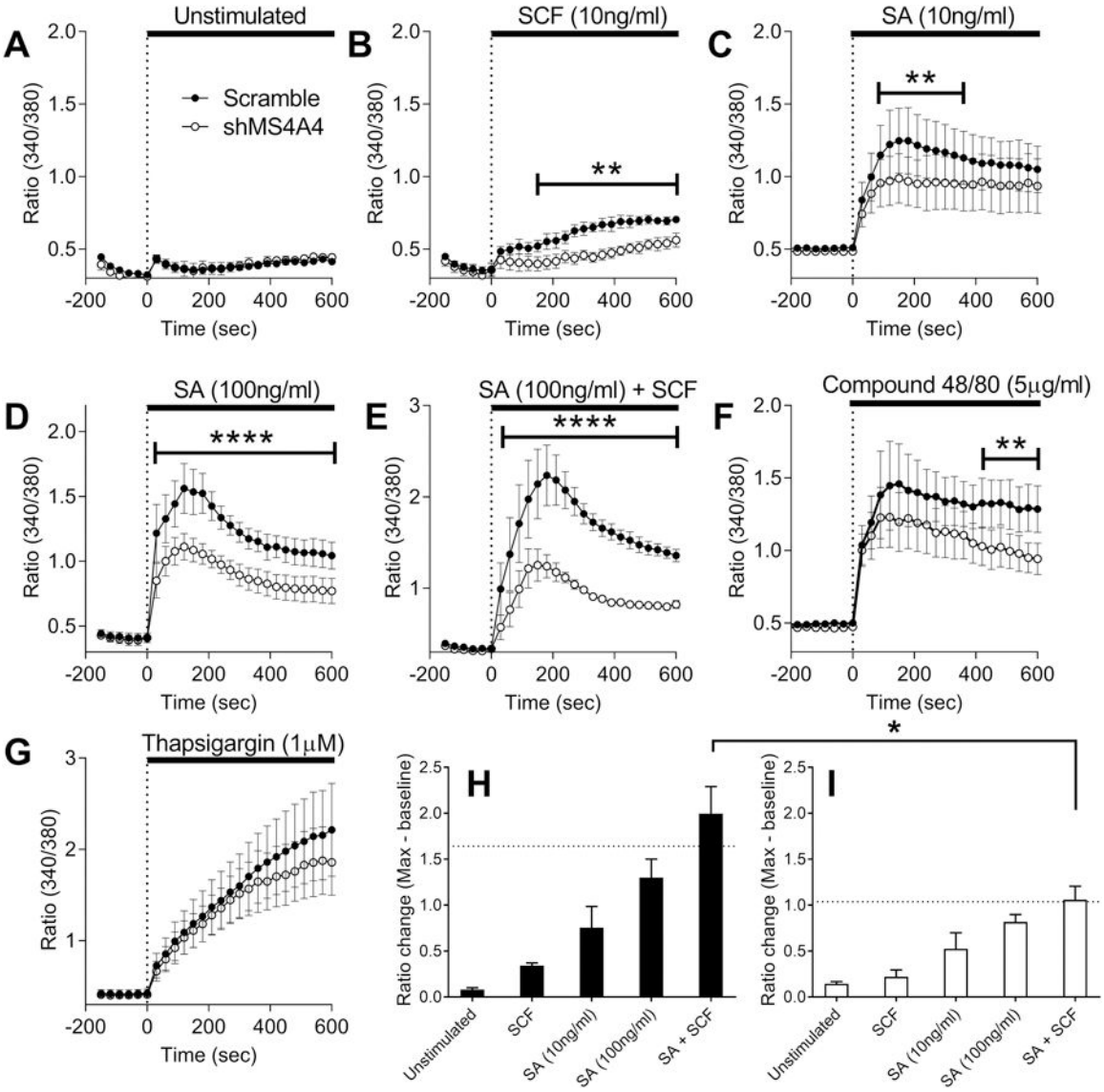


**Figure 3. MS4A4A promotes FcεRI-dependent and KIT-mediated $Ca^{2+}$ conductance promoting synergy in $Ca^{2+}$ responses.**

(A-G) Ratiometric calcium signaling compare calcium conductance between scramble control and shMS4A4A following incubations of various stimuli. (A) medium alone, (B) SCF, (C) streptavidin 10 ng/ml, (D) streptavidin 100 ng/ml, (E) compound 48/80 5 μg/ml, (F) thapsigargin 1 μM, (G) streptavidin 100 ng/ml + SCF 10ng/ml. (H) Comparison in scramble control treated cells of fluorescence ratio pre-stimulation to maximum ratio post-stimulation; dashed line indicates additive fluorescence between SCF and streptavidin 100 ng/ml. (I) Comparison of fluorescence ratio in shMS4A4A treated cells. Data are the mean ± SEM from three to seven independent experiments. *p<0.05, **p<0.01, ****p<0.0001. ANOVA with Sidak's posttest.

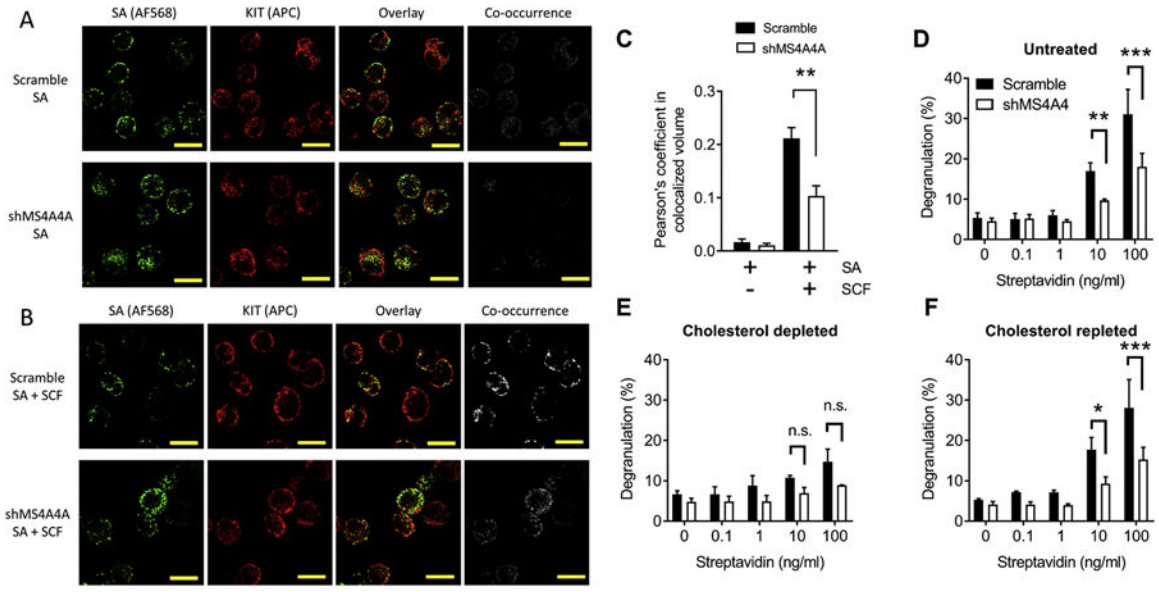


**Figure 4. MS4A4A promotes KIT and FcεRI colocalization after co-stimulation of receptors and MS4A4A likely functions in lipid rafts.**

(A-B) Confocal microscopy of human LAD2 MCs show effect of MS4A4A on colocalization of crosslinked FcεRI (green; AF568) and KIT (red; Allophycocyanin) when MCs were stimulated by biotinylated IgE plus AF568 streptavidin (A) compared with stimulation by biotinylated IgE plus AF568 streptavidin in the presence of SCF (B). Top panels show representative images of scramble control treated cells and bottom panels show representative images of shMS4A4A treated cells. (C) Quantification of colocalization assessed by Pearson's coefficient in colocalized volume above threshold of each stack of high power images. (D) Knockdown of MS4A4A results in reduced degranulation. (E) Depletion of cholesterol with MβCD reduces degranulation and the difference between scramble control and shMS4A4A is no longer significant. (F) Depletion of cholesterol, followed by repletion of cholesterol restores the phenotype to that of untreated cells. Data from D-F are the mean ± SEM from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ANOVA with Sidak's posttest.

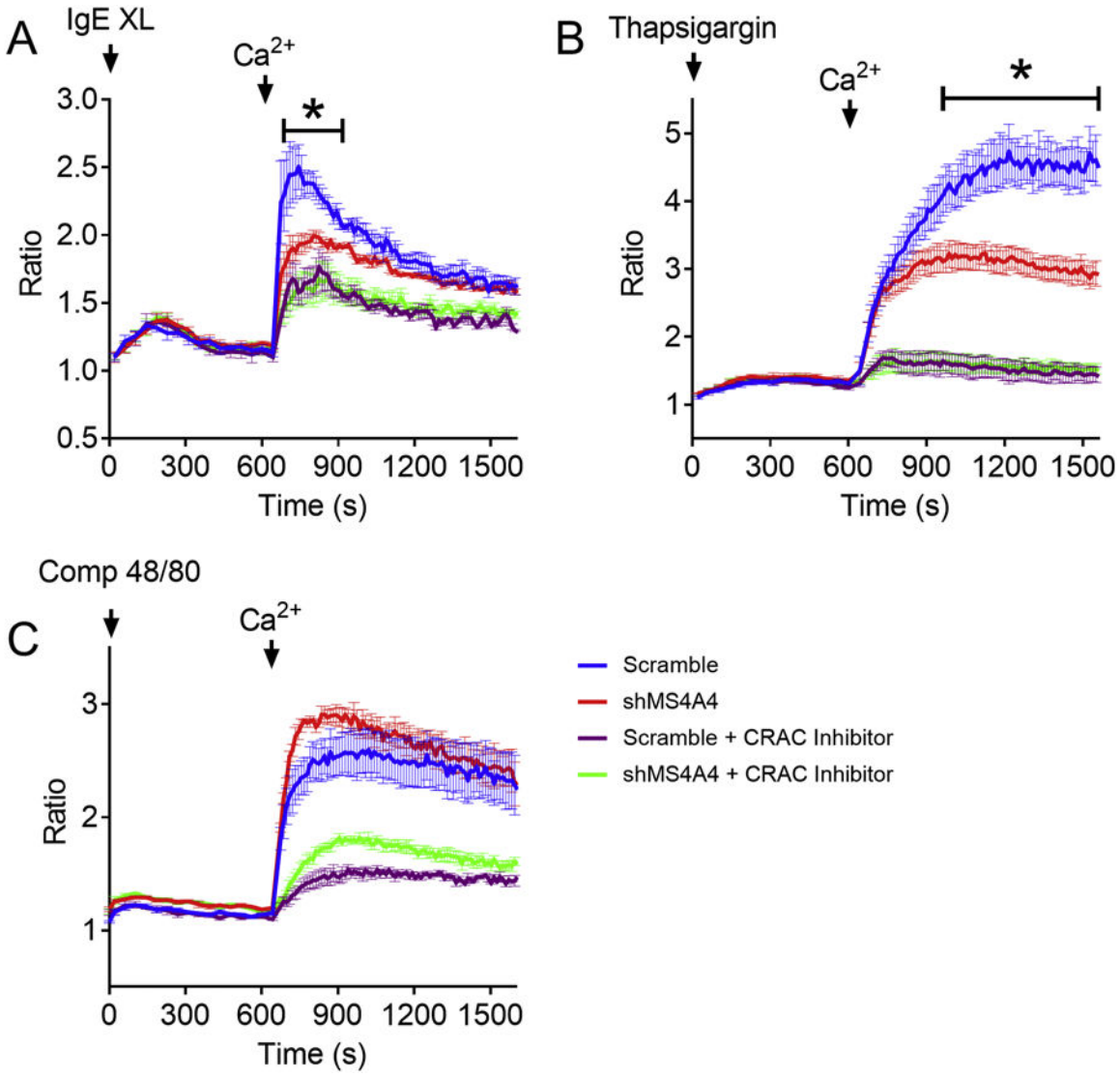


**Figure 5. MS4A4A functions in SOCE.**

Store-operated calcium entry measured by ratiometric $Ca^{2+}$ assays in the absence of $Ca^{2+}$, followed by addition of 2mM $Ca^{2+}$. The first peak before the addition of $Ca^{2+}$ is release from stores and the peak after $Ca^{2+}$ is SOCE. (A) Streptavidin and SCF, (B) thapsigargin, and (C) Compound 48/80. Arrows indicate the introduction of stimulus and $Ca^{2+}$. Data from are the mean ± SEM from three independent experiments. ***p<0.001, ****p<0.0001, ANOVA with Sidak's posttest.

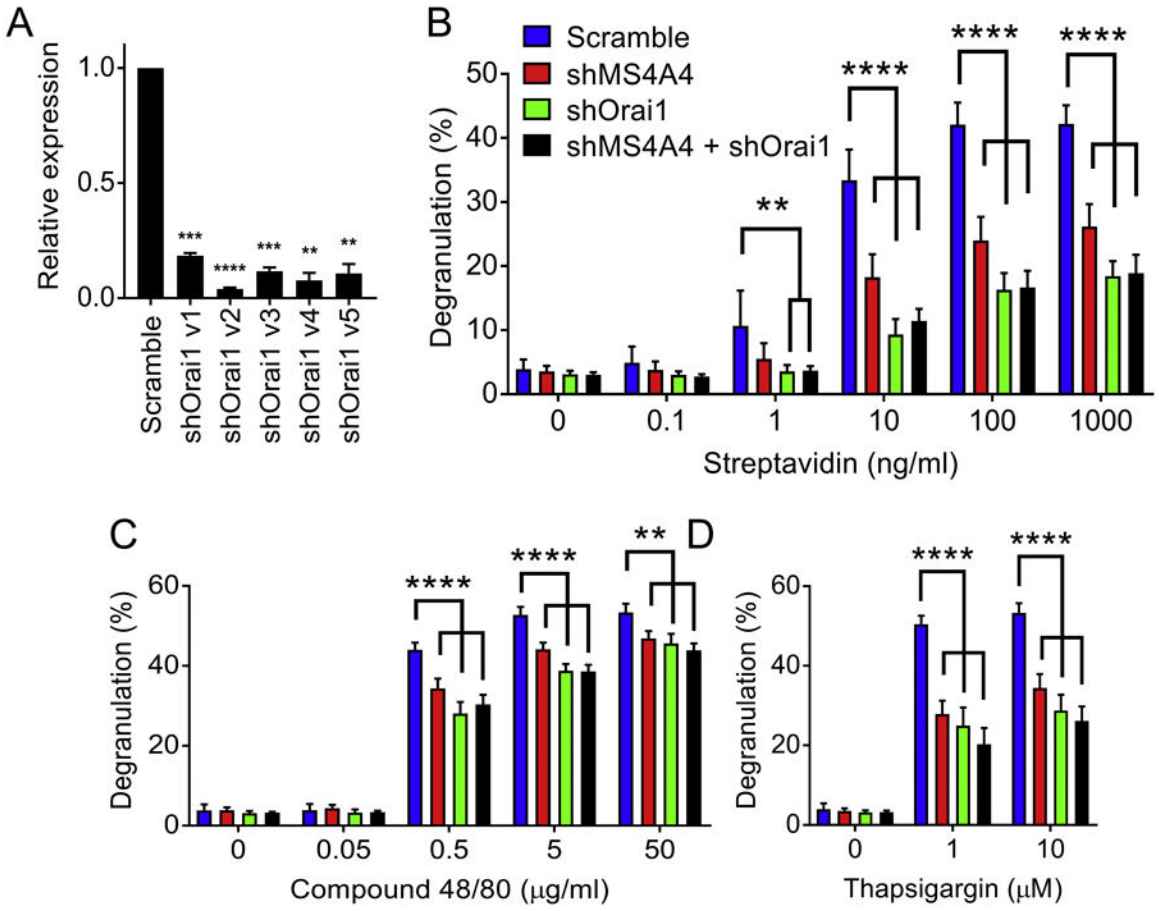


**Figure 6. MS4A4A function in SOCE is through regulation of Orai1.**

(A) qRT-PCR for Orai1 mRNA in LAD2 cells using five shRNA constructs targeting Orai1. (B-D) Comparison of β-hexosaminidase release from LAD2 cells transduced with scramble, shMS4A4A, shOrai1 (v2, the most effective construct) or shMS4A4A plus shOrai1 (v2). Stimulants of degranulation included biotinylated IgE with streptavidin (B), compound 48/80 (C), and thapsigargin (D). Data are the mean ± SEM from three (A) or five (B-D) independent experiments. **p<0.01, ***p<0.001, ****p<0.0001, ANOVA with Sidak's posttest.

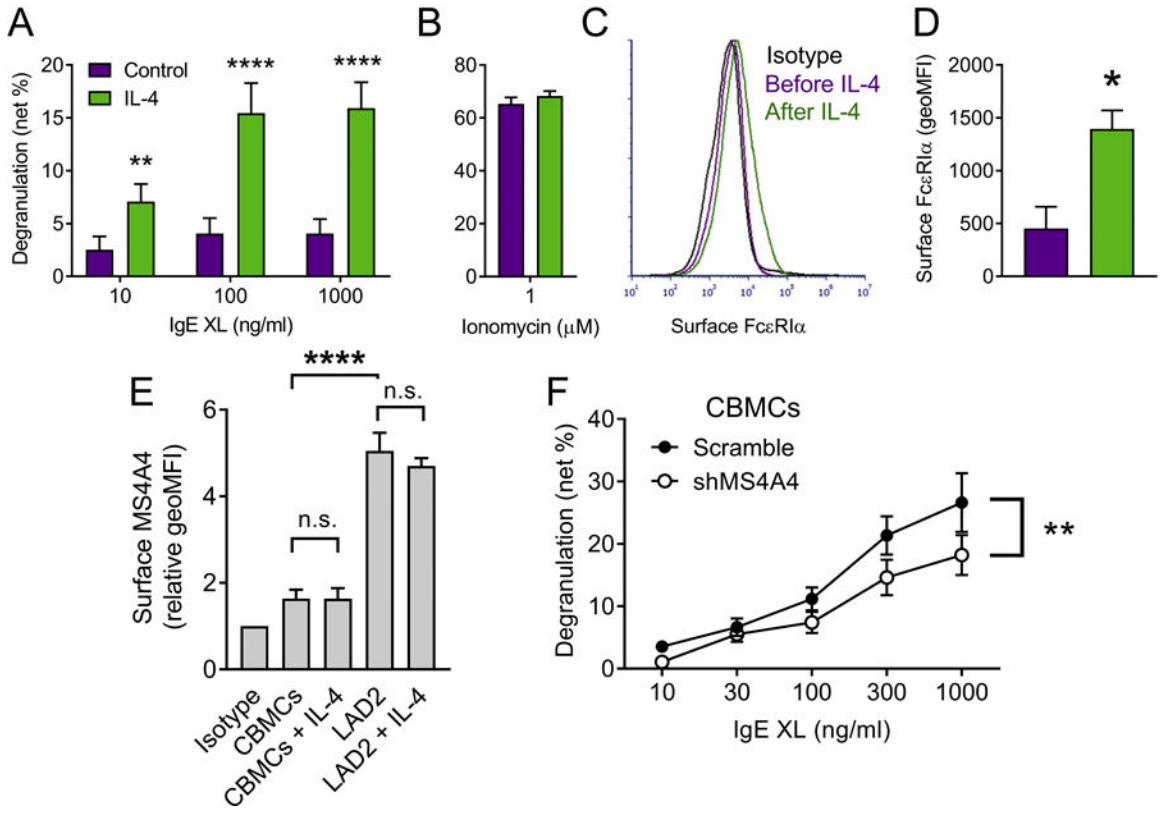


**Figure 7. MS4A4 is expressed in primary cord blood-derived MCs and function is conserved.**

(A) CBMCs degranulate when primed with IL-4. Dose response of IgE crosslinking (XL) with anti-IgE in the absence and presence of IL-4. (B) Ionomycin degranulation was unaffected by IL-4. (C) Histograms of flow cytometry analysis of FcεRIα surface expression. (D) combined data from multiple different donors shows an increase in surface FcεRIα expression with IL-4 stimulation. (E) CBMCs and LAD2 MCs stained for surface MS4A4A expression with and without IL-4 stimulation. (F) Degranulation of CBMCs with increasing concentrations of anti-IgE. Data are the mean ± SEM from four (A-E) or five (F) independent experiments. *p<0.05, **p<0.01, ****p<0.0001, n.s. = not significant. ANOVA with Sidak's posttest was used for (A), t-test for (D), and ANOVA with or without post-test for (E & F, respectively).